ANTIMICROBIAL AGENTS AND RESISTANCE

Identification of catalase-like activity from *Mycobacterium leprae* and the relationship between catalase and isonicotinic acid hydrazide (INH)

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As *Mycobacterium leprae* proliferate inside macrophages, it has been speculated that catalase encoded by *katG* may protect the bacilli from deleterious effects of peroxide generated from the macrophage and may also play a crucial role in the survival of *M. leprae* *in vivo*. However, unlike that of *M. tuberculosis*, the *katG* of *M. leprae* has been reported to be a pseudogene, implicating that isoniazid, which is activated to a potent tuberculocidal agent by catalase, is unlikely to be of therapeutic benefit to leprosy patients. These results raise a question as to how *M. leprae* avoids H₂O₂-mediated killing inside macrophages. To understand the survival of *M. leprae* in macrophages, the present study attempted to detect catalase-like activity in *M. leprae*. Catalase-like activity was found in *M. leprae* cell lysate by the diaminobenzidine (DAB) staining method with non-denaturing polyacrylamide gel electrophoresis. An ammonium sulphate precipitation study revealed that the catalase-like activity was precipitable with 80% ammonium sulphate. The effect of isoniazid (INH) on *M. leprae* growth was also tested by RT-PCR and radiorespirometric assay to examine catalase-like activity in *M. leprae*, because INH was activated by catalase. It was found that the viability of *M. leprae* was decreased at a concentration of 20 µg/ml by radiorespirometric assay and it was inhibited at higher concentrations as determined by RT-PCR. These data suggest that a catalase-like activity other than that encoded by *katG* is present in *M. leprae*.

Introduction

In order to survive and proliferate, intracellular pathogens have defence mechanisms to deal with microbial killing mechanisms of host cells. Several mechanisms for resistance to intracellular killing have been proposed, including the scavenging of free radicals produced by mononuclear phagocytes. One protein with known free-radical scavenging activities, catalase (KatG), metabolises H₂O₂, a toxic oxygen metabolite [1]. Thus, the presence of catalase in mycobacteria probably enhances their pathogenicity, as it has been reported that *Escherichia coli* becomes resistant to killing by H₂O₂ by the expression of a catalase gene.

Isoniazid (isonicotinic acid hydrazide, INH) is one of the principal antituberculosis drugs. Catalase activates INH to a toxic oxidised derivative, which inhibits the biosynthesis of cell wall mycolic acids by linkage to other enzymes of the mycolic acids biosynthetic pathway [2].

Although several genes (*inhA*, *katG* and *oxyR*) are involved in the mechanism of INH resistance in mycobacteria, the development of resistance in *M. tuberculosis* is often associated with a loss of catalase-peroxidase activity through mutations in the sequence of its *katG* gene [3, 4]. To gain more insight into the mechanism of action of INH, attempts were made to isolate and characterise catalase from *M. leprae*, as some workers have reported that the organism is susceptible to INH and the drug was used in the 1960s and 1970s to treat leprosy [5–7]. However, due to the unacceptable incidence of hepatic toxicity, INH was not recommended. Recently, INH was used with rifampicin in the treatment of borderline tuberculoid

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leprosy patients, resulting in the regression of nerve abscesses [8].

The *katG* gene of *M. leprae* is thought to be a pseudogene, an inactive, non-functional gene, unlike that of *M. tuberculosis*, because it contains multiple deletions. This implies that isoniazid is unlikely to be of therapeutic benefit to leprosy patients [9].

These contradictory observations raise a question about how *M. leprae* avoids H₂O₂-mediated killing; they suggest that there is a protein that confers protection against H₂O₂-mediated damage even in the absence of adequate catalase activity.

To investigate catalase-like activity in *M. leprae*, this study examined protein preparations by non-denaturing gel electrophoresis and diaminobenzidine (DAB) staining [10]. It also tested the effect of INH on *M. leprae*.

Materials and methods

Cell lysate preparation, non-denaturing gel electrophoresis and biochemical assay

Bacterial strains used for these studies were as follows: *M. leprae* Thai53, *M. smegmatis* mc²155 and *E. coli*. *M. leprae* was obtained from infected nude mouse footpads by the methods described by Nakata *et al.* [11]. *M. leprae* bacilli from host tissues were purified at 4°C. Mouse footpads were minced and homogenised in phosphate-buffered saline (Sigma) and centrifuged at 100 *g* for 10 min to remove host cell components and the supernate was then centrifuged at 3500 *g* for 20 min. The bacteria were enumerated by the procedure of Shepard and McRae [12]. *M. smegmatis* mc²155 and *E. coli* were grown in M7H9 broth (Difco) containing Tween 80 20% and LB broth, respectively. The bacteria were harvested and washed with PBS. The resulting pellet consisted of purified bacterial cells.

Bacteria were suspended in sonication buffer (50 mM Tris-HCl, 10 mM MgCl₂, sodium azide 0.02%, pH 7.4) and treated ultrasonically for 10 min at 30 W for *E. coli* and *M. smegmatis* mc²155, and for 45 min at 75 W for *M. leprae* with a Sonifier 250 (Branson Ultrasonic, USA) in an ice-water bath. The sonicated materials were centrifuged at 12 000 *g* for 30 min and supernates were stored at -20°C as cell lysates. Completeness of destruction was confirmed by microscopic examination of the pellet. Protein concentrations were estimated by the method of Bradford with bovine serum albumin fraction V as the standard.

Catalase activity was determined by monitoring the decomposition of H₂O₂ at 240 nm with a spectrophotometer (Hewlett Packard, Palo Alto, CA, USA) by a modification of the method described by Beers and Sizer [13]. Then 300 µl of 50 mM potassium phosphate buffer (Sigma) and 290 µl of H₂O₂ solution (Sigma)

0.036% were pipetted into a cuvette; 100 µl of buffered bacterial lysates were added to the cuvette and mixed by pipetting. The time to required for the absorbance at 240 nm (A₂₄₀) to decrease from 0.45 to 0.40 absorbance units was recorded.

Catalase activity was also determined by the DAB staining method [10]. Non-denaturing gel electrophoresis of crude lysate (40 µg) combined with loading buffer, containing neither SDS nor mercaptoethanol, was performed in a mini-gel system (Hoefer, Mighty Small II, San Francisco, CA, USA) at 150 V. After completion of electrophoresis, the gel was stained in 3 ml of diaminobenzidine (Sigma) 0.5 mg/ml and 3 ml of horseradish peroxidase (Sigma) 50 µg/ml in 50 mM potassium phosphate buffer 50 ml, pH 7.0, at 23°C, for 45 min. The gel was then rinsed and soaked in H₂O₂ 0.006% in the phosphate buffer until staining was completed.

Ammonium sulphate precipitation

M. leprae lysate (1 mg/ml) was combined with (NH₄)₂SO₄ 0.113 g to make 1 ml of *M. leprae* lysate (20% saturation). When the ammonium sulphate (Sigma) was completely dissolved, the suspension was centrifuged at 10 500 *g* for 20 min at 4°C. Further amounts of (NH₄)₂SO₄ sulphate were added to the resulting supernates as follows: 0.121 g (40% saturation), 0.130 g (60% saturation), 0.140 g (80% saturation), 0.152 g (100% saturation), respectively. The protein precipitate was collected by centrifugation (10 500 *g*, 20 min, 4°C) and resuspended with 50 mM phosphate buffer. Each of the fractions was used to determine catalase activity by the DAB staining method.

Macrophage preparation, infection and drug treatment

Peritoneal macrophages were harvested from BALB/c mice as described previously [14]. Briefly, cells were collected by lavage with 10 ml of cold DPBS containing heparin (Sigma) 10 U/ml, washed twice by centrifugation at 150 *g* for 10 min at 4°C, and suspended in RPMI 1640 (Sigma) containing heat-inactivated fetal bovine serum (Hyclone Lab, Logan, UT, USA) 10% and gentamicin (Sigma) 25 µg/ml. Cell viability was consistently >95% in the trypan blue (Sigma) exclusion test. Peritoneal macrophages were plated in six-well tissue-culture plates (Becton Dickinson Labware, Franklin Lakes, NJ, USA) at 2 × 10⁶ cells/well and allowed to adhere for 2 h at 37°C in a humidified atmosphere containing CO₂ 5% in air. Non-adherent cells were removed by washing with fresh medium and the plates were incubated overnight. In the morning, non-adherent cells were again removed by washing. The monolayers were overlaid with 6 × 10⁷ bacilli of *M. leprae* in RPMI 1640 containing fetal bovine serum 10% adjusted to yield a bacter-

ium:macrophage ratio of 30:1 and incubated at 37°C. After 4 h, the non-phagocytosed bacteria were removed by washing with warm DPBS. Cells were treated with RPMI 1640 containing serial dilutions of INH (10–320 µg/ml; Sigma) and then incubated at 37°C.

RNA isolation

INH-treated *M. leprae* in the macrophages were placed in Trizol reagent (Sigma) for RNA isolation. After macrophages infected with *M. leprae* were homogenised and lysed with 1 ml of Trizol reagent, samples were transferred into 1.5-ml sterilised microcentrifuge tubes. After extraction with chloroform (Sigma), RNA was precipitated with isopropanol (Merck, Darmstadt, Germany) and resuspended with DEPC (Sigma)-treated water. To remove the contaminant DNA, samples were treated with DNaseI (Boehringer Mannheim, Germany) and RNAase inhibitor (Promega, Madison, WI, USA) for 1 h at 37°C.

cDNA synthesis

cDNA was synthesised as described previously [15]. RNA was transcribed into cDNA by avian myeloblastosis virus reverse transcriptase (Promega) in a 20-µl reaction volume containing 50 mM Tris-HCl, 8 mM MgCl₂, 30 mM KCl, 0.1 M dithiothreitol, 10 mM concentration of each deoxynucleoside triphosphate, 1 nM random hexamers (Sigma) and 400 U of RNAase inhibitor at 42°C for 1 h. The mixture was then heated at 95°C to inactivate the enzymes, cooled and stored at –20°C.

RT-PCR

cDNA was subjected to 40 cycles of amplification by PCR with *M. leprae*-specific primers that amplify a 360-bp segment of the 18-kDa protein gene. PCR was performed in a total volume of 10 µl of buffer obtained from Boehringer Mannheim (10 mM Tris-HCl, 1.5 mM MgCl₂, 50 mM KCl) containing 1.25 mM dNTP, 1 µM of each primer, primer 1 (5'-ATTCGTCGTCGAGTTC GACCTTCCT-3'), primer 2 (5'-CTTAGCTTGTTGCG CAAACAACAGT-3'), template 1.5 µl and AmpliTaq DNA polymerase 1 U. Negative controls, which contained all reaction components except the template, were included in all experiments to detect contamination. The cycling profile comprised denaturation at

94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 90 s for 40 cycles, followed by final extension at 72°C for 10 min. Positive control DNA was processed under the same PCR conditions. PCR products were analysed by electrophoresis in an agarose 2% gel and ethidium bromide staining.

Radiorespirometry

A 0.3-ml volume of 0.1 N NaOH (Sigma) was added to INH-treated *M. leprae* and lysed macrophages infected by *M. leprae*. *M. leprae* suspensions were added to 6-ml screw-cap vials containing 7H9 broth 1 ml and [1-¹⁴C]palmitic acid (Amersham) 1 µCi. Non-infected macrophages and cell-free media were added to replicate vials as control. Culture vials, with caps loosened, were placed within wide-mouthed liquid scintillation vials containing a 2 × 5-cm strip of Whatman filter paper (Whatman International, Maidstone, Kent) to which 500 µl of 2 N NaOH had been added. The double-vial assemblies were then incubated for 24 h at 33°C. The inner culture vials were removed, and 5 ml of cocktail solution were added to the scintillation vials containing the filter papers. The counts/min (cpm) were determined in a liquid scintillation counter (Beckman LS-6000 Series, Palo Alto, CA, USA).

Results

Catalase activity

To examine catalase-like activity in *M. leprae in vitro*, cell lysate was obtained by sonication of *M. leprae* (1 × 10⁹ bacilli/ml). Spectrophotometry for catalase activity revealed enzyme activity in the three bacterial strains tested. Catalase-like activity was determined by monitoring the decomposition of H₂O₂ at 240 nm with a spectrophotometer with bovine liver catalase as control. Catalase activity was found in sonicated *E. coli*, *M. smegmatis* and *M. leprae*, although the catalase activity of *M. leprae* was weaker than that of the other bacteria (Table 1). DAB staining of non-denaturing gel electrophoresis for catalase activity also revealed catalase-like activity (Fig. 1). Catalase activity was visible almost instantly as an achromatic band against a brown background. This method showed one band with catalase activity in *M. leprae* similar to that in *E. coli* and *M. smegmatis* mc²155. To determine

Table 1. Mobility and specific activity of catalase in bacterial lysates following non-denaturing polyacrylamide 7.5% gel electrophoresis and spectrophotometric assay

Source	Concentration (mg/ml)	Specific activity (unit/mg of protein)	Mobility (Rf)
Bovine catalase	10	20 090	0.399
Mouse catalase	10	690	0.175
<i>E. coli</i> lysate	1.25	16.8	0.892
<i>M. smegmatis</i> mc ² 155	1.26	15.3	0.901
<i>M. leprae</i>	1.2	11	0.910

Fig. 1. Non-denaturing PAGE analysis of various bacterial cell lysates in non-denaturing polyacrylamide 7.5% gels stained by DAB methods. The band in *M. leprae* was believed not to be of host tissue origin because of the difference in location between *M. leprae* and mouse band. Lane 1, bovine catalase; 2, *E. coli* cell extract; 3, *M. smegmatis* cell extract; 4, *M. leprae* cell extract; 5, mouse liver catalase.

Fig. 2. Non-denaturing PAGE analysis of $(\text{NH}_4)_2\text{SO}_4$ -fractionated *M. leprae* cell lysates. The precipitating extract of 80% $(\text{NH}_4)_2\text{SO}_4$ in *M. leprae* extract revealed one band with catalase-like activity. Lane 1, bovine liver catalase; 2, *M. leprae* cell lysate; 3, 20% fraction; 4, 40% fraction; 5, 60% fraction; 6, 80% fraction; 7, 100% fraction; 8, mouse liver catalase.

whether the band of *M. leprae* was derived from host cells, mouse liver catalase was tested as control. Catalase activity in *M. leprae* was believed not to be of host tissue origin, because of the difference in location between *M. leprae* and mouse catalase. The mobility of each of these bands and the corresponding total activity for each of the samples tested are listed in Table 1.

Ammonium sulphate precipitation

Bacterial extracts precipitated at different salt concentrations. Each precipitating extract was tested for catalase activity by the DAB staining method. The precipitating extract of $(\text{NH}_4)_2\text{SO}_4$ 80% in the *M. leprae* extract revealed one band with catalase activity (Fig. 2), in contrast to *E. coli* and *M. smegmatis* mc²155 which had bands in the 60% precipitant (data not shown).

Effects of INH on *M. leprae* analysed by RT-PCR and radiorespirometric assay

The study also tested the effect of INH on the growth of *M. leprae* by RT-PCR and radiorespirometric assay, to examine the presence of catalase-like activity in *M. leprae*. AFB/methylene blue stain and DNA-PCR, respectively, were used to determine whether *M. leprae* used in these experiments was phagocytosed efficiently and consistently (Fig. 3a). It was found that the viability of *M. leprae* was decreased depending on drug concentrations and it was inhibited at INH 160 $\mu\text{g}/\text{ml}$ as determined by RT-PCR (Fig. 3b), whereas in the radiorespirometric assay, *M. leprae*

Fig. 3. (a) DNA-PCR analysis of the effect of INH on *M. leprae*. Analysis of PCR products from 40 cycles of PCR with primers for 360-bp product; 10 μl of PCR product DNA were separated by electrophoresis through agarose 2% gel for 1 h at 80 V and stained with ethidium bromide. (b) Analysis of the effect of INH on *M. leprae* by RT-PCR: the 360-bp fragment of *M. leprae* DNA and 117-bp fragment of pLLIII DNA as control were seen on agarose 2% gel and stained with ethidium bromide. Lane 1, M Φ without *M. leprae*; 2, INH 0 $\mu\text{g}/\text{ml}$; 3, 10 $\mu\text{g}/\text{ml}$; 4, 20 $\mu\text{g}/\text{ml}$; 5, 40 $\mu\text{g}/\text{ml}$; 6, 80 $\mu\text{g}/\text{ml}$; 7, 160 $\mu\text{g}/\text{ml}$; 8, 320 $\mu\text{g}/\text{ml}$; 9, DNA ladder (100 bp).

growth was inhibited at 20 $\mu\text{g/ml}$ (Fig. 4). The result obtained by RT-PCR required higher concentration than that obtained by radiorespirometric assay. Although there is a difference between results obtained by the two methods, the viability of *M. leprae* was inhibited by INH as determined by both methods.

Discussion

Various mechanisms employed by mycobacteria to escape from the antimicrobial actions of mononuclear phagocytes have been reported. Possible mechanisms include inhibition of phagosome–lysosome fusion [16], inhibition of acidification of the phagosome [17] and scavenging or inhibition of bactericidal agent, such as reactive radicals. *M. leprae* is traditionally considered an obligate intracellular pathogen and proliferates within macrophages. The adaptation mechanisms that enable the human pathogens *M. tuberculosis* and *M. leprae* to thrive in the intracellular environment of susceptible mononuclear phagocytic cells are not completely understood, despite several recent advances. Of potential significance for the successful parasitism of host macrophages is the response of mycobacteria to oxidative stress, as reactive oxygen and nitrogen species are likely to be encountered during interactions with phagocytic cells. Previous studies have suggested that oxygen and reactive oxygen intermediates (ROI) produced by the host cells contribute to the control of mycobacterial growth and survival. ROIs include superoxide, H_2O_2 and hydroxyl radicals. Protection against ROIs is provided by antioxidant enzymes, such as superoxide dismutase, catalase and peroxidase. Superoxide dismutase was shown to be present in *M.*

leprae, but attempts to detect catalase in *M. leprae* have so far been unsuccessful [18, 19]. Catalase and peroxidase activities in *M. leprae* have been debated for a long time, since they were detected in human-derived *M. leprae* in 1967.

Recently, several workers have demonstrated that a *katG* homologue of *E. coli* is also present in *M. leprae* and described nucleotide sequence analysis of the *M. leprae* DNA region homologous to those of other bacterial *katG* gene encoding a catalase. However, as discussed previously, the *katG* gene of *M. leprae* is a pseudogene, which has probably been inactivated by multiple mutation events [11]. No characteristic of catalase-peroxidase activity could be detected in cell-free extracts of armadillo-derived *M. leprae* in biochemical studies by Wheeler and Gregory, who found weak catalase activity, which was believed to be of host tissue origin [19]. Lygren *et al.* also failed to find catalase activity in *M. leprae* [18]. In their report, unlike the other extracts, the *M. leprae* preparation had been exposed to irradiation to inactivate the bacilli. It is possible that this treatment may have inactivated a peroxidase-catalase activity. There are probably proteins and genes that confer protection against H_2O_2 -mediated damage even in the loss of catalase activity, thus promoting survival of the *M. leprae* in the environment of the phagocytic oxidative burst. To survive during infection, isoniazid-resistant *katG* mutations have apparently compensated for the loss of KatG catalase-peroxidase activity by expression of another gene. INH-resistant, KatG-negative strains over-expressed alkyl hydroperoxidase (Ahp)C to a varying extent, whereas *katG* wild-type strains produced the AhpC protein at the low wild-type levels. The strict

Fig. 4. The effect of INH on *M. leprae* as analysed by radiorespirometric assay. Radiorespirometric assay for *M. leprae* viability with the oxidation of [1-C^{14}] palmitic acid to CO_2 by *M. leprae* as measured in Buddemeyer system. The viability of *M. leprae* was decreased at a concentration of 20 $\mu\text{g/ml}$.

correlation between loss of KatG function and over-expression of AhpC has also been noted recently by others [20, 21]. AhpC is similar to a family of bacterial and eukaryotic antioxidant proteins with Ahp and thioredoxin-dependent peroxidase (Tpx) activities [22]. Although *ahpC* can be induced by peroxide and involved in isoniazid resistance of the *M. tuberculosis*, it is not clear that *ahpC* can be involved in protection of *M. leprae* from H₂O₂.

However, unlike the previous reports, the present study has shown a catalase-like activity in cell lysate of *M. leprae* and demonstrated the effect of INH on the growth of *M. leprae*. Furthermore, there is a report that isoniazid was used with rifampicin in the treatment of borderline tuberculoid leprosy patients with the result that nerve abscesses totally regressed [8]. These results suggest that a catalase-like activity is present in *M. leprae* and it was expected that Ahp and Tpx may play a key role in the survival of *M. leprae* in the presence of a high concentration of oxygen reactive intermediates produced by macrophages.

The DAB staining procedure was applied to polyacrylamide gel electrophoresis of crude cell lysates [10, 23]. It is a simple and reliable method and successfully localises catalase in 200–0.2 units/band, so it was possible to detect *M. leprae* catalase activity that was weaker than that of other bacteria. There is another method available – a double-staining method that mixes DAB and ferricyanide to test catalase activity [24]. Mycobacteria produce two classes of catalase, the heat-labile T-catalase, which also has a peroxidase-like function, and the heat stable M-catalase, which does not act as a peroxidase. It seems that the double-staining method is needed to distinguish between the two classes in the gels. It is believed that the double-staining procedure is very useful in characterising the catalase activity in *M. leprae*.

The present study used (NH₄)₂SO₄ precipitation to purify proteins that have catalase-like activity. The concentration of 80% (NH₄)₂SO₄ precipitated a single band with catalase-like activity.

M. leprae has not only defied all attempts at cultivation *in vitro*, but also exhibits the longest generation time of all bacteria, requiring 13 days to double in experimentally infected mice. Because of that, it was difficult to isolate and purify the protein. To further identify and purify this activity, large quantities of protein from cell lysates had to be obtained so that the fraction that contained the enzymic activity in cell lysate could be purified and analysed.

The mechanism of action of isoniazid against *M. tuberculosis* involves conversion of the inactive pro-drug, INH, to an active form in a peroxidatic reaction catalysed by catalase, KatG, encoded by the *katG* gene. Isoniazid specifically targets a long-chain enoyl-acyl

carrier protein reductase (InhA), an enzyme essential for mycolic acid biosynthesis in mycobacteria [2]. INH-resistant strains soon develop and this was often associated with concomitant loss of catalase activity and loss of virulence for guinea-pigs. Genetic confirmation that development of INH resistance is associated with loss of catalase came from experiments in which susceptibility to INH was restricted to INH-resistant strains of *M. smegmatis* and *M. tuberculosis* by transforming them with a functional catalase gene (*katG*) [25].

The 18-kDa PCR identified only the presence of *M. leprae* because it could not determine the viability of the organisms. Reverse transcription (RT)-PCR for 16S rRNA of *M. leprae* in clinical specimens was developed recently [15]. This assay had 10 organisms as the lower limit of detection. The RT-PCR assay indicates the presence of viable organisms. The present study attempted to perform RT-PCR for mRNA for the 18-kDa protein to ascertain the viability, and compared it with RT-PCR for 16S rRNA (data not shown). Amplified cDNA for the 18-kDa protein was readily visible when 10² organisms were present. RT-PCR for the 18-kDa mRNA represented clearer changes of bands than those of the 18-kDa PCR (data not shown). This means that the 18-kDa mRNA is one of the well-conserved RNAs like 16S rRNA. RT-PCR was used to follow the efficacy of drug treatment. The viability of *M. leprae* was inhibited at INH 160 µg/ml in RT-PCR (Fig. 3b), growth of *M. leprae* was inhibited at 20 µg/ml in the radiorespirometric assay (Fig. 4).

To better understand the molecular basis of leprosy pathogenesis, the identification of many of the major components of mouse-derived *M. leprae* is required. This approach was undertaken in the belief that proteins expressed by the pathogen *in vivo* would reflect its expression within the host cell environment, thereby allowing a better understanding of the mycobacterium–host cell relationship. Future studies will undoubtedly include a careful analysis of candidate genes and proteins of *M. leprae*. The fraction that conferred the enzymic activity in this study will be further characterised.

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