Dapsone resistance in *Mycobacterium leprae*

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Summary The *folP*1 gene of *Mycobacterium leprae*, which encodes dihydropteroate synthase (DHPS), was studied for the presence of mutations associated with resistance to dapsone (DDS). When the *folP*1 of several DDS-resistant clinical isolates of *M. leprae* were sequenced, two missense mutations were identified. One mutation occurred at codon 53, substituting isoleucine for threonine in DHPS-1, and a second mutation occurred in codon 55, substituting arginine for proline. DNA sequencing of strains of *M. leprae* resistant to 0·01 g% DDS in the mouse diet revealed that 13 of 14 strains contained either the 53 or 55 *folP*1 mutation. None of the susceptible strains and only one of five strains resistant to 0·001 g% DDS revealed a mutation in *folP*1, suggesting that only high-level DDS resistance is associated with the mutations identified in *folP*1. Development and application of simple molecular tests to assess drug-related mutations in *M. leprae* could establish current levels of drug resistance in leprosy as a reference point for future monitoring of drug resistance at the global level.

Introduction

In this era of declining leprosy prevalence, control measures could be in jeopardy. Potential threats that could undermine current gains toward the global elimination of leprosy are both operational and technical. Operationally, maintenance of dedicated personnel and expertise in diagnosis and treatment of leprosy may become more difficult as vertical programs become integrated into general health systems. Technical issues remaining unsolved or poorly understood in the struggle to control leprosy globally include the lack of a highly effective vaccine, the rise of immunodeficiency diseases in populations harbouring leprosy, and the potential for emergence of drug-resistant strains of *Mycobacterium leprae*.

Resistance to dapsone (DDS) was the first indication that new drug therapies were needed.[1,2] The multidrug therapy (MDT) regimen recommended by the World Health Organization (WHO) for the treatment of multibacillary leprosy was designed to prevent emergence of drug resistance, while providing shortened and affordable treatment schedules required in developing countries. Now, despite our having implemented MDT for more than 15 years in various parts of the world, we do not have reliable measurements of drug resistance in the remaining pool of *M. leprae*.

Recent advances in the elucidation of molecular events responsible for drug resistance in mycobacteria have allowed the development of new tools for screening for drug

resistance,[3–5] and application of these tools has revealed the existence of both monoresistant[6] and multidrug-resistant[7] strains of *M. leprae*. Recently, point mutations in the putative *M. leprae* gene for dihydropteroate synthase (*folP*) have been identified in DDS-resistant strains of *M. leprae*,[8,9] and definitive evidence linking these mutations with DDS resistance and proof of enzymatic activity of the putative dihydropteroate synthase (DHPS) of *M. leprae* have been obtained.[10] A more complete understanding of the mechanism of action of DDS and the modes of resistance available to *M. leprae* should facilitate the development of new tools for monitoring DDS resistance, and initiate investigations into new strategies to circumvent this phenomenon. In this study we extend our earlier findings with respect to mutations associated with DDS resistance, and discuss the need for developing and implementing new molecular tests for screening *M. leprae* for drug resistance.

Materials and methods

BACTERIAL STRAINS

DDS-resistant and susceptible strains of *M. leprae* were obtained originally from leprosy patients from: the Anandaban Leprosy Hospital, Kathmandu, Nepal; Leprosy Research Center, National Institute of Infectious Disease, Tokyo, Japan; Schieffelin Leprosy Research and Training Center, Karigiri, India; and G. W. Long Hansen's Disease Center, Carville, LA (see Table 1). Resistance to DDS was determined in the mouse footpad system by Shepard's kinetic method,[11] and all dapsone-resistant strains were propagated thereafter in the footpads of BALB/c mice fed mouse chow containing appropriate concentrations of DDS *ad libitum*. DDS-resistant strains grew in footpads of mice administered DDS in a concentration of either 0·001 or 0·01 g per 100 g mouse chow. These concentrations represent 10-fold and 100-fold, respectively, the minimal effective dose (MED) for susceptible strains of *M. leprae*. Thai-53, a DDS-susceptible strain of *M. leprae*, was the kind gift of Dr M. Matsuoka, Leprosy Research Center, Tokyo, Japan.

Bacterial DNA was harvested from ethanol-fixed tissues containing *M. leprae* following rehydration for 60 min in 10 mM Tris, 1 mM EDTA buffer, pH 7·4 (TE). The rehydrated tissue was minced with scissors to a gelatinous consistency, resuspended in 0·3 ml TE buffer, frozen in liquid N_2, and thawed at 95°C, after which the freezing/thawing treatment was repeated two more times. The tissue was then digested for 18 h at 60°C with proteinase K (2·5 mg per ml) in 100 mM Tris, 150 mM NaCl, 10 mM EDTA, pH 7·4 digestion buffer. Proteinase K was heat-inactivated at 95°C for 10 min, and DNA was extracted by phenol/chloroform/isoamyl alcohol as described previously.[12] The precipitated DNA was resuspended in 30 μl TE buffer.

DNA SEQUENCING OF DDS RESISTANT AND SUSCEPTIBLE STRAINS OF *M. LEPRAE*

The entire *folP*1 was amplified by PCR from DNA preparations of DDS-susceptible and resistant strains of *M. leprae* with the aid of folP1-1 and folP1-2 primer sets.[10] PCR fragments were purified, and the DNA sequence of *folP*1 was obtained using folP1-1, folP1-2, folP1-9 and folP1-20 primers.[10] The sequence of *folP*1 from each strain was compared to that of a DDS-susceptible strain of *M. leprae* found in the Sanger Centre database, Cambridge, England (www.sanger.ac.uk).

Table 1. Mutations in *M. leprae folP*1 associated with DDS resistance

Strain[a]	DDS susceptibility[b]	Origin	Codon[c]
2262	≥0·01	USA	Thr53Ala
CDC-DDS-1	≥0·01	USA	Thr53Ala
61–00	≥0·01	India	Thr53Ala
India 2	≥0·01	India	Thr53Arg
63–00	≥0·01	India	Thr53Arg
2898	≥0·01	USA	Thr53Ile
Airaku-3	≥0·01	Japan	Thr53Ile
Zensho-4	≥0·01	Japan	Thr53Ile
591	≥0·01	Nepal	Pro55Arg
India 4	≥0·01	India	pro55Arg
62–00	≥0·01	India	Pro55Arg
64–00	≥0·01	India	Pro55Leu
Zensho-2	≥0·01	Japan	Pro55Leu
Airaku-2	≥0·001, <0·01	Japan	Pro55Leu
H101	≥0·001, <0·01	USA	None
H103	≥0·001, <0·01	USA	None
H107	≥0·001, <0·01	USA	None
NIH-1	≥0·001, <0·01	USA	None
Thai-53	<0·0001	Thailand	None
19-F-1	<0·0001	USA	None
H105	<0·0001	USA	None

[a] Designation of strain of *M. leprae*.

[b] Susceptibility to DDS, determined in the mouse footpad system, in terms of the minimal concentration of DDS in the mouse chow that inhibits multiplication of *M. leprae*. Strains designated as <0·0001% are fully susceptible to DDS; those designated as ≥0·01% are fully resistant, whereas those designated ≥0·001, <0·01% are moderately resistant.

[c] Designation of the mutation in *folP*1 associated with dapsone resistance.

Results and discussion

The dihydropteroate synthase (DHPS) of *M. leprae* is encoded by *folP*1, which is located in an operon encoding three other genes (*folE*, *folB* and *folK*) putatively involved in folate biosynthesis (see Figure 1). The amino acid sequence of the DHPS of *M. leprae*, deduced from the DNA-sequence of *fol*P1, identified a protein of approximately 31 kDa molecular weight, with two signature regions (PS00792 and PS00793) associated with *folP* enzymes from diverse bacterial species, including *Bacillus subtilis* and *Neisseria meningitides*.[10] The second signature motif is the region in which mutations have been shown to induce resistance to sulphonamide and sulphone compounds in bacteria, including *M. leprae*.[10,13,14] Mutations at amino-acid residues 53 and 55 in this region of *M. leprae folP*1 have been shown to be associated with DDS resistance, and to confer increased resistance to DDS by recombinant *Escherichia coli* that express only mutant DHPS obtained from *M. leprae*.[10]

Further analysis of DDS-resistant strains of *M. leprae* has confirmed our earlier findings, showing mutations in codons representing amino acids 53 (threonine) and 55 (proline) of the DHPS of *M. leprae*, as shown in Table 1. Missense mutations found in codon 53, resulting in a substitution of alanine, arginine or isoleucine for threonine, were found only in DDS-resistant strains that multiplied in mice administered DDS in 100-fold the MED.

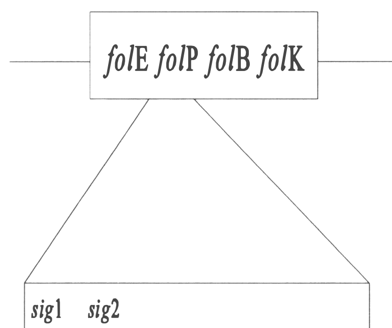

Figure 1. Schematic drawing of the segment of *M. leprae* cosmid MLCB 2548 that contains the putative folate operon. The expanded region of the operon shows the signature regions 1 and 2 of dihydropteroate synthase. Signature region 2 encompasses amino acids 53 and 55, in which mutations were found associated with DDS resistance. Genes in the operon are: *fol*E, which encodes GTP cyclohydrolase; *fol*P, which encodes hydydropteroate synthase; *fol*B, which encodes dihydroneopterin aldolase; and *fol*K, which encodes dihydropteridine pyrophosphokinase.

Missense mutations at codon 55, resulting in substitutions of either arginine or leucine for proline, were most often identified in strains that grew at 100-fold the MED for DDS. Only one strain of DDS-resistant *M. leprae*, which multiplied in mice administered DDS in 10-fold the MED, contained a missense mutation at codon 55. All other strains resistant at 10-fold the MED showed the fully susceptible genotype. Although analysis of these strains is as yet incomplete, it is interesting to speculate why most of these phenotypes do not have mutations in *folP*1. In the pre-MDT era, clinical experience showed that patients infected with strains of *M. leprae* resistant to low and moderate levels of DDS responded to DDS monotherapy. It may be that low and moderate levels of DDS resistance are artefacts of the mouse footpad assay, and lack clinical significance. Alternatively, these low and moderately resistant strains may represent 'mutants' that foster the development of high-level resistance upon selection of the *folP*1 mutations identified above. Finally, the significance of these strains in terms of 'real' DDS resistance remains to be elucidated.

That the resistant strains analysed in this study originated from at least three different parts of the world suggests that DDS resistance exists in many areas of the world. The most recent surveys for DDS resistance were done by de la Cruz *et al.*[15] and Butlin *et al.*[16] In both instances, treatment of the patient population with MDT had been in place for 10–12 years, despite which significant levels of high-level DDS resistance was observed. In Cebu, the Philippines, the frequency of DDS resistance at all three levels has been increasing over the last 20 years.

In addition to DDS-resistant strains of *M. leprae*, monoresistant strains resistant to rifampicin have been identified.[17] In most cases, these were the result of drug-trials in which rifampicin was administered as monotherapy. A recent report has also identified a multidrug-resistant strain of *M. leprae* resistant to DDS, rifampin, and ofloxacin.[7] These strains were selected under pressure from drugs now in common use for treating leprosy. As drug regimens continue to be shortened in an attempt to find the minimal duration of chemotherapy that is capable both of effecting cure and preventing the selection of drug-resistant mutants, we must remain alert to the possibility of emerging drug resistance in leprosy. New molecular tests based on mutations associated with drug resistance are needed to permit simplified monitoring in many regions of the world. This is especially important as the number of

laboratories carrying out the mouse footpad technique is diminishing around the world, and an atmosphere of complacency surrounds leprosy control programmes as prevalence falls.

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DISCUSSION

Professor Britton: What is the effect of introducing mutations at both positions 53 and 55 *in vitro*?

Dr Gillis: Resistance is increased some 50-fold, in terms both of enzyme-kinetics and of multiplication of the organisms.

Dr Ginsberg: I don't understand why your data are inconsistent with the notion of step-wise resistance. Couldn't the mutation at position 53 or 55 be the last of a series of mutations required to confer high-level resistance?

Dr Gillis: There are no other mutations in this gene. If there are other mutations, we don't know where to search for them. Perhaps there are mutations in genes related to other pathways; for example, there is some evidence that PABA is overproduced in some cases of sulphone or sulphonamide resistance.