30、32、34 ヶ月目の末梢リンパ球について、4種のペプチドで誘導されるサイトカインを調査した。

B. 研究方法

独立行政法人医薬基盤研究所・霊長類医科学研究センターで繁殖育成された6ー8ヶ月齢の幼若カニクイザル6頭を3群に分け、らい菌を鼻腔内、鼻先端部、左手根部にそれぞれ 2 頭ずつ接種した。らい菌接種および感染動物の維持は、医薬基盤研究所・動物実験委員会の承認を得た後 P2感染実験施設内でおこなった。らい菌接種前、接種後三年間にわたり 2 ヶ月月間隔で採血し、定法に従ってリンパ球を分離した。

$2x10^5$ のリンパ球を蛍光色素標識マウスモノクローナル抗体で染色し、FACS により主要リンパ球サブセットレベルを測定した。用いた抗体の組み合わせは、

PE-CD20/FITC-CD3,

PE-CD8/ FITC-CD16

Cy5-CD8/PE-CD4/FITC-CD29

Cy5-CD8/PE-CD4/FITC-CD69

の 4 種類である。

サイトカインの誘導は、リンパ球を $2x10^6$/ml の濃度で 10%FCS-RPMI-1640 培地に浮遊させ、4 種のらい菌由来ペプチド(MMP-II; 0.1μg/ml、LpK; 0.1μg/ml、LipoK;0.1μg/ml、FAP; 1μg/ml)と混合して 4 日間培養した。培養 4 日目に培養液を回収し、遠心上清を -80℃で凍結保存した。培養上清中のサイトカイン(IL-2、IL-4、IL-6、IL-12、IFNγ、TNFα)は市販の Monkey Cytokine Assay ELISA Kit を用いて測定した。

C. D.研究結果および考察

らい菌接種後 26ヶ月から 34ヶ月の間では、主要リンパ球サブセットレベルには特に著しい変化が認められなかった。

図 1 にらい菌を接種したカニクイザルの末梢リンパ球を4種のペプチドで刺激した培養上清中の IL-12 を測定した結果を示す。らい菌接種後 28ヶ月から 34ヶ月の間では、6頭すべてで、MMP-II 以外の LpK、LipoK、FAP の 3 種のペプチドで刺激した場合に高レベルの IL-12 が産生されることが判明した。この結果から、少なくともMMP-IIはらい菌の持続感染を評価する指標とは成り得ないこと、また、IL-12 の検出では、らい菌感染で誘導される特異性の高い免疫の標的となるペプチド絞り込むことが不可能であると判断した。

図 2 に 4 種の抗酸菌由来ペプチドで刺激したリンパ球培養上清中の IFNγを測定した結果を示す。らい菌接種後 32 ヶ月目および34ヶ月目では、LpKとFAPで刺激した場合にのみ IFNγの産生が誘導されること、#002 と#007 の2頭でのみ高レベルの IFNγ産生が認められることが明らかとなった。一方、FAP で誘導される IFNγは、#007 でのみ 28ヶ月目から34ヶ月目まで継続して高レベルの IFNγが検出された。

#002 の IFNγ産生に関しては、らい菌感染で誘導された免疫と考えるよりも非定型抗酸菌の感染により誘導された免疫で生じた結果である可能性が高い。昨年度の報告書でも考察しているが、今回実験に供試した 6 頭の内 3 頭(#001、#002、#009)では、らい菌接種前(Pre)のリンパ球を3種のペプチドで刺激した場合に、陽性のリンパ球幼若化反応が認められている。このことから、これら3頭ではらい菌接種前に飼育環境中に存在する非定型抗酸菌に感染していた可能性が

さらに、接種後二年目で幼若化反応が高進していることが確認されている。

図 2 の結果が、らい菌の持続感染で誘導された結果か否かを確認する目的で、らい菌接種前(Pre)のリンパ球を LpK および FAP で刺激した場合の幼若化反応(SI : Stimulation Index)と図 2 の 28ヶ月目の IFNγ産生量との相関を調べてみた(図 3)。その結果図 3 に示すように、#002 と#007 ではいずれも Lpk で誘導される IFNγ産生量が高いが、#002 では Pre での幼若化反応が高いレベルにあることが判る(図 3 上図)。一方、#007 は Pre での幼若化反応が陰性(SI<2.0)で、かつ LPK 刺激および FAP 刺激で高レベルの IFNγを産生する唯一の個体であることが明らかとなった。これらの結果から、FAP で誘導される IFNγ産生がらい菌の持続感染を特異的に評価する最も信頼性の高い指標であると判断した。

昨年の報告書に記載したが、#007 では、らい菌接種後3種のペプチド(LpK、MMP-II、FAP)すべてに対するリンパ球幼若化反応が二年間にわたり持続すると同時に、感染直後から休止期記憶 CD4 陽性 T 細胞と考えられる CD29high 細胞レベルが増加し、二年間高レベルを維持した。このことから、CD29high/CD4 細胞がペプチドで誘導される幼若化反応と IFNγ産生を担う細胞集団である可能性が考えられる。さらに、#007 は、唯一三年間にわたり低レベルではあるがらい菌特異抗体が検出されることから、持続感染の可能性が高い。今後はらい菌ペプチドで誘導されるリンパ球および抗原提示細胞の反応性を詳細に検討する予定である。らい菌を接種した手根部のバイオプシーによりらい菌の持続感染を確認する実験も実施し、らい菌の持続感染を成立させる接種条件として、手根部接種法の有用性を明らかにする必要がある。

E. 結論

幼若カニクイザルにらい菌を鼻腔内、鼻尖端、左手根部の異なった経路で接種し、らい菌の持続感染モデル成立の有無を抗酸菌由来ペプチドで誘導される末梢リンパ球のサイトカイン産生能を指標として調査した。その結果、IL-12 と IFNγがペプチド刺激した培養上清中に検出されたが、らい菌感染で誘導される免疫の標的エピトープとしては FAP が最も特異性の高いペプチドであることが判明した。今後は FAP で誘導される IFNγ産生量を指標として持続感染の経過を追跡調査するとともに、らい菌が持続感染している可能性の高いカニクイザルについてリンパ球及び抗原提示細胞の機能を解析し、ヒト患者の免疫応答との類似性を明らかにしてゆく必要がある。

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G. 知的所有権の出願・登録状況

なし

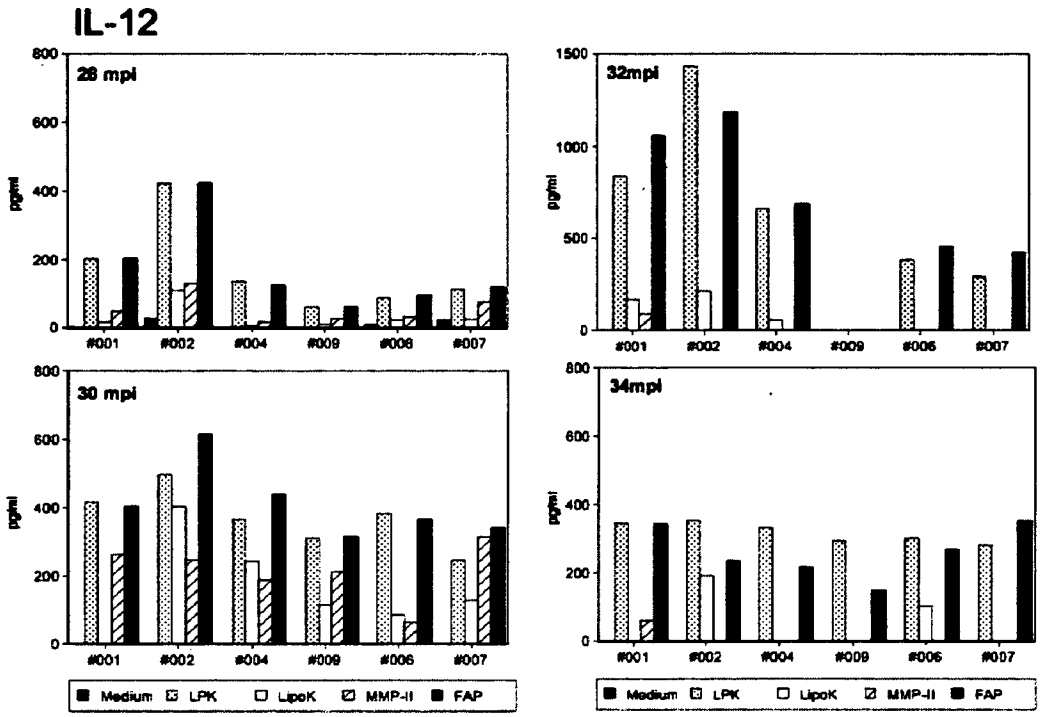

図 1：らい菌を接種した 6 頭のカニクイザルから接種後 28、30、32、34 ヶ月目に分離した末梢リンパ球を 4 種の抗酸菌由来ペプチドで刺激して培養上清中に産生された IL-12 量

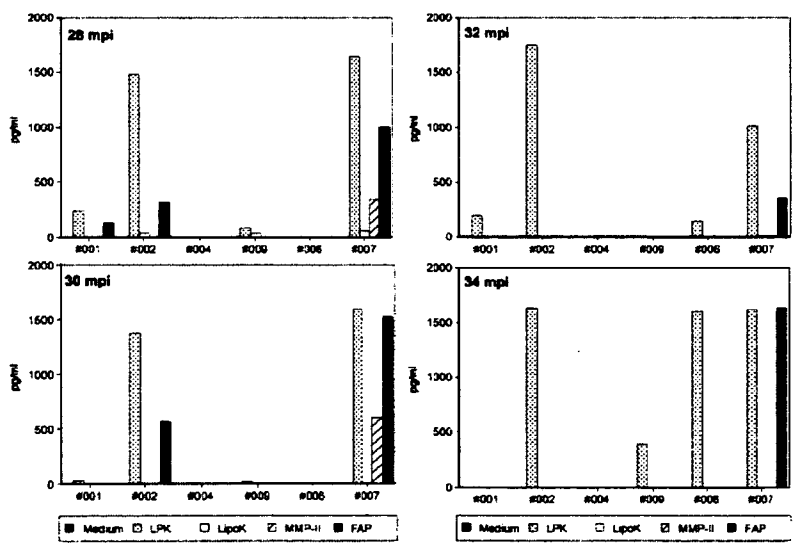

図 2：らい菌を接種した 6 頭のカニクイザルから接種後 28、30、32、34 ヶ月目に分離した末梢リンパ球を 4 種の抗酸菌由来ペプチドで刺激して培養上清中に産生された IFNγ 量

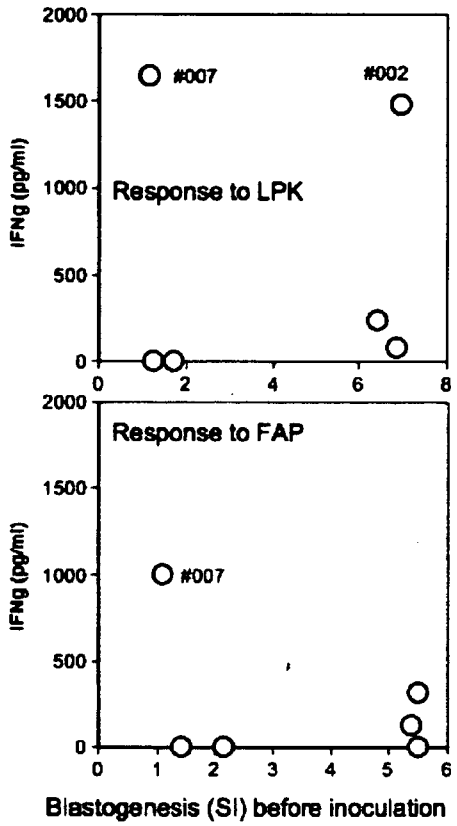

図 3：らい菌接種前のリンパ球を LPK および FAP で刺激した場合のリンパ球幼若化反応（SI: Stimulation Index；X 軸）と感染後 28 ヶ月目のリンパ球を LPK および FAP で刺激した培養上清中の IFNγ量（Y 軸）との相関

厚生労働科学研究費補助金（新興・再興感染症研究事業）

ハンセン病診療のネットワーク構築に関する研究

平成19年度　分担研究報告書

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分担研究報告書

ハンセン病診療のネットワーク構築に関する研究

分担研究者　石井則久 国立感染症研究所ハンセン病研究センター生体防御部　部長

研究要旨:日本におけるハンセン病診療がスムースに行われるようにネットワーク構築を目指した。54名の皮膚科医・医師にハンセン病の講習会・実習（皮膚スメア検査、神経肥厚触診、病理組織検査など）を行い、ハンセン病患者・回復者の診療体制を依頼した。さらに、ハンセン病回復者が安心して一般病院を受診できるように作成したパンフレットを日本皮膚科学会、日本ハンセン病学会などで配布し、ハンセン病診療がスムースに行われるようにした。

A. 研究目的

日本におけるハンセン病診療がスムースに行われるようにネットワークの構築を目指す。

B. 研究方法

ハンセン病診療に欠けている要素を抽出し、それらを補う資料や情報を提供する。また、ハンセン病の新規患者については、実際に診療方法、検査方法を指導し、主治医がハンセン病を理解し、自ら診療可能になるようにした。

C. 研究結果

ハンセン病患者の減少のため、皮膚科医が診療する機会が殆ど無い。ハンセン病診療するにあたり、回復者の心情を理解し、皮膚スメアテスト検査実施は必須であるため、62名に対して講習会を実施した（参加者：皮膚科医51人、皮膚科以外の医師3人、回復者1人、県のハンセン病担当者3人、資料のみの参加皮膚科医4人）。ハンセン病の知識、回復者の心情、皮膚スメア検査実習、神経肥厚の触診、病理組織検査を実施し、知識・技術の伝達を行った。ハンセン病診療の座右の書として作成した「ハンセン病アトラス 診断のための指針」も配布し、当事者の他、医局員や若い皮膚科医の教育に活用するようにした。

ハンセン病回復者は、過去の偏見・差別の歴史から、なかなか一般医療機関に受診する勇気がない。一般医療機関受診のチャンスを広げるため、昨年度作成したハンセン病患者（回復者）向けパンフレットと医療者向けパンフレットを大学皮膚科、学会（日本皮膚科学会総会、日本ハンセン病学会など）、療養所（施設、ケースワーカーなど）、関係機関に配布し活用を依頼した。

2007年には11名の新規ハンセン病患者がいた。全ての患者について、主治医に対して診療及び検査の指導を行った。9名の新規患者については主治医に対して、実際の検査の実技指導、治療の指導を行い、ハンセン病を確実に診療できる体制を確立した。

D. 考察

ハンセン病患者が減少し、診療する機会が減少し、教育を受けていない、一度も診療機会がない皮膚科医が大多数を占めるようになっている。また、ハンセン病の歴史やハンセン病回復者の心情なども理解できていない。それらを解決するために、講習会を開催し、意識向上に努めた。皮膚科医は知識吸収の意欲はあり、講習会には51名の皮膚科医が参集した。さらに他の皮膚科医の教育も必要と考え、「ハンセン病アトラス」を配布した。この意欲を持続させるために、年に一回程度の継続した教育機会を設けることが必要である。

ハンセン病回復者を一般医療機関に受診させる（インテグレーション）事は難しいが、一歩でもそれに近づける努力は必要である。そのため、気軽に相談できる皮膚科医を回復者向けパンフレットに記載し、全国学会などを中心に配布した。これらの皮膚科医を起点として他の診療科などに受診できることを期待したい。

ハンセン病の新規患者は減少しているが、外国人患者については鑑別にハンセン病が入っているので、診断に迷うことは多くないようである。一方、日本人患者については、ハンセン病を鑑別に入れることは難しく、診断が遅れる場合がある。数年に１名程度は日本人新規患者も登録されることがあり、必ず鑑別に「ハンセン病」を入れることが必要である。

E. 結論

ハンセン病診療を皮膚科医が主体的に実施するためのネットワーク作りは、まだ始まったばかりであるが、皮膚科医の教育、ハンセン病回復者の一般医療機関への受診の動きを、引き続き行うことが重要である。

F. 健康危機情報

なし

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なし

研究成果の刊行に関する一覧表

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The frequency of drug resistance mutations in *Mycobacterium leprae* isolates in untreated and relapsed leprosy patients from Myanmar, Indonesia and the Philippines

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Summary

Introduction The magnitude of drug resistance in *Mycobacterium leprae* to dapsone, rifampicin, and ofloxacin was studied in three Southeast Asian countries with a high prevalence of leprosy.

Methods *M. leprae* from the skin of leprosy patients was collected in North Maluku and North Sulawesi in Indonesia, Yangon in Myanmar, and Cebu in the Philippines. Mutations in the drug resistance determining regions in the *folP1*, *rpoB*, and *gyrA* genes, which have been proven to confer resistance, were analysed. In addition, samples from 51 newly diagnosed cases and 13 patients with leprosy relapse in Cebu were submitted for susceptibility testing in the mouse footpad.

Results Of 252 isolates obtained from new cases, 3% were dapsone resistant and 2% were rifampicin resistant. In samples taken from patients with relapsed leprosy ($n = 53$), significantly more resistance mutations were detected: 15% had dapsone resistance mutations, and 8% had rifampicin resistance mutations. Two patients

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with relapsed leprosy had mutations for both dapsone and rifampicin resistance. No mutations conferring quinolone resistance were detected. No mutations were detected in the *folP1* gene of *M. leprae* isolates with a low degree of resistance to dapsone.

Discussion Detection of drug-resistant cases by mutation detection in the drug resistance determining region of the genome is a practical method for monitoring resistance. A comparison of the results obtained in this study with previous data obtained prior to the use of multidrug therapy (MDT), does not indicate clearly whether the magnitude of drug resistance has changed. Larger studies of resistance mutations in *M. leprae* isolated from patients with relapsed leprosy are needed to confirm our results.

Conclusion We recommend monitoring the magnitude of drug resistance globally, by testing *M. leprae* DNA from relapse cases and a representative sample of new cases.

Introduction

Multidrug therapy (MDT) was introduced for leprosy control to minimise the development of drug resistance in *Mycobacterium leprae*.[1] Implementation of MDT in leprosy control markedly decreased the global prevalence of the disease during the last two decades, as expected,[2] but isolates with resistance to one or more antibiotics have been detected in many areas.[3–9] Comprehensive data on the magnitude of drug resistance is crucial to evaluate the efficacy of MDT and to maintain the effectiveness of the current leprosy control strategy; the mouse footpad method for drug susceptibility testing is not, however, applicable for large-scale surveillance of the global level of resistance, because it is cumbersome, time-consuming, and available in only a few laboratories in the world. Also, although knowledge of the drug susceptibility of the causative organism of individual patients initiating treatment may be beneficial, the footpad method is impractical for this purpose. Resistance to the anti-leprosy drugs, dapsone, rifampicin and ofloxacin, evolves by amino acid substitution at the binding sites of these drugs. The elucidation of mechanisms for resistance enables us to examine susceptibility to these drugs by a DNA-based assay of PCR-direct DNA sequencing.[4–16]

In the present study, the frequency of *M. leprae* mutations in the drug resistance determining region (DRDR) in the *folP1*, *rpoB*, and *gyrA* genes, which have been proven to confer resistance to dapsone, rifampicin, and ofloxacin, respectively, were examined. With this methodology, a large number of isolates were tested to obtain useful data for exact analysis of drug resistance levels, and the frequency of drug resistance was determined by pertinent DNA sequencing of *M. leprae* isolates from new and relapse cases in three Southeast Asian countries, namely, Indonesia, Myanmar, and the Philippines.

Materials and Methods

M. leprae from the skin of leprosy patients was collected in North Maluku and North Sulawesi in Indonesia (2000–2005), Yangon in Myanmar (2003–2005), and Cebu in the Philippines (2001–2006). The samples were obtained from patients before starting MDT (new cases), from patients treated with MDT for up to 4 months (recent cases), and from patients with relapse (defined as patients who developed new skin lesions after the completion of MDT and whose BI had increased by more than 2 log units at any site[17]).

Bacterial specimens were obtained from the skin lesions of patients by the standard slit skin smear method commonly utilised for assessment of the bacterial index (BI), using a disposable scalpel blade.[18] The material remaining on the blade after doing the smear was used for the study, the blade being soaked in 1 ml of 70% ethanol and kept in a separate vial at room temperature until analysis.

Additionally, *M. leprae* from skin biopsies from 64 patients, including 51 newly diagnosed and 13 relapse cases in Cebu, was submitted for susceptibility testing in the mouse footpad.[19] Groups of mice were infected in both hind footpads with 5000 *M. leprae* and fed continuously a diet containing either no drug, dapsone 0·01%, dapsone 0·001%, dapsone 0·0001%, or clofazimine 0·001%, while other mice received rifampicin 10–20 mg/kg/5 times weekly by gastric gavage. Six months after footpad inoculation *M. leprae* was enumerated from those footpads. Drug resistance was deemed to be present when the number of *M. leprae* exceeded 100,000 viable bacilli in drug treated mice. Sequences in the DRDR of each gene were analysed, in DNA recovered from bacilli which grew in the footpads of mice treated with dapsone.

For the analysis of drug resistance by mutation detection, the blades were sent to Japan in separate, labeled tubes and the bacilli-containing tissues were removed from the tip of the blade using a sterile toothpick. One toothpick was used for each blade to avoid cross-contamination. DNA templates were prepared using a previously described method.[20] Mutations in the *folP1*, *rpoB*, and *gyrA* genes were analysed by PCR-direct DNA sequencing. DNA fragments containing codons known to be associated with resistance for dapsone, rifampicin, and ofloxacin were amplified by nested PCR. Nested PCR was carried out using a G mixture of the FailSafe PCR System (EPICENTRE, Madison, WI, USA) in a 25 μl volume.

Primers were designed according to the sequence of *folP1* (accession No. AL583917, Gene ML0224), *rpoB* (accession No. AL583923, Gene ML1891), and *gyrA* (accession No. AL583917, Gene ML0006) of *M. leprae*. The sequences of the primers are listed in Table 1.

DNA fragments corresponding to the whole *folP1* gene of isolates found to be dapsone resistant to a low degree, were sequenced as described by Kai *et al.*[15] The PCR programme consisted of one hold cycle of 2 min at 94 °C linked to a three-step cycle of 30 s at 94 °C, and 30 s at 56 °C, and 30 s at 72 °C for 30 cycles followed by a final hold cycle of 5 min at 72 °C. PCR fragments were purified and sequenced according to the same protocol as previously described.[20]

Table 1. Sequences of oligonucleotide primers for *M. leprae*

		Primer	Sequence (5′-3′)
folP1 gene	Outer primers	folP1-F1	CTTGATCCTGACGATGCTGT
		folP1-R1	CCACCAGACACATCGTTGAC
	Inner primers	folP1-F2	GATCCTGACGATGCTGTCCAG
		folP1-R2	ACATCGTTGACGATCCGTG
rpoB gene	Outer primers	rpoB-F1	ACGCTGATCAATTATCCGTCC
		rpoB-R1	GTATTCGATCTCGTCGCTGA
	Inner primers	rpoB-F2	CTGATCAATATCCGTCCGGT
		rpoB-R2	CGACAATGAACCGATCAGAC
gyrA gene	Outer primers	gyrA-F1	ATGACTGATATCACGCTGCCA
		gyrA-R1	ATAACGCATCGCTGCCGGTGG
	Inner primers	gyrA-F2	GATGGTCTCAAACCGGTACATC
		gyrA-R2	ACCCGGCGAATTGAAATTG

Isolates with mutations at codons 53 and 55 in the *folP1* gene, at codons 407, 410, 420, 425, 427, in the *rpoB* gene, and at codon 91 in the *gyrA* gene were defined as resistant to dapsone, rifampicin, and ofloxacin, respectively. These mutations have been confirmed to confer resistance to the drug, dapsone,[5,6,8,9,15,16] rifampicin,[4–8,10,13] or quinolone[4–6,8] by the mouse footpad susceptibility test and by mutation detection in the DRDR for each drug. Frequencies of resistance were compared by the Fisher's exact test.

The study was approved by the institutional ethics committee of the National Institute of Infectious Diseases, Japan, and the three local institutional review boards. Informed consent was obtained prior to the collection of bacterial samples.

Results

Biopsies and slit skin smears were analysed from 121 new or recent cases and 10 relapse cases from Indonesia, 54 new or recent cases and 24 relapse cases from Myanmar, and 77 new or recent cases and 19 relapse cases from Cebu. All newly detected cases were treated with WHO MDT.[21] Almost all patients in Indonesia who relapsed were retreated with same WHO regimen. The MB patients who relapsed in Cebu, were retreated with monthly doses of rifampicin 600 mg, ofloxacin 400 mg and minocycline 100 mg for a total of 12 doses. In Myanmar, when susceptibility test results were known after relapse, patients with susceptible bacilli were treated with same WHO regimen. If dapsone resistance was found, patients were treated with clofazimine 300 mg monthly, clofazimine 50 mg daily, and rifampicin 600 mg monthly for one year. In general, these patients have responded well to the alternative treatment, although final follow-up details are not yet available.

The frequency of drug resistance to the three antibiotics studied varied between countries, and between new and relapse cases (Table 2).

In Indonesia, dapsone resistance mutations was found in 1/121 (1%) new and recent cases and 1/10 (10%) relapse cases; in Myanmar, in 4/54 (7%) new and recent cases and 2/24 (8%) relapse cases; and in the Philippines in 2/77 (3%) new cases and 5/19 (26%) relapse cases. In Indonesia, 4/121 (3%) of *M. leprae* isolates from new and recent cases were found to have rifampicin resistant mutations, while 2/10 (20%) relapse cases were found to have rifampicin resistant mutations. In Myanmar, 1/54 (2%) *M. leprae* isolates from new and recent cases were found to have rifampicin resistance mutations, while isolates from 2/24 (8%) relapse cases had rifampicin resistance mutations. In the Philippines, 0/77 (0%) *M. leprae* from new and recent cases had rifampicin resistance mutations and 0/19 (0%) relapse cases had

Table 2. Prevalence of drug resistance in *M. leprae* isolates from Asian countries

New or recent case			Relapse case		
Dapsone	Rifampicin	Ofloxacin	Dapsone	Rifampicin	Ofloxacin
Indonesia (North Maluku and North Sulawesi)					
1/121 (0·8%)	4/121 (3·3%)	ND	1/10 (10%)	2/10 (20%)	ND
Myanmar (Yangon)					
4/54 (7·2%)	1/54 (1·8%)	0/54	2/24 (8·3%)	2/24 (8·3%)	0/24
Philippines (Cebu)					
2/77 (2·6%)	0/77	0/77	5/19 (26%)	0/19	0/19

rifampicin resistance mutations. The frequency of resistance mutations for both dapsone and rifampicin was consistently higher in patients with leprosy relapse than in new cases, in each of the areas studied. In fact, the frequency of both dapsone and rifampicin resistance mutations was significantly higher in the full cohort of relapse cases than in new and recent cases, $P < 0{\cdot}001$ and $P < 0{\cdot}05$ respectively. Ofloxacin resistance was not evaluated in patients in Indonesia, and was found in no new cases (131) or patients with relapse (43) in Myanmar or the Philippines.

Dapsone resistance mutations in isolates in new or recent cases were detected in all three areas. Four isolates with rifampicin resistance mutations were detected in Indonesia and one in Myanmar, among new or recent cases. An isolate with dapsone resistance mutations was found in an Indonesian patient treated for 2 months. Of four patients with dapsone resistance mutations in Myanmar, three were collected before the start of MDT, and one was from a patient treated for 2 months. Two isolates with dapsone resistance mutations were obtained from patients in Cebu before starting MDT. Of four new or recent cases in Indonesia with rifampicin resistance mutations, one sample was obtained before the start of MDT, two were from patients treated for 2 months, and one was from a patient treated for 4 months with MDT. One isolate from a newly diagnosed case in Myanmar had rifampicin resistance mutations. One isolate in Myanmar and another in Indonesia, both from patients with relapse, had both dapsone and rifampicin resistance mutations. No Multidrug resistance was detected other than these two cases, in all three areas.

The mutations detected were as follows. Mutations in the *folP1* gene included one case of ACC to GTC (Thr → Val) and one case ACC to AGA (Thr → Arg) at codon 53; seven cases of CCC to CTC (Pro → Leu), two cases to TCC (Pro → Ser), two cases to CGC (Pro → Arg), and two cases to CGT (Pro → Arg) at codon 55. Mutations in the *rpoB* gene included one case of GAT to TAT (Asp → Tyr) at codon 410, one case of CAC to GAC (His → Asp) at codon 420; six cases of TCG to TTG (Ser → Leu) and one case of TCG to ATG (Ser → Met) at codon 425. The high frequency of the mutation TCG to TTG at codon 425 is the same result as previously observed in other areas.[10,12] No mutation was demonstrated in the *gyrA* gene of isolates from any area.

Of 64 isolates tested by the mouse footpad method, one isolate had dapsone resistance mutations to a high degree (HD), two had dapsone resistance mutations to an intermediate degree (ID), and 5 had dapsone resistance mutations to a low degree (LD) (Table 3).

Table 3. The results of susceptibility testing for dapsone by the mouse footpad method and sequencing of the *folP1* gene in *M. leprae*

	Mutation			Mouse footpad method results			
Isolate	DHPS substitution	*folP1* mutation	Degree of resistance	0·0001%	0·001%	0·01%	Controls
01Mat02	Thr53Val	ACC53GTC	High	5/5	5/5	5/5	5/5
NCR	Thr53Arg	ACC53AGA	Intermediate	5/5	6/6	0/6	7/7
02Mat47	Pro55Leu	CCC55CTC	Intermediate	5/6	4/4	0/5	6/6
01Mat01	None	None	Low	5/5	0/3	0/6	3/6
01Mat03	None	None	Low	5/8	0/7	0/7	8/8
02Mat25	None	None	Low	4/5	0/5	0/5	5/5
EER	None	None	Low	5/7	0/5	0/8	11/11
MMR	None	None	Low	3/5	0/6	0/6	6/6

The mutation ACC to GTC at codon 53 in the *folP1* gene was detected in the HD isolate, while mutations ACC to AGA at codon 53, and CCC to CTC at codon 55 were detected in the ID isolates. No mutation was demonstrated at either codon in the *folP1* gene of the five isolates with LD in Cebu.

Discussion

The proportion of isolates with dapsone resistance mutations among new and recent cases was 0·8%, 7·2% and 2·6% in Indonesia, Myanmar and the Philippines, respectively. These frequencies of primary dapsone resistance, though of some concern in Myanmar, are generally low, as previously found in San Francisco[22] and the Philippines,[23] and are far lower than the almost 1/3 of cases found in Louisiana,[24] Ethiopia[25] and later in the Philippines,[26] the latter groups being almost entirely LD resistance without known mutation of the *folP1* gene. While the number of patients with leprosy relapse assessed for dapsone resistance in this study was small, fully 8/53 (15%) were found to harbour dapsone-resistant genes. Though this frequency is high, except for the two relapse cases with both dapsone and rifampicin resistance, the reinstitution of WHO MDT, containing the only bactericidal agent in that regimen, rifampicin, as well as clofazimine, currently recommended by the WHO for leprosy relapse following MDT,[21] would likely prove effective. It is unclear whether or not the frequency of dapsone resistance has declined since the wide implementation of MDT, since prior to that time the majority of patients with isolates with dapsone resistance mutations harboured LD strains in many areas[24–26] for which there is no identifiable mutation in the *folP1* gene.

Dapsone resistance in *M. leprae* is known to be the result of specific mutations in codons 53 and 55 within the *folP1* gene coding dihydropteorate synthase (DHPS).[5,6,8,9,15,16] Cambau *et al.* showed that of 10 HD or ID isolates with dapsone resistance mutations, 9 isolates harboured mutations at codon 53 or 55, while one ID isolate showed no mutation in the *folP1* gene.[9] Of 6 LD dapsone resistant isolates, five isolates showed no mutation and one showed a mutation at codon 53.[9] No mutation was detected in 22 susceptible isolates.[9] In other studies, all 15 HD isolates with dapsone resistance mutations showed mutations at codon 53 or 55, while 7 susceptible strains showed no mutation in the *folP1* gene.[5,6,8,15,16] Five LD isolates from the Philippines in our study harboured no mutation in the *folP1* gene. These were all resistant in the mouse foot pad to 0·0001% dapsone in the diet, but not to higher levels. Therefore, almost all isolates identified as dapsone resistance by mutation detection are resistant to dapsone to a high or intermediate degree. However almost no low degree isolates with dapsone resistance mutations could be detected as resistant by mutation detection. Though Shepard[27] found that *M. leprae* obtained from untreated leprosy patients in an earlier era were consistently inhibited by 0·0001% dapsone in diet, Rees[28] found a few were not inhibited at that level. The finding that isolates in the mouse with resistance to dapsone at a concentration of 0·0001% is not associated with a mutation in the *folP1* gene suggests perhaps that resistance to that level of dapsone is found at the far extreme of the dapsone-sensitive *M. leprae* distribution. This concept is important as the vast majority of previously identified dapsone resistance, both primary and secondary, was found resistant only to this level and not higher ones. In any event, it is considered that such cases have no clinical significance, since administration of 0·0001 g DDS per 100 g mouse diet is of the same order as that observed in humans receiving 1 mg DDS daily[29] and the usual dosage of DDS in MDT is 100 mg daily.

As all isolates with HD or ID isolates with dapsone resistance mutations exhibited mutations at codon 53 or 55, the PCR direct sequencing method will detect all clinically significant dapsone resistant cases and the method is feasible for detecting isolates with dapsone resistance mutations.

Although two patients with dapsone resistance mutations among new or recent cases were treated for 2 months with MDT, they can be classified as primary dapsone resistant cases, since the multiplication of the bacilli is very slow. Resistant strains could not replace susceptible strains in the patient within such a short time.

A striking finding of the study is the detection of isolates with rifampicin resistance mutations amongst patients newly or recently detected and a greater frequency amongst relapse cases. Though in the areas studied the rate of primary rifampicin resistance, 2%, is reasonably low, the rate in patients with leprosy relapse, 8%, is of concern, as well as the two relapse cases who were resistant to both dapsone and rifampicin. In these two instances, retreatment with WHO MDT[21] would need to be prolonged for the improvement of condition since MDT for these cases is monotherapy with clofazimine. Though the number of cases with leprosy relapse in this study is small and as previously mentioned, rifampicin is the key and sole bactericidal component of MDT, perhaps reconsideration of an alternative treatment for those who relapse after MDT is in order; this might reasonably include minocycline[30] and moxifloxacin.[31] Larger studies of rifampicin resistance mutations in relapse cases are needed to ascertain if our current results are generally representative.

Rifampicin resistance is conferred by mutations in the beta subunit of RNA polymerase coded by the *rpoB* gene. Mutations at codon 407, 410, 420, 425 and insertions between 408 and 409 have been confirmed as associated with rifampicin resistance.[4–8,10,11,13,14] Mutations at codons 401,[7] 416,[32] and 427[5,7] have also been found but it has not been revealed clearly whether these mutations confer rifampicin resistance in *M. leprae*. Although mutations at 401 were detected in the *rpoB* gene of rifampicin resistance isolates,[7] it is not proven whether this mutation is associated with rifampicin resistance or not, since the mutation occurred simultaneously with a mutation at codon 420 which is known to be associated with rifampicin resistance. Mutations at 416 were also detected but no confirmatory data from the mouse foot pad susceptibility tests were available,[32] although it is known that this mutation confers rifampicin resistance in *Mycobacterium tuberculosis*.[31] A mutation at codon 427 was detected in one clinical isolate[5] and one rifampicin resistant isolate.[7] The former case was not confirmed by the mouse footpad method and the latter one was detected concordantly with a mutation at 425, although the mutation at this position is known to be associated with rifampicin resistance in *Mycobacterium tuberculosis*.[33] Clarification of the association between these mutations and rifampicin resistance by the mouse footpad method is highly recommended. In studies so far reported on the association of rifampicin resistance with mutations in the *rpoB* gene, only one rifampicin resistant isolate showed no mutation.[5] Taking these results into consideration, detecting rifampicin resistant cases by mutation detection in the *rpoB* gene is a practical method for monitoring resistance to rifampicin.

The prevalence of rifampicin resistance mutations in cases with leprosy relapse was higher than the incidence in new cases, so this also must be monitored carefully. A previous study showed that among a total of 404 multibacillary patients who had been treated with various rifampicin containing regimens, 22 (5·4%) were resistant to rifampicin.[34] Although a small sample size, the prevalence of rifampicin resistance mutations in Indonesia and Myanmar is higher than that found previously, before MDT was implemented. Two possible

reasons for a high prevalence of drug resistance are poor compliance, both with self-administration of drugs and premature discontinuation of therapy,[35] and prior monotherapy with rifampicin, either for leprosy or as part of the standard chemotherapy for tuberculosis which would also expose leprosy patients to rifampicin monotherapy.

Of the two patients with leprosy relapse with doubly resistant mutations (dapsone and rifampicin), the one from Myanmar had previously received monotherapy with dapsone (1973–1977), followed by monotherapy with rifampicin (1982–1986).[36] Of the five patients with isolates with dapsone resistance mutations in the Philippines, three had received prior dapsone, one as monotherapy, and two others either as the sole agent or in one instance combined with clofazimine and in another combined with clofazimine and rifampicin. Though the other relapse patients in the Philippines, as well as those from Indonesia and Myanmar, were treated with WHO MDT, it is unclear whether patients had adhered to the regimen and completed therapy.

Five isolates with ofloxacin resistance mutations have been reported,[4–8] all isolates having the mutation GCA to GTA (Ala → Val) at codon 91 (numbering system as used for *M. leprae*) in *gyrA* gene. A strain with the mutation GGC to TGC (Gly → Cys) at codon 89 was reported previously,[5] but resistance to ofloxacin was not confirmed by the mouse footpad method. Two other amino acid changes, Ser at 91, and Asp at 94 (numbering system as used for *M. tuberculosis*), in the *gyrA* gene of *M. tuberculosis* are associated with quinolone resistance.[37] It seems mutations at the codons 89, 92, and 95 in the *gyrA* gene of *M. leprae* also cause quinolone resistance. No mutation at these codons, 89, 91, 92, and 95, was detected in the samples tested. Thus the level of quinolone resistance in the areas investigated is still very low.

The study indicated the existence of primary and secondary resistance to dapsone and rifampicin in countries where many leprosy cases are still detected. A comparison of the results obtained in this study with previous data obtained prior to the use of MDT, does not indicate clearly whether the magnitude of drug resistance has changed.[23,26,33] We consider this study as a first effort to assess the magnitude of drug resistance in the MDT era. In order to preserve the efficacy of MDT and prevent the spread of drug resistant bacilli, carefully designed global studies are recommended, as suggested previously.[38] Monitoring the susceptibility of isolates from each case of leprosy relapse allows optimal treatment to be chosen, by avoiding ineffective drugs and choosing effective compounds. The longitudinal surveillance of levels of drug resistance in new cases in some areas with a high prevalence of leprosy might contribute to predicting the spread of drug resistant strains. The application of the susceptibility test by mutation detection should be attempted, especially in cases where treatment failure seems a possibility.

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