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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.

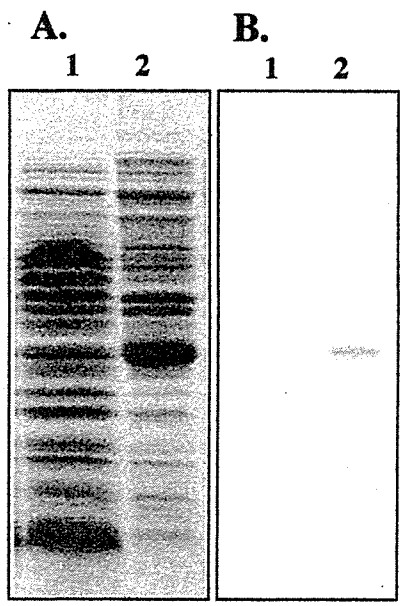

Fig. 1 : Expression and detection of *M. leprae* LpK in *E. coli*, A. Coomassie stain : 1, mock transformed and 2. *lpk* transformed *E. coli* extract. B. Western blot of the same, using monoclonal anti-His tag antibody.

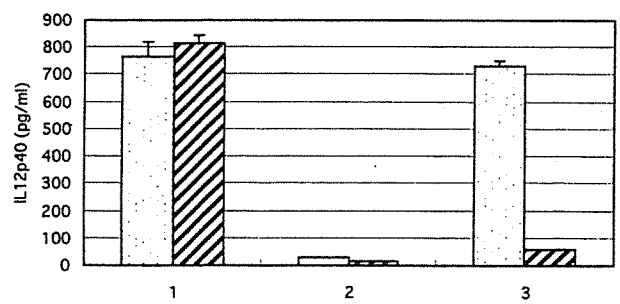

Fig. 2 : IL-12 p40 production is induced by *M. leprae* lipoprotein LpK : IL-12 p40 cytokine induction from human blood monocytes was observed using 1-LpK, 2-gene product of ML1699, 3-LPS. Hatched bar indicates the production of IL-12 p40 in the presence of polymyxin B.

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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	*folP* gene		*rpoB* 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, *folP* and *rpoB* 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 *folP* gene encoding dihydropteroate synthase (DHPS) for dapsone (5), and the *rpoB* gene for rifampin resistance (4, 8). The polymerase chain reaction (PCR) conditions and primers for *folP* and *rpoB* 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 *folP* gene from 39 biopsy specimens was 18. Among amplified samples, four samples showed *folP* mutations (22.2%). The *folP* 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 *folP* gene, primer pair for *rpoB* 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 *folP*

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 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

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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 *Hin*d 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 *Hin*d 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 *Hin*d 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 *Hin*d 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 *Hin*d 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,

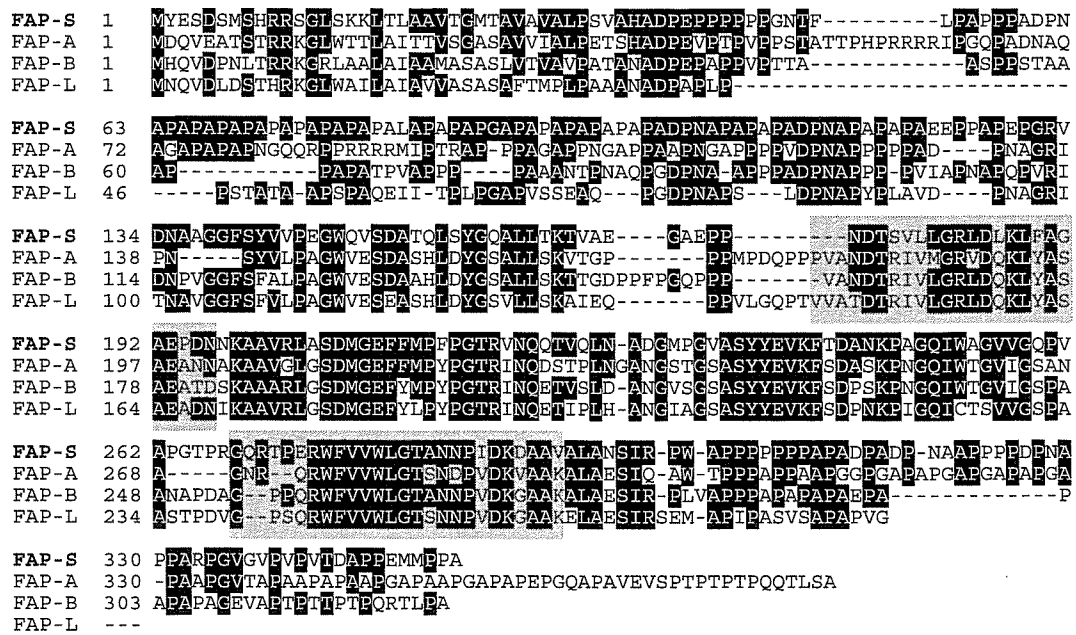

Fig. 1. Alignment of deduced amino acid sequences of *fapS* gene product (FAP-S) with FAP-A (*M. avium*), FAP-B (*M. bovis* BCG) and FAP-L (*M. leprae*). The amino acids identical to those of FAP-S are indicated by black boxes. The putative sequences of fibronectin-binding region are shown in gray background.

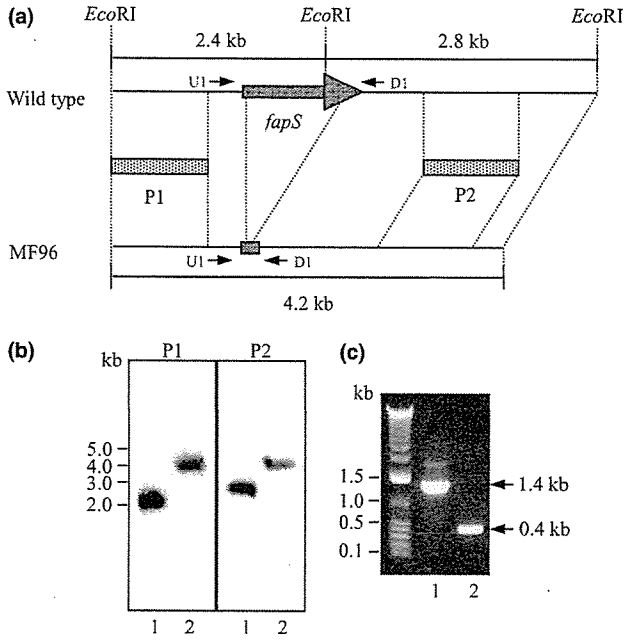

Fig. 2. Disruption of *fapS* gene. (a) Schematic diagram of the *fapS* region on the chromosome of wild type *M. smegmatis* (mc^{2}155) and the mutant strain MF96. The position of the two hybridization probes, P1 and P2, are also shown. The arrows indicate the primers (U1, D1) used for PCR analysis. (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.

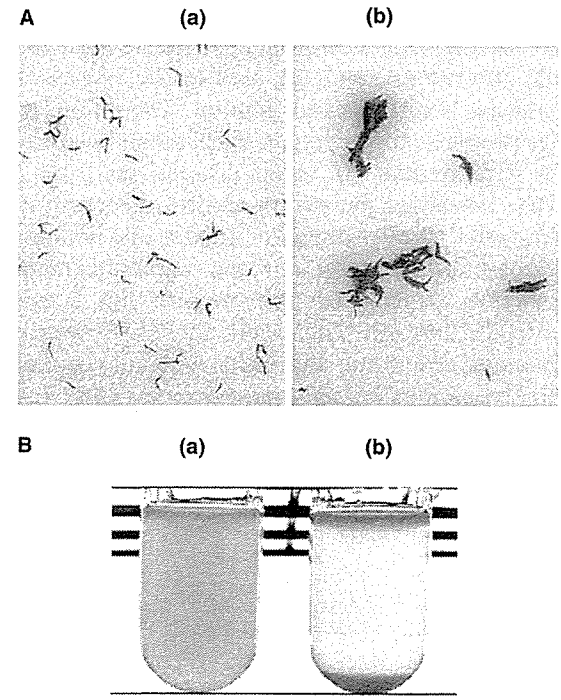

Fig. 3. Morphological observation of wild type (a) and mutant strain MF96 (b). (A) Light microscopic observation of cultured broth diluted 1:100 in PBS containing 0.2% skim milk followed by Ziehl-Neelsen staining. Magnification, 1000×. (B) Cultured broths left for 1 h without shaking.

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

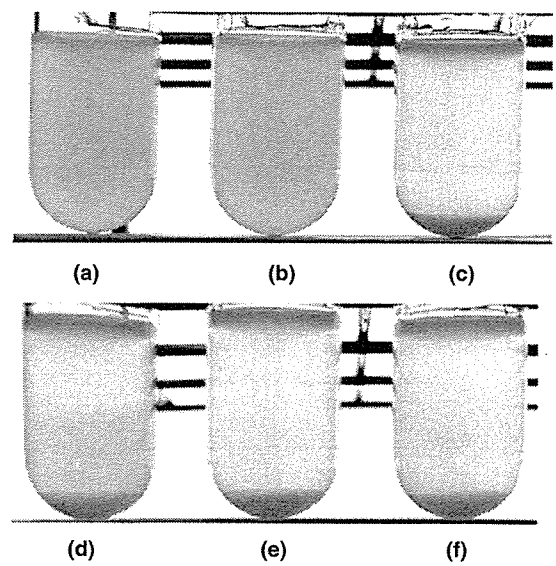

Fig. 4. Complementation experiment. Observation of wild type (a) and mutant strain MF96 transformed with pFAPS (b), pMV306kan (c), pFAPL (d), pFAPB (e) and pFAPA (f). All cultured broths were left for 1 h without shaking.

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*.

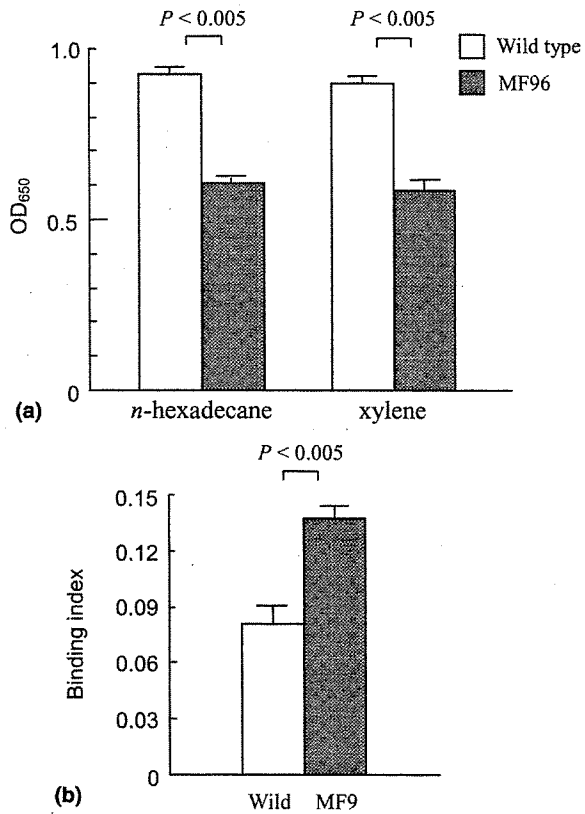

Fig. 5. (a) The cell surface hydrophobicity based on the degree of adherence to two hydrocarbons (*n*-hexadecane and xylene), assessed by the reduction in absorbance of aqueous phase separated from hydrocarbon mixture including cells of wild type (open square) and mutant strain MF96 (filled square). (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

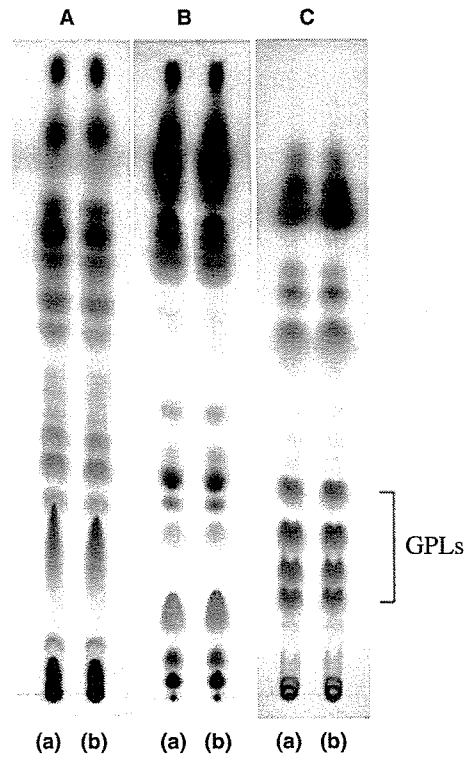

Fig. 6. Comparison of the lipid composition of wild type (a) and mutant strain MF96 (b). (A) Total lipids developed with $CHCl_3$/CH_3OH (9:1, v/v). (B) Total lipids developed with $CHCl_3$/CH_3OH/H_2O (30:8:1, v/v/v). (C) Total lipids subjected to mild alkaline hydrolysis and developed with $CHCl_3$/CH_3OH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

Acknowledgements

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Upregulation of T-Cell-Stimulating Activity of Mycobacteria-Infected Macrophages

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Abstract

Macrophages are one of the most abundant host cells to come in contact with mycobacteria. However, the infected macrophages less efficiently stimulate autologous T cells *in vitro*. We investigated the effect of the induction of phenotypic change of macrophages on the host cell activities by using *Mycobacterium leprae* as a pathogen. The treatment of macrophages with interferon-γ (IFN-γ), GM-CSF and interleukin-4 deprived macrophages of CD14 antigen expression but instead provided them with CD1a, CD83 and enhanced CD86 antigen expression. These phenotypic features resembled those of monocyte-derived dendritic cells (DC). These macrophage-derived DC-like cells (MACDC) stimulated autologous $CD4^+$ and $CD8^+$ T cells when infected with *M. leprae*. Further enhancement of the antigen-presenting function and CD1a expression of macrophages was observed when treated with IFN-γ. The *M. leprae*-infected and -treated macrophages expressed bacterial cell membrane-derived antigens on the surface and were efficiently cytolysed by the cell membrane antigen-specific $CD8^+$ cytotoxic T lymphocytes (CTL). These results suggest that the induction of phenotypic changes in macrophages can lead to the upregulation of host defence activity against *M. leprae*.

Introduction

The extent of mycobacterial spread is closely associated with the disease severity and is controlled by bacterial antigen-specific cell-mediated immunity. The activation of $CD4^+$ and $CD8^+$ T-cell subsets is induced by cell–cell contact with antigen-presenting cells (APC), which is therefore the most critical host defence component against mycobacterial infection [1–3]. The mycobacteria reside in phagosomes of macrophages but restrict the capacity of the phagosomes to fuse with late endosomes/lysosomes [4] and avoid getting being processed. These observations suggest that mycobacteria-infected macrophage less efficiently express the bacterial antigens on their surface. Furthermore, macrophages generally lack the activity to load peptide derived from exogenous protein or bacterial protein to major histocompatibility complex (MHC) class I molecules. Therefore, it is assumed that macrophages are resistant to MHC-restricted-killing activities of cells such as CTL allowing mycobacteria to reside and hide in macrophages.

Here, we tried to upregulate the APC function of macrophages in order to enhance the host defence activity. Leprosy, caused by *Mycobacterium leprae* infection, provides a useful model to evaluate immunoregulatory mechanism against an intracellular pathogen, because the disease extent is closely associated with the potential of host defence activity. *M. leprae* preferentially infects macrophages and Schwann cells [5–7] and induces clinical manifestation mainly in skin and peripheral nerves. However, the disease shows broad spectrum with the various skin manifestations from single lesion (tuberculoid leprosy) to almost entirely disseminated one (lepromatous leprosy). The mechanism that produces this broad spectrum involves the extent of cell-mediated immune response to *M. leprae*. We previously reported that macrophages phagocytosed *M. leprae*, but their antigens are not fully expressed on the surface of macrophages, which results in reduction or avoidance of their contact with T cells [8]. In addition to macrophages, monocyte-derived dendritic cells (DC) are well-characterized subset of professional APC, capable of stimulating both naïve and memory type autologous $CD4^+$ and $CD8^+$ T cells [9–11]. We also demonstrated that, in contrast to macrophages, the DC expressed *M. leprae*-derived antigens on the surface and stimulated

both T-cell subsets to produce interferon-γ (IFN-γ) which is a representative type 1 cytokine and is considered to be associated with the killing of intracellular mycobacteria [2, 12]. Thus, DC can evoke the activation of type-1 T cells and control the multiplication of *M. leprae* [8, 13–15].

Macrophages are the most abundant primary cell type to come in contact with *M. leprae* and are frequently seen in inflamed tissues. These macrophages are thought to play a role in initial antimycobacterial immune responses for the better control of bacterial spread. Therefore, we tried to induce phenotypic changes in *M. leprae*-infected macrophages to DC-like cells and examined their antigen-presenting activities such as stimulation and differentiation of T cells into type-1 cells. Furthermore, we examined their susceptibility to killing activities of *M. leprae*-derived antigen-specific CTL.

Materials and methods

Preparation of cells and bacteria. Peripheral blood was obtained under informed consent from healthy, but PPD-positive, individuals. Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll–Paque Plus (Pharmacia, Uppsala, Sweden) and cryopreserved in liquid nitrogen until use, as previously described [16]. Macrophages were differentiated by culturing plastic adherent $CD14^+$ monocytes with RPMI 1640 medium containing M-CSF (R&D Systems, Minneapolis, MN, USA) [17]. For preparation of the monocytes, $CD3^+$ T cells were removed from either freshly isolated heparinized blood or cryopreserved PBMC using immunomagnetic beads coated with anti-CD3 monoclonal antibody (Dynabeads 450, Dynal, Oslo, Norway), and the plastic adherent cells were used as monocytes [16]. Monocyte-derived DC were differentiated from the monocytes [16, 18]. Briefly, the monocytes were cultured for 5 days in the presence of 50 ng of recombinant GM-CSF (Pepro Tech EC LTD, London, UK) and 10 ng of rIL-4 (Pepro Tech) per ml. rGM-CSF and rIL-4 were supplied every 2 days. Macrophage-derived DC (MACDC) were differentiated from macrophages as follows. Macrophages were treated with 300 U/ml of IFN-γ during last 24 h of 3-day culture. On day 3, macrophages were washed, and the media were replaced with media containing rGM-CSF and rIL-4. The production of MACDC was conducted by using the same protocol to that of DC. In some cases, *M. leprae*-infected or -uninfected macrophages, MACDC and DC were further treated with indicated doses of maturation and activation factors for DC including CD40 ligand (L) (Pepro Tech) or lipopolysaccharide (LPS) (*Escherichia coli* 0111: B4, Difco Laboratories, Detroit, MI, USA).

M. leprae (Thai-53) was obtained from footpads of BALB/*c-nu/nμ* mice. The isolated bacteria were counted by Shepard's method [19] and were frozen at −80 °C until use. The viability of *M. leprae* was assessed by using fluorescent diacetate/ethidium bromide test [20]. The macrophages and DC were infected with *M. leprae* by coculturing at indicated multiplicity of infection (MOI). The MOI was determined upon an assumption that all macrophages and DC were susceptible to infection with *M. leprae*. The macrophages and DC were previously shown to be similarly susceptible to the bacterial infection *in vitro* [8].

Analysis of cell-surface and intracellular antigens. The expression of cell-surface antigens on macrophages, MACDC and DC was analysed using FACScalibur (Becton Dickinson Immunocytometry System, San Jose, CA, USA). About 1×10^4 live cells were analysed. For analysis of cell-surface antigens, the following monoclonal antibodies were used: FITC-conjugated monoclonal antibodies against HLA-ABC (G46-2.6, BD Biosciences, San Jose, CA, USA), HLA-DR (L243), CD14 (Leu-M3, Becton Dickinson) and CD86 (FUN-1, BD Biosciences) and phycoerythrin-labelled monoclonal antibodies against CD83 (HB15a, Immunotech, Marseille, France). Murine-unlabelled monoclonal antibody to CD1a (NA1/34, Serotec, Oxford, UK) was also used and was visualized by FITC-labelled goat $F(ab')_2$ antimouse immunoglobulin G (IgG) (Tago-immunologicals, Camarillo, CA, USA). Purified rabbit polyclonal antibodies (pAbs) to cell-wall proteins, cell membrane fractions and cytosol fractions of *M. leprae* each depleted of lipoarabinomannan (provided by Drs J. Spencer and P. J. Brennan, Colorado State University) were used. The details of the preparation are available at http://www.cvmbs.colostate.edu/mip/leprosy. Among the *M. leprae*-derived fractions, membrane fraction was the most antigenic in terms of activation of adaptive immunity [21]. We also used pooled sera from 10 untreated lepromatous leprosy patients who were classified according to clinical criteria (WHO) based on skin smears (given by Dr H. Minagawa, Leprosy Research Center, Tokyo, Japan) [8]. FITC-conjugated murine antihuman Ig (Tago-immunologicals) was used as secondary antibody for detection. The optimal concentrations of monoclonal antibodies, pAbs and patient's pooled sera were determined in advance.

Assessment of APC functions of macrophages, MACDC and DC. The ability of macrophages, MACDC and DC infected with *M. leprae* to stimulate autologous T cells was assessed using an autologous stimulator T-cell-mixed reaction as previously described [8, 18]. The stimulators such as macrophages, MACDC and DC were treated with 50 μg/ml of mitomycin C, washed extensively to remove extracellular bacteria and were used as a stimulator. $CD4^+$ and $CD8^+$ T cells purified using immunomagnetic beads coated with monoclonal antibodies to CD8 and CD4, respectively, were used as a responder population. Responder cells (1×10^5/well) were plated in 96-well, round-bottom tissue culture plates, and stimulators were added to give an indicated stimulator responder T-cell

ratio. The T-cell proliferation during the last 10 h of a 4-day culture in the presence of 4% heat-inactivated human serum was quantified by incubating the cells with 1 μCi/well of [^{3}H]-thymidine.

Assessment of cytokine production. Levels of the following cytokines were measured: IFN-γ and IL-10 produced by $CD4^+$ and $CD8^+$ T cells stimulated for 4 days with macrophages, MACDC or DC and IL-12 p70 produced by *M. leprae*-infected or -uninfected macrophages and MACDC upon a stimulation with soluble form CD40L for 24 h. The concentrations of IFN-γ, IL-12 p70 and IL-10 were quantified using OptEIA ELISA Set available from BD Biosciences.

Assessment of cytotoxic activity of $CD8^+$ T cells. The susceptibility of *M. leprae*-infected macrophages and MACDC to the cytokilling activity of $CD8^+$ T cells was evaluated. Ten thousand macrophages and MACDC, either uninfected or infected with *M. leprae* at MOI 5, were used as a target cell. As an effector population, $CD8^+$ CTL activated by stimulation with autologous DC, which were pulsed with 15 μg/ml of *M. leprae*-derived cell membrane fractions, were used. The targeT cells were cocultured for 5 h with the effector cells at various effector/target cell ratios. The supernatant was collected for lactate dehydrogenase (LDH) release assay. The concentration of LDH released by target cell death was measured according to the instructions of the assay kit (Cyto Tox 96® Non-Radioactive Cytotoxicity Assay, Promega, Madison, WI, USA). The percent specific killing was calculated as follows:

$$\frac{\text{LDH (test sample)} - \text{LDH (spontaneous effector cell level)} - \text{LDH (spontaneous target cell level)}}{\text{LDH (total target cell lysis level)} - \text{LDH (spontaneous target cell level)}}$$

Statistical analysis. Student's *t*-test was applied to demonstrate statistically significant differences.

Results

Upregulation of APC function of *M. leprae*-infected macrophages

Phenotypic characterization of MACDC was carried out by analyzing the surface expression of various molecules on macrophages and MACDC (Fig. 1). There were no

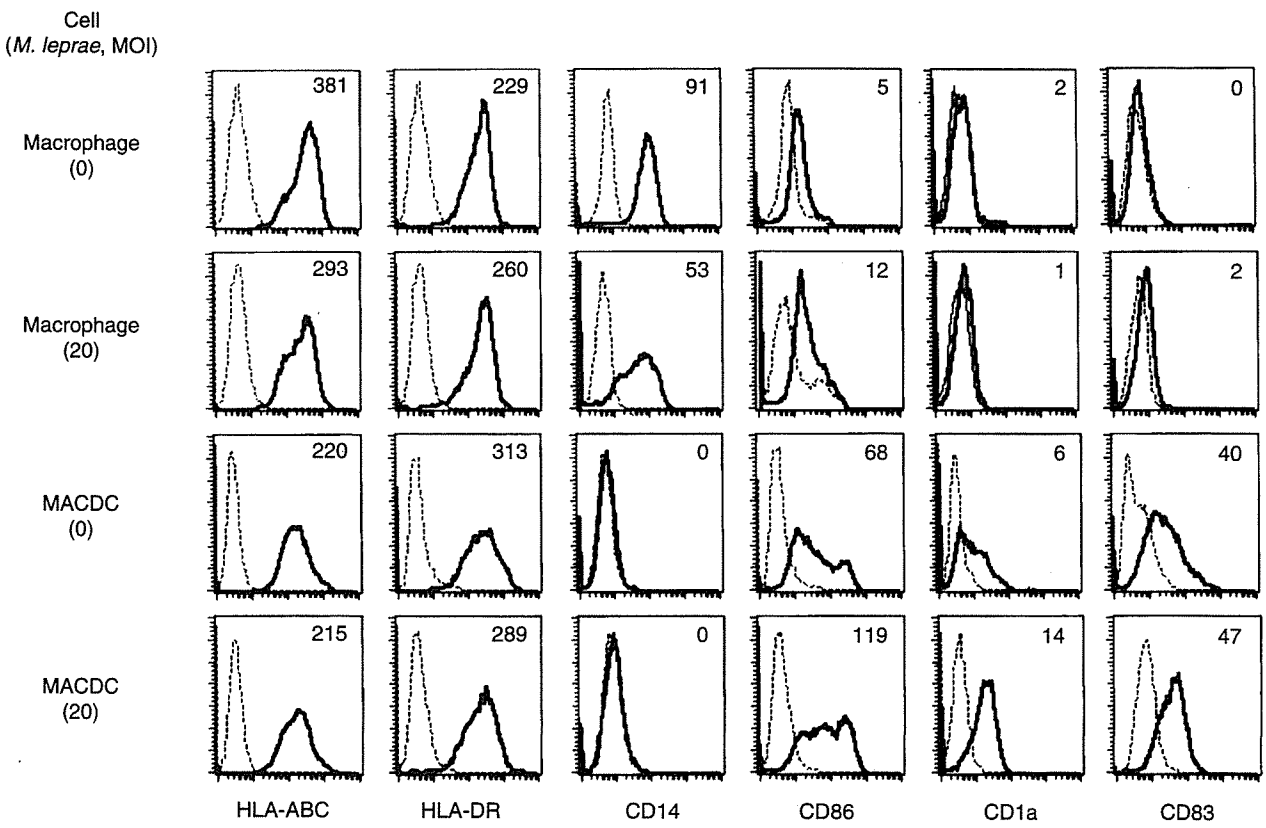

Figure 1 Expression of various molecules on macrophages and macrophage-derived dendritic cell-like cells (MACDC) either uninfected or infected with *Mycobacterium leprae* [multiplicity of infection (MOI) 20]. Both macrophages and MACDC were infected with *M. leprae* for 5 days. The number represents the difference in mean fluorescence intensity between dotted and solid lines. A representative of three independent experiments is shown. Dotted line, control monoclonal antibody; solid line, monoclonal antibody.

apparent difference in the expression of HLA-ABC and HLA-DR antigens between uninfected macrophages and MACDC. Also, no significant upregulation of expression of these molecules was induced by *M. leprae* infection. While macrophages expressed CD14 antigen, the surface CD14 expression on MACDC was completely lost. On the contrary, the expression of CD86 antigen on MACDC was higher than that on macrophages. The expression of CD1a and CD83 antigens which is DC-specific marker was induced on MACDC, both of which level was further upregulated by *M. leprae* infection. These results indicated that MACDC showed phenotypic characteristics similar to monocyte-derived DC, and MACDC were at least partially activated by *M. leprae* infection. Further characterization of MACDC was conducted by comparing the APC function of MACDC with that of monocyte-derived DC (Table 1). Both DC and MACDC induced proliferation of autologous $CD4^+$ and $CD8^+$ T cells in a bacterial dose-dependent manner, but a higher T-cell proliferation was induced by MACDC rather than DC. More than 75% of the MACDC-induced T-cell proliferation was suppressed by monoclonal antibody to MHC or CD86 antigens (data not shown). Previously, we showed that monocyte-derived DC were resistant to exogenous factors such as LPS and CD40L in terms of APC function [8], but using MACDC, both factors upregulated the T-cell-stimulating function of *M. leprae*-infected, but not uninfected, MACDC (Table 2). The proliferation of both $CD4^+$ and $CD8^+$ T-cell subsets were upregulated in a manner dependent on the dose of *M. leprae*.

Table 1 Proliferative response of autologous T cells to *Mycobacterium leprae*-infected macrophage-derived dendritic cell (DC)-like cells (MACDC)*

		CD4		CD8	
M. leprae (MOI)	Stimulator T/DC	20	40†	10†	20
0	DC	1.9 ± 0.6	1.6 ± 0.5	1.8 ± 0.4	1.5 ± 0.3
	MACDC	2.1 ± 0.6	1.7 ± 0.5	1.7 ± 0.4	1.3 ± 0.2
5	DC	3.8 ± 0.9‡	2.7 ± 0.9‡	3.7 ± 1.2‡	2.6 ± 0.7‡
	MACDC	14.6 ± 2.3‡	8.0 ± 2.0‡	13.9 ± 3.3‡	10.6 ± 2.8‡
20	DC	6.9 ± 1.2‡	7.6 ± 1.3‡	10.1 ± 2.1§	8.1 ± 2.0‡
	MACDC	22.3 ± 5.2‡	17.9 ± 5.0‡	21.6 ± 4.1§	18.2 ± 4.3‡

*The responder $CD4^+$ and $CD8^+$ T cells (1×10^5/well) were stimulated for 4 days with an indicated dose of autologous monocyte-derived DC (DC) or MACDC. MACDC were differentiated from macrophages, either uninfected or infected for 5 days with an indicated dose of *M. leprae*, by using IFN-γ, rGM-CSF and rIL-4. The proliferation of responder cells was quantified by an incorporation of [^{3}H]-thymidine. Representative of three separate experiments is shown. Assays were done in triplicate, and results are expressed as mean ± SD.
†[^{3}H]-thymidine uptake ($\times 10^3$ cpm).
‡$P < 0.05$.
§$P < 0.01$.

Table 2 Effect of exogenous factors on the antigen-presenting cell function of macrophage-derived dendritic cell (DC)-like cells (MACDC)*

		CD4		CD8	
M. leprae (MOI)	Tested factor T/DC	20	40†	10†	20
0	None	1.0 ± 0.0	0.8 ± 0.0	1.3 ± 0.2	0.9 ± 0.1
	LPS	1.5 ± 0.3	1.2 ± 0.2	1.7 ± 0.3	1.2 ± 0.2
	CD40L	2.8 ± 0.2	1.9 ± 0.2	2.3 ± 0.4	1.9 ± 0.3
5	None	5.7 ± 0.8‡	4.5 ± 0.7‡**	4.9 ± 0.4¶	3.9 ± 0.3‡§
	LPS	16.5 ± 2.0‡	11.7 ± 2.0**	13.8 ± 0.7¶	10.9 ± 0.9‡
	CD40L	11.2 ± 0.3‡	8.3 ± 0.4‡	10.4 ± 0.7¶	7.6 ± 0.8§
20	None	9.3 ± 1.2‡	6.9 ± 0.8‡§	7.7 ± 0.6‡¶	6.4 ± 0.6‡¶
	LPS	23.3 ± 2.4‡	15.7 ± 2.1§	21.7 ± 2.0‡	16.3 ± 1.1¶
	CD40L	16.5 ± 0.5‡	12.3 ± 0.5‡	15.0 ± 0.8¶	11.9 ± 1.0‡

*The responder $CD4^+$ and $CD8^+$ T cells (1×10^5/well) were stimulated for 4 days with autologous MACDC. MACDC were differentiated from macrophages, either uninfected or infected, for 5 days with an indicated dose of *Mycobacterium leprae*, by using IFN-γ, rGM-CSF and rIL-4 and were further treated with exogenous factors including LPS (30 ng/ml) and soluble form of CD40L (1 μg/ml). The proliferation of responder cells was measured by an incorporation of [^{3}H]-thymidine. Representative of three separate experiments is shown. Assays were done in triplicate, and results are expressed as mean ± SD.
†[^{3}H]-thymidine uptake ($\times 10^3$ cpm).
‡$P < 0.005$.
§$P < 0.01$.
¶$P < 0.001$.
**$P < 0.05$.

Next, the role of exogenous IFN-γ on macrophages and its effect on MACDC production was determined (Fig. 2). While no apparent alterations were induced by 24 h IFN-γ treatment in the expression of HLA-ABC and HLA-DR antigens (data not shown), the expression of CD1a was significantly upregulated by IFN-γ treatment or by *M. leprae* infection. The highest CD1a expression on macrophages was achieved when both *M. leprae* infection and IFN-γ treatment were conducted. Similar upregulation was observed in the expression of CD86 antigen. Moreover, when the IFN-γ treatment was assessed from the functional aspect, it upregulated APC function of *M. leprae*-infected MACDC (Table 3). The IFN-γ treatment on macrophages significantly upregulated activities of MACDC to stimulate both $CD4^+$ and $CD8^+$ T-cell subsets, and 300 U/ml of IFN-γ provided the optimal upregulation. However, a sole IFN-γ treatment was not sufficient and both GM-CSF and IL-4 were required for upregulating the APC function of macrophages (data not shown).

Then, we examined whether MACDC activated type-1 T cells by measuring IFN-γ production by T cells (Table 4). While both the bacteria-infected and -uninfected macrophages, even after being stimulated with an exogenous IFN-γ, did not stimulate autologous $CD4^+$ and $CD8^+$ T cells, MACDC derived from *M. leprae*-infected macrophages did stimulate both subsets of T cells to produce IFN-γ. However, neither IL-4 nor IL-10 was produced by T cells (data not shown). The IFN-γ production by T cells stimulated with MACDC exhibited a bell-shape phenomenon. When up to MOI 20 of *M. leprae* was infected to precursor macrophages, T cells were activated in a bacterial dose-dependent manner, but when more than MOI 20 of the bacteria was infected, the APC function of MACDC decreased. Similar T-cell activation pattern was also observed with heat-killed *M. leprae* (data not shown). Furthermore, MACDC attained an ability to

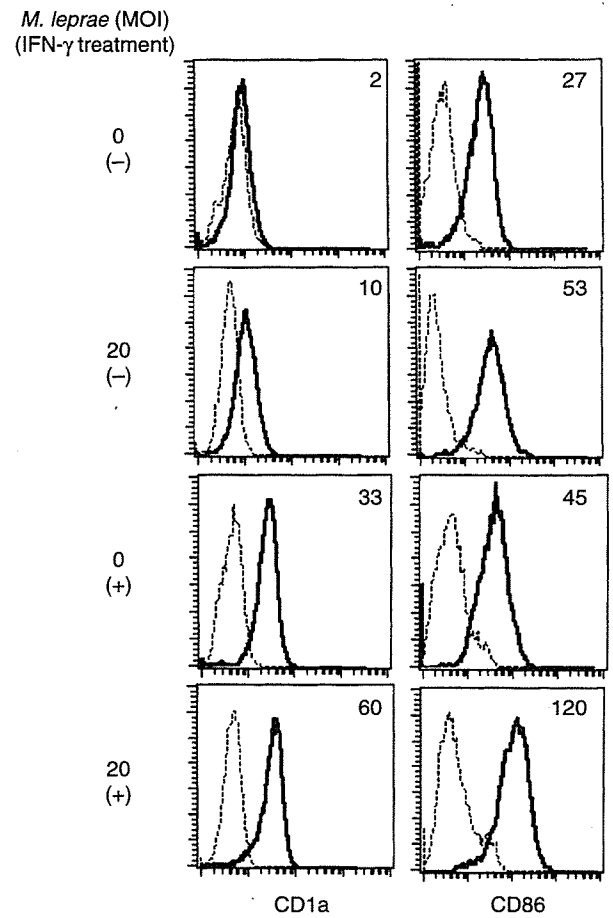

Figure 2 Expression of CD1a and CD86 antigens on macrophages. Macrophages were uninfected or infected with *Mycobacterium leprae* [multiplicity of infection (MOI 20)] on day 2 of culture and were subsequently treated with IFN-γ (300 U/ml) for 24 h on day 3 of culture. The number represents the difference in mean fluorescence intensity between dotted and solid lines. A representative of three independent experiments is shown. Dotted line, control monoclonal antibody; solid line, monoclonal antibody.

Table 3 Effect of exogenous IFN-γ on the T-cell-stimulating activity of macrophage-derived dendritic cell (DC)-like cells (MACDC)*

		CD4		CD8	
M. leprae (MOI)	IFN-γ (U/ml) T/DC	20	40†	10†	20
0	0	4.7 ± 1.2	3.2 ± 1.1	4.9 ± 2.0	3.6 ± 1.8
20	0	15.5 ± 3.0‡	11.5 ± 2.1§	14.9 ± 1.9‡§	11.4 ± 1.6§
20	100	25.7 ± 2.8‡	18.9 ± 2.7§	23.2 ± 2.2‡	18.4 ± 2.4§
20	300	33.6 ± 2.9‡	26.1 ± 3.0§	32.7 ± 3.0§	26.3 ± 3.0§
20	1000	11.1 ± 4.5	0.9 ± 0.6	11.9 ± 1.6	8.8 ± 0.7

*The responder $CD4^+$ and $CD8^+$ T cells (1×10^5/well) were stimulated for 4 days with an indicated dose of autologous MACDC. MACDC were produced from macrophages, either uninfected or infected with *Mycobacterium leprae* for 5 days at multiplicity of infection (MOI) 20 and treated with an indicated dose of IFN-γ. The proliferation of responder cells was measured by an incorporation of [^{3}H]-thymidine. Representative of three separate experiments is shown. Assays were done in triplicate, and results are expressed as mean ± SD.
†[^{3}H]-thymidine uptake ($\times10^3$ cpm).
‡$P < 0.001$.
§$P < 0.005$.