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Effects of Overexpression of the Alkyl Hydroperoxide Reductase AhpC on the Virulence and Isoniazid Resistance of *Mycobacterium tuberculosis*

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Mutations to the regulatory region of the *ahpC* gene, resulting in overproduction of alkyl hydroperoxide reductase, were encountered frequently in a large collection of isoniazid (INH)-resistant clinical isolates of *Mycobacterium tuberculosis* but not in INH-susceptible strains. Overexpression of *ahpC* did not seem to be important for INH resistance, however, as most of these strains were already defective for catalase-peroxidase, KatG, the enzyme required for activation of INH. Transformation of the INH-susceptible reference strain, *M. tuberculosis* H37Rv, with plasmids bearing the *ahpC* genes of *M. tuberculosis* or *M. leprae* did not result in a significant increase in the MIC. Two highly INH-resistant mutants of H37Rv, BH3 and BH8, were isolated in vitro and shown to produce no or little KatG activity and, in the case of BH3, to overproduce alkyl hydroperoxide reductase as the result of an *ahpC* regulatory mutation that was also found in some clinical isolates. The virulence of H37Rv, BH3, and BH8 was studied intensively in three mouse models: fully immunocompetent BALB/c and Black 6 mice, BALB/c major histocompatibility complex class II-knockout mice with abnormally low levels of CD4 T cells and athymic mice producing no cellular immune response. The results indicated that *M. tuberculosis* strains producing catalase-peroxidase were considerably more virulent in immunocompetent mice than the isogenic KatG-deficient mutants but that loss of catalase-peroxidase was less important when immunodeficient mice, unable to produce activated macrophages, were infected. Restoration of virulence was not seen in an INH-resistant *M. tuberculosis* strain that overexpressed *ahpC*, and this finding was confirmed by experiments performed with appropriate *M. bovis* strains in guinea pigs. Thus, in contrast to catalase-peroxidase, alkyl hydroperoxide reductase does not appear to act as a virulence factor in rodent infections or to play a direct role in INH resistance, although it may be important in maintaining peroxide homeostasis of the organism when KatG activity is low or absent.

Isoniazid (INH) has been a first-line antituberculosis drug since 1952. It is unusual in that it has a very narrow spectrum of activity, being active against some species of mycobacteria, especially the *Mycobacterium tuberculosis* complex, but not against all members of the genus (3). In recent years, details of the mechanism of action of INH have begun to emerge. The INH susceptibility of *M. tuberculosis* is thought to result from the conversion of the drug from an inactive to an active form by the bacterial catalase-peroxidase, KatG, encoded by the *katG* gene (13, 35). One of the reaction intermediates (14) affects a second mycobacterial protein, InhA, which is an acyl carrier protein-enoyl reductase involved in mycolic acid synthesis (1, 6); the modified InhA is no longer active, and fatty acid synthesis is affected. This hypothesis is supported by the findings that mutations in *katG* which affect catalase-peroxidase activity result in high levels of INH resistance and that mutations in the *inhA* gene confer low or intermediate resistance (10, 12, 15, 19). Some INH-resistant clinical isolates have been found to have mutations in both *katG* and *inhA* (12).

In addition to its role in the activation of INH, KatG is thought to play another key role in infection with the tubercle bacillus. As intracellular pathogens capable of surviving and multiplying within host macrophages, mycobacteria have to survive exposure to macrophage killing mechanisms, including the oxidative stress response. The catalase-peroxidase KatG is a component of the bacterial OxyR response, which is induced in response to oxidative stress (9, 31) and protects the bacteria against oxidative killing. Thus, the loss of a functional KatG in INH-resistant strains of *M. tuberculosis* would be expected to lead to a reduced ability of the pathogen to survive within the intracellular environment; indeed, it was reported many years ago that INH resistance was associated with loss of virulence in the guinea pig (18). It is important to understand more about the relationship between INH resistance and virulence, as this could be of great relevance to the epidemiology and prognosis of tuberculosis in immunodepressed individuals.

Recently, it was found that the gene encoding the OxyR transcription factor of *M. tuberculosis* is defective as a result of a number of mutations, and with the exception of KatG, *M. tuberculosis* does not have a peroxide-inducible response (5, 28). However, some INH-resistant *katG* mutants compensate for their loss of catalase-peroxidase activity by overexpression of the alkyl hydroperoxide reductase AhpC (27). Thus, it has been claimed that during infection with INH-resistant, KatG-deficient *M. tuberculosis*, there is a selection for compensatory *ahpC* promoter mutations, enabling the organisms to grow intracellularly (27). Furthermore, data from several laborato-

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TABLE 1. Properties and genotypes of the principal *M. tuberculosis* strains studied[a]

Strain	Genotype	*ahpC* mutation	Catalase sp act (U/mg of total protein)	INH MIC (mg/ml)	Reference
23	*katG1* (Arg463Leu) *inhA*$^+$	−141	0.36	1	10
28	*katG1* (Arg463Leu) *inhA*$^+$	−72	0.36	0.2	10
33	Δ*katG inhA2* (209C → T)	−48	<0.01	>20	10
34	Δ*katG inhA*$^+$	−81	<0.01	>5	10
S43	Δ*katG inhA*$^+$	−54	ND	10	32
S48	*katG*$^+$ *inhA*$^+$	−51	ND	1	32
S50	*katG*$^+$ *inhA*$^+$	−48	ND	1	32
S149	*katG9* (Ala202Thr) *inhA*$^+$	−48	ND	1	32
S165	*katG10* (Asp487Ala) *inhA*$^+$	−81	ND	0.2	32
S195	*katG11* (A → G −24) *inhA*$^+$	−74	ND	10	32
BH3	*katG7* (ΔG123) *inhA*$^+$	−81	<0.01	>10	This report
BH8	*katG8* (Ala144Glu) *inhA*$^+$	wt	0.10	>10	This report
H37Rv	*katG*$^+$ *inhA*$^+$	wt	0.36	0.05	

[a] ND, not determined (strains are not viable); wt, wild type.

ries suggest that overproduction of AhpC may also contribute to INH resistance (5, 7, 33). In this study, we have evaluated the contribution of *ahpC* overexpression to INH resistance and compared the virulence of isogenic mutants of *M. tuberculosis* which either are defective in KatG or lack KatG but overproduce AhpC, using normal and immunologically deficient mice.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Of the 104 isolates examined in this study, the principal strains presented are listed in Table 1, together with the salient phenotypic and genotypic properties. Strains 23, 28, 33, 34, S43, S50, S149, S165, and S195 are independent INH-resistant clinical isolates of *M. tuberculosis*, with INH MICs of ≥0.2 μg/ml, that were described in detail elsewhere (10, 12, 32). Strains BH3 and BH8 are in vitro-selected INH-resistant mutants of *M. tuberculosis* H37Rv, the wild-type reference strain. They were selected in a single step by plating about 10^{10} CFU of *M. tuberculosis* H37Rv on 7H11 agar plates supplemented with oleic acid, dextrose, catalase, and albumin (OADC) and containing 10 μg of INH per ml and were then purified on the same medium. Among several INH-resistant clones, BH3 and BH8 were retained because they displayed the lowest detectable catalase activities and were clearly mutated in *katG* but not in *inhA*. It is conceivable that the *ahpC* mutation present in BH3 was acquired during the purification step following initial isolation of this INH-resistant mutant.

For control purposes, 20 clinical isolates of *M. tuberculosis* which were fully susceptible to INH (MICs of ≤0.05 μg/ml) were screened for modifications in the region between *oxyR′* and the *ahpC* gene; in all 20 strains, the nucleotide sequence corresponded absolutely to the wild-type sequence of the H37Rv reference strain (data not shown). All *M. tuberculosis* strains were grown either in liquid Middlebrook 7H9 medium supplemented with OADC enrichment or on solid Middlebrook 7H11 medium supplemented with OADC. Drug susceptibility testing and MIC determinations were performed by standard procedures. All manipulations of *M. tuberculosis* strains were done in a biosafety level 3 laboratory.

Plasmids pPD11 and pPD12 are derivatives of pMD31 (16) that carry the *ahpC* genes from *M. leprae* and *M. tuberculosis*, respectively, on 1.3-kb *Bam*HI and 2.1-kb *Pvu*II fragments. After electrotransformation of *M. smegmatis* mc^{2}155 (29) and selection for kanamycin-resistant colonies on LB plates, transformants were purified and tested for their susceptibility to H_2O_2 (100 μM) and cumene peroxide (20 μM), using the filter paper disk overlay method (24).

Nucleic acid techniques. To analyze the *oxyR-ahpC* intergenic regions of 104 strains, fluorescent primers TB90 (5′-6-fam-CCGATGAGAGCGGTGAGCTG) and TB91 (6-fam-ACCACTGCTTTGCCGCCACC) were used in standard PCRs (32) containing appropriate DNA templates (denaturation at 94°C for 2 min, annealing at 60°C for 2 min, elongation at 72°C for 2 min, and final chain elongation at 72°C for 10 min). The resultant 236-bp fragment was processed for single-strand conformation polymorphism analysis (SSCP) as described previously (32) and submitted to overnight electrophoresis (16 h) on an Applied Biosystems 373A automated sequencer, using a 6% polyacrylamide gel containing 1× Tris-borate-EDTA buffer and 10% glycerol under the run conditions recommended by Applied Biosystems. The electrophoretic mobility of each strand was determined with respect to Rox-labeled internal Genescan size markers (Applied Biosystems catalog no. 401100), using the GENESCAN software of Applied Biosystems, which enables exact comparisons between different samples to be made. Strains showing abnormal PCR-SSCP profiles were subjected to fluorescent cycle sequencing to identify mutations in the *ahpC* regulatory region. In most cases, the complete sequences of *katG* and the *inhA* regulatory region were also determined (12, 13).

Use of *lacZ* reporter gene technology to monitor expression of *ahpC*. The regulatory region was amplified by PCR, processed as described above, and then cloned into the *Bam*HI site upstream of the promoterless *lacZ* gene on the mycobacterial shuttle vector pYUB76 (2). After electrotransformation of *M. smegmatis* mc^{2}155 (29), β-galactosidase activity was measured as described elsewhere (33).

Protein purification and antibody production. Recombinant KatG was purified to homogeneity from cell-free lysates of *Escherichia coli* UM262/pKat (14), using a fast protein liquid chromatography system (Pharmacia) equipped with Hi-Trap Q anion-exchange and Superdex 200 gel filtration chromatography columns. Three samples (0.2 mg) in Freund's incomplete adjuvant were injected intramuscularly into New Zealand White rabbits at weekly intervals. Serum was taken after 40 days and stored as frozen aliquots. Two samples (0.25 mg) of alkyl hydroperoxide reductase prepared by J. A. Marcinkeviciene and J. S. Blanchard (Albert Einstein College of Medicine, New York, N.Y.) were injected intramuscularly into New Zealand White rabbits 3 weeks apart. Serum was taken 3 weeks later and stored as frozen aliquots before being freeze-dried.

Protein studies, activity gels, and immunoblotting (Western blotting). Bacilli from 100-ml cultures were concentrated by centrifugation after about 14 days of incubation and then washed twice with sodium phosphate buffer (pH 7.0, 50 mM). After the second wash, the cells were resuspended in 600 μl of buffer, mixed with 1 ml of acid-washed glass beads (600 μm), and then lysed by shaking for 10 min in a Mickle apparatus. The suspension was centrifuged (10 min at 5,000 rpm), and the supernatant was transferred to a new microtube and then recentrifuged for 30 min at 15,000 rpm. The pellet was discarded, and the supernatant was used for protein concentration determination, specific activity testing, and immunoblotting. Activity gels to detect catalase and peroxidase activity were prepared as described previously (11). Immunoblotting involved electrotransfer of proteins to a nitrocellulose membrane (Hybond C+; Amersham) followed by incubation with an anti-KatG antibody (diluted 1:2,000) or with an anti-AhpC antibody (diluted 1:1,000) and detection of bound immunoglobulins with an enhanced chemiluminescence-peroxidase system (Amersham).

Virulence studies in mice. All mice were locally bred (The National Institute for Medical Research, London, United Kingdom). Major histocompatibility complex (MHC) class II-knockout mice were originally obtained as a breeding nucleus from D. Gray, Hammersmith Hospital, London, United Kingdom, with permission from D. Mathis, Institut National de la Santé et de la Recherche Médicale. Mice were inoculated intraperitoneally with approximately 10^6 CFU of *M. tuberculosis* and then sacrificed at various intervals; their spleens and lungs were removed aseptically. The infected tissue was weighed and homogenized in 1.0 ml of chilled saline, using a BeadBeater (Biospec Products, Bartesville, Okla.) and 2.5-mm-diameter glass beads for 10 s. Serial 10-fold dilutions of the homogenate were made in saline, and viable counts were determined on Middlebrook 7H11 agar.

RESULTS

Prevalence of mutations in the *ahpC* regulatory region of clinical isolates of *M. tuberculosis*. Since genetic arguments suggested that there may be a relationship between overexpression of the *ahpC* gene and the INH resistance of *M. tuberculosis* (5, 33), a panel of 104 independent clinical isolates was screened for mutations in the regulatory region of the gene by

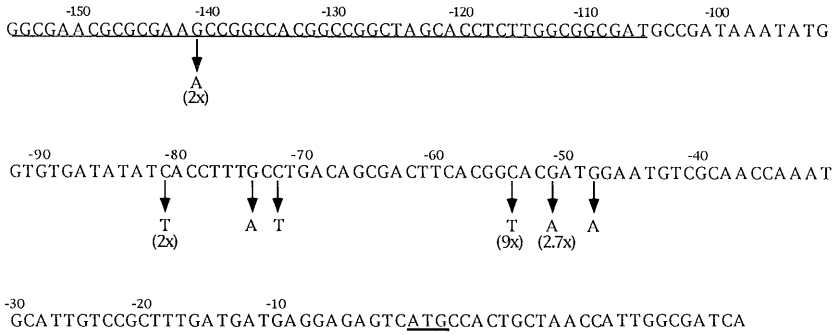

FIG. 1. Summary of mutations detected in the *ahpC* regulatory region of *M. tuberculosis*. The base changes found in the various INH-resistant clinical isolates are shown below the wild-type sequence from H37Rv (GenBank accession number U16243). The corresponding promoter activity of each fragment is expressed (in parentheses) relative to that of the wild-type promoter activity as determined by measurement of *lacZ* expression levels in *M. smegmatis*. The residual *oxyR* sequences and the *ahpC* start codon are underlined.

automated PCR-SSCP (32). No alterations were found in the 27 susceptible strains examined, whereas abnormal PCR-SSCP profiles were detected in 10 of the 77 drug-resistant samples, displaying MICs ranging from 0.2 to >10 μg of INH per ml. The DNA sequences of the corresponding PCR fragments were determined and revealed the existence of seven different transition mutations, three of which have not been described previously (Fig. 1). All strains with complete deletions of *katG* were found to have altered *ahpC* regulatory regions.

Overexpression of *ahpC* is associated with mutations. To establish whether some of the mutations observed were responsible for overexpression of *ahpC*, suitable PCR fragments were obtained from strains 23, 34, S48, and S58 and cloned in front of the promoterless *lacZ* reporter gene on shuttle plasmid pYUB76 (2). After transformation of *M. smegmatis*, the corresponding constructs were found to produce between two- and ninefold more β-galactosidase activity (Fig. 1) than the control plasmid harboring the wild-type *ahpC* regulatory region, thus indicating that the mutations did indeed result in increased levels of transcription in this surrogate host.

To confirm, in turn, that the AhpC protein was overproduced, protein extracts were prepared from *M. tuberculosis* H37Rv, and from two INH-resistant clinical isolates with alterations at *ahpC*, and subjected to immunoblot analysis using polyclonal antisera specific for the AhpC and KatG proteins. The AhpC protein, which was not detectable in the control strain, H37Rv, was found to be highly abundant in strain 34, a high-level INH-resistant mutant that lacked KatG as the result of a *katG* deletion (Fig. 2A, lane 3). In contrast, strain 23, which displays low-level INH resistance, behaved like H37Rv, suggesting that the alteration seen in its *ahpC* promoter region, which was in the residual *oxyR* coding sequence, did not cause overexpression in *M. tuberculosis* although a modest increase in promoter activity was seen in *M. smegmatis* (Fig. 1). This discrepancy may reflect the very different responses to oxidative stress employed by slow- and fast-growing mycobacteria (7).

Relationship between *ahpC* overexpression and INH resistance. Details of the INH resistance levels measured with clinical isolates harboring the various putative *ahpC* regulatory mutations are given in Table 1, where it can be seen that there is no clear correlation between overexpression of *ahpC* and resistance. However, the interpretation is complicated by the fact that most of the strains also have mutations in *katG* which reduce, or abolish, catalase-peroxidase activity. Three strains (23, 28, and S165) with some of the less severe *katG* missense mutations were resistant to low levels of INH and had altered *ahpC* regulatory regions, while one highly resistant isolate (S195) with a putative *ahpC* up-promoter mutation was genotypically *katG*$^+$ but also had a possible regulatory mutation (Table 1).

If overexpression of *ahpC* is responsible for a significant level of INH resistance, then transformation of *M. tuberculosis* with a multicopy plasmid carrying the gene should have the same effect. To test this possibility, plasmids pPD11 and pPD12, carrying the *ahpC* genes of *M. tuberculosis* and *M. leprae*, were introduced into H37Rv, and the MICs for the transformants were determined. In both cases, there was only a slight increase in the MIC of INH, from 0.05 to 0.1 μg/ml, a level which is of no clinical significance.

Although this result is consistent with previous findings for the *M. tuberculosis* complex (33), it is in contrast to the situation described by several authors for transformants of *M. smegmatis* with plasmids carrying the *M. tuberculosis ahpC* gene, where increased levels of INH resistance were found (7, 33). This result was confirmed in our study when the same plasmids carrying the *ahpC* genes of *M. tuberculosis* and *M. leprae* were introduced into *M. smegmatis*, as the MIC increased two- to fourfold. However, this increase probably resulted from re-

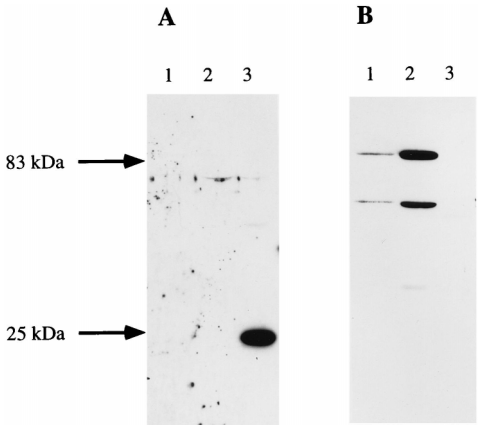

FIG. 2. Western blot analysis of protein extracts from various *M. tuberculosis* strains. Proteins (~50 μg) were separated by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels (8%, wt/vol), transferred to Hybond C+ membranes, and incubated with a polyclonal antiserum to AhpC (A) or KatG (B). Samples were from strain H37Rv (lanes 1), low-level INH-resistant clinical isolate 23 (*katG1 ahpC141*) (lanes 2), or high-level INH-resistant clinical isolate 34 (Δ*katG ahpC81*) (lanes 3). The positions of KatG (83 kDa) and AhpC (25 kDa) are indicated. The second KatG-derived species probably corresponds to a proteolytic product that is observed in some samples (B).

TABLE 2. MICs of INH and sensitivity to peroxides of various mycobacteria

Strain	INH MIC (mg/ml)	Diam of zone of inhibition (mm)[a]		Catalase sp act (% of control activity)
		H_2O_2	Cumene peroxide	
mc²155/pMD31	32	21	26	100
mc²155/pPD11 (*M. leprae ahpC*)	128	30	36	24
mc²155/pPD12 (*M. tuberculosis ahpC*)	128	30	34	46
H37Rv	0.05	ND[b]	ND	ND
H37Rv/pPD11	0.1	ND	ND	ND
H37Rv/pPD12	0.1	ND	ND	ND

[a] Mean of three independent tests.
[b] ND, not determined.

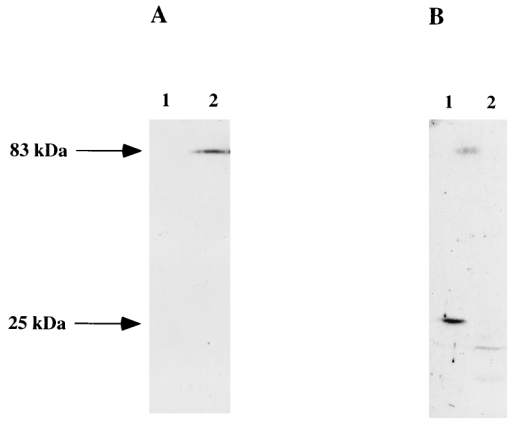

FIG. 3. Comparative Western blot analysis of *M. tuberculosis* BH3 and BH8. Immunodetection was performed with polyclonal antisera to KatG (A) and AhpC (B). Lanes 1, BH3; lanes 2, BH8.

pression of *katG* and not from overexpression of *ahpC*, as the transformants were found to be considerably more susceptible to H_2O_2 by means of a plate assay (Table 2). To determine whether this increased susceptibility to H_2O_2 was indeed due to a reduction in catalase levels, the enzyme activities of the corresponding transformants were measured. This determination revealed that introduction of the *M. leprae ahpC* gene led to lower catalase activity than was observed in transformants with the *M. tuberculosis* gene and that, in turn, these transformants produced less activity than the control *M. smegmatis* strain (Table 2).

Isolation and characterization of INH-resistant mutants of *M. tuberculosis*. As previous studies of the effect of INH resistance on the virulence of *M. tuberculosis* had been performed with clinical isolates (18, 22) that were not genetically defined, we attempted to isolate mutants of the well-characterized reference strain, H37Rv, which could grow in the presence of 10 μg of INH per ml. All INH-resistant mutants were screened for lesions in *katG*, *inhA*, and *ahpC*, genes that are known, or believed, to be associated with resistance, and two suitable derivatives of H37Rv, BH3 and BH8, were retained for further study. When protein extracts of BH3 and BH8 were tested for catalase-peroxidase by means of activity gels and Western blotting, BH8 was found to produce significantly reduced amounts of the enzyme, while BH3 did not appear to produce any KatG (Table 1; Fig. 3).

DNA sequence analysis revealed that the *katG* gene in BH8 had acquired a C-A mutation in codon 144, leading to replacement of alanine by glutamic acid, while the other genes were unchanged. By contrast, not only had the *katG* gene of BH3 incurred a frameshift mutation, resulting from loss of a cytosine residue in codon 123, but the *ahpC* gene had acquired a putative up-promoter mutation. As the same mutation was found in the regulatory region of strain 34 (Table 1; Fig. 1) and shown to be responsible for overproduction of AhpC (Fig. 2), it was probable that BH3 also overproduced the enzyme. This was confirmed by Western blotting. Figure 3 shows that while the levels of alkyl hydroperoxide reductase in BH8 were below the limits of immunodetection, BH3 produced large amounts of this enzyme.

Enzymological studies indicated that the amount of catalase-peroxidase activity in BH3 was negligible whereas that measured in BH8 was about 25% of the wild-type level (Table 1). Although the Ala144Glu substitution has not been found previously in bacterial catalase-peroxidases, its possible effects can be understood with the help of a structural model based on yeast cytochrome *c* peroxidase (10). Position 144 is occupied by alanine in all catalase-peroxidases for which sequences are available, and this is predicted to be situated in a highly conserved α-helix. In the *E. coli* enzyme, substitution of the neighboring leucine (Leu141) by phenylalanine radically lowered enzyme activity and stability (17), properties which were also clearly affected in KatG from strain BH8.

Roles of *katG* and *ahpC* in the virulence of *M. tuberculosis* in immunocompetent mice. To assess the respective contributions of catalase-peroxidase and alkyl hydroperoxide reductase to the virulence of *M. tuberculosis*, we performed a series of experiments using different mouse models of experimental disease. The results of infection of BALB/c mice with H37Rv, the KatG-deficient mutant (BH8), and the KatG-minus, AhpC-overproducing mutant (BH3) are shown in Fig. 4. The two mutant strains BH3 and BH8 behaved similarly and showed reduced counts compared to the wild-type strain H37Rv over the course of the infection in both lungs and spleens of both BALB/c (Fig. 4) and Black 6 (data not shown) mice. The most apparent difference in growth pattern between the wild-type strain and the INH-resistant mutants occurred after approximately 18 days of infection, when there was a marked reduction in the viability of the mutant strains. At this stage of the infection, there is induction of an acquired, cell-mediated immune response (23), involving the activation of macrophages by *M. tuberculosis*-reactive T cells. Since activated macrophages produce elevated levels of bactericidal peroxide (21), this finding is consistent with the idea that an effective catalase-peroxidase response would be particularly important at this stage of the infection. Overexpression of *ahpC* did not appear to confer a selective advantage on BH3 with respect to BH8, indicating that alkyl hydroperoxide reductase probably does not act as a virulence factor. This conclusion is supported by other work with isogenic INH-resistant strains of *M. bovis* in the guinea pig model of tuberculosis (see Discussion).

To examine whether the *M. tuberculosis* strains recovered from the spleens of mice, 80 days after infection, had the same genotype as those initially inoculated, 10 colonies of each strain were screened by fluorescent PCR-SSCP. In all three cases, no differences were observed, indicating that the lesions in *katG* did not revert to a significant extent and that BH8 had not acquired mutations in the *ahpC* regulatory region during passage through the mice.

Roles of *katG* and *ahpC* in the virulence of *M. tuberculosis* in immunodeficient mice. To investigate further the virulence of

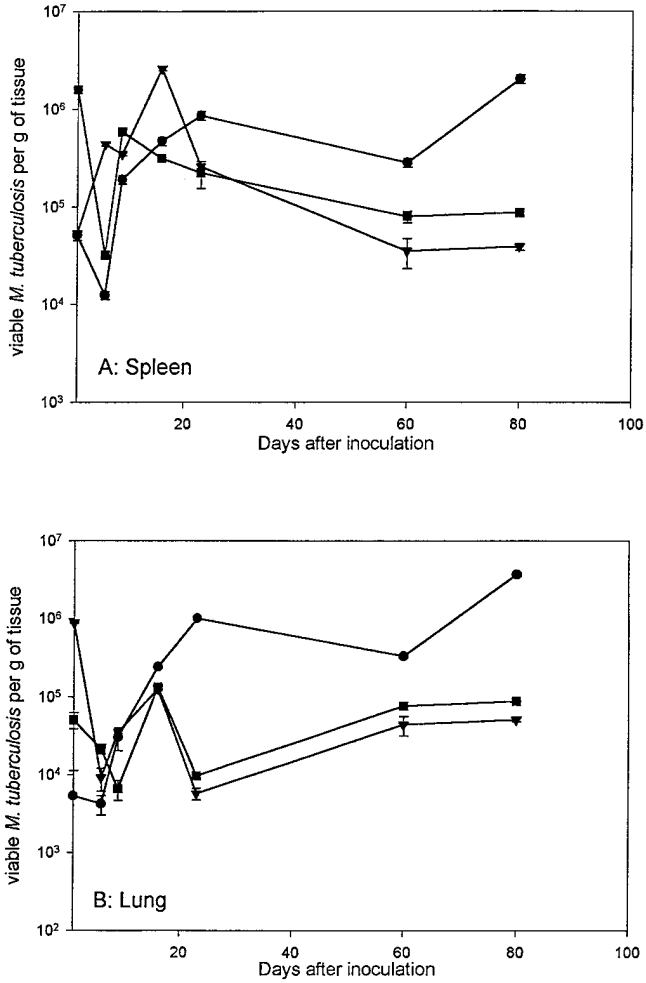

FIG. 4. Growth of *M. tuberculosis* H37Rv (circles), BH3 (triangles), and BH8 (squares) in the spleens (A) and lungs (B) of immunocompetent BALB/c mice. Each point represents the mean of results obtained from three to five mice, plus or minus the standard deviation.

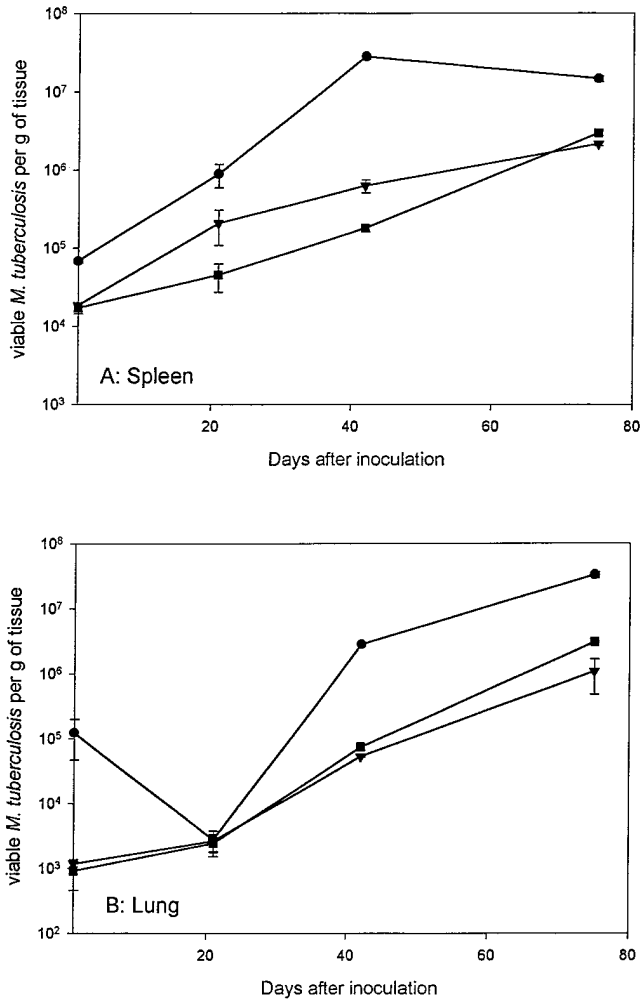

FIG. 5. Growth of *M. tuberculosis* H37Rv (circles), BH3 (triangles), and BH8 (squares) in the spleens (A) and lungs (B) of MHC class II-knockout mice. Each point represents the mean of results obtained from three to five mice, plus or minus the standard deviation.

the catalase-peroxidase-deficient mutants, we then infected mice with defective cell-mediated immunity. MHC class II-knockout mice have a targeted deletion in the Aβ gene (4); $A\beta^{-/-}$ mice fail to express MHC class II molecules and have a drastically reduced number of highly abnormal CD4 cells (4). These mice show a greatly elevated level of susceptibility to infection with *M. tuberculosis* (30) but retain some ability to control the infection, presumably through non-CD4-mediated activation of macrophages. In these mice all three strains grew, although the catalase-peroxidase mutants grew somewhat less rapidly (Fig. 5). Thus, in the absence of an effective CD4/class II-mediated response, the difference between catalase-peroxidase-deficient strains of *M. tuberculosis* and the wild type is less apparent.

This finding was confirmed when growth of the double mutant BH3 was compared to that of the wild-type H37Rv in congenitally athymic mice (Fig. 6). Athymic mice lack any effective T-cell mechanism for controlling *M. tuberculosis*, and both strains grew rapidly in lungs and spleen, such that by day 40 the mice were moribund, with an overwhelming infection and large tuberculoma at the site of inoculation. The growth curve of the mutant strain BH3 was essentially identical to that of the wild type; thus, when there is no effective immune response, the lack of catalase-peroxidase activity does not affect the virulence of the organism.

DISCUSSION

In the last few years there has been considerable interest in the role of alkyl hydroperoxide reductase in the INH resistance of *M. tuberculosis* following the observations of Rosner (24) and Rosner and Storz (25) that certain strains of *E. coli*, a bacterium that is normally insensitive, became mildly susceptible to the drug if they had knockout mutations in *oxyR* or the OxyR-dependent *ahpC* gene. Subsequently, it was found that the *oxyR* gene of *M. tuberculosis* harbored multiple lesions and that the organism appeared to be unable to mount an effective oxidative response (5, 28). Since the *oxyR* gene, which is transcribed divergently from *ahpC*, is believed to encode the transcriptional activator for *ahpC*, it was reasonable to assume that the exquisite INH susceptibility of the tubercle bacillus resulted from limiting concentrations of alkyl hydroperoxide reductase, an enzyme that may nullify the effects of the activated form of the drug. It was shown here by immunoblotting (Fig. 2), and by others (7), that the levels of AhpC are indeed low in

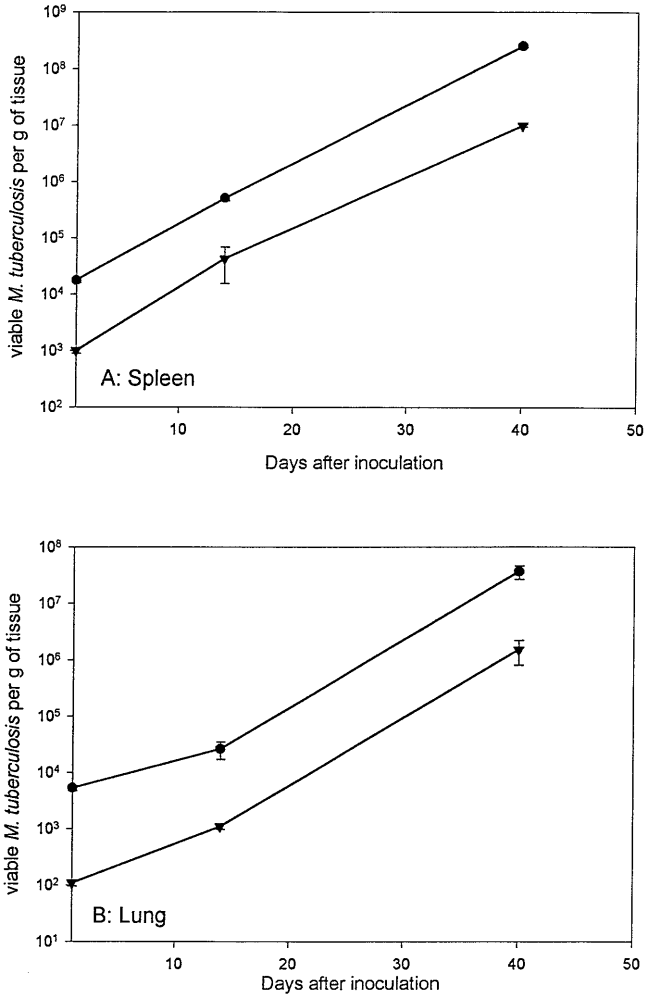

FIG. 6. Growth of *M. tuberculosis* H37Rv (circles) and BH3 (triangles) in the spleens (A) and lungs (B) of athymic mice. Each point represents the mean of results obtained from three mice, plus or minus the standard deviation.

wild-type *M. tuberculosis* but can be increased substantially as a result of mutations to the *ahpC* regulatory region of INH-resistant strains of both clinical and laboratory origin. While this work was in progress, Sherman et al. (27) published convincing evidence that showed *ahpC* overproduction to be due to regulatory mutations in INH-resistant clinical isolates that lacked KatG as had also been reported for some INH-resistant *M. bovis* strains (33). Our results are consistent with their findings, as those strains that produced undetectable levels of KatG generally had *ahpC* up-mutations whereas others, with some of the commonly occurring missense mutations in *katG* (e.g., Ser315Thr [10, 20, 26]), did not. Monitoring the *ahpC* regulatory region might thus represent a useful surrogate marker for the presence of some of the more severe *katG* mutations.

Preliminary studies with *M. smegmatis* transformed with plasmids bearing cloned mycobacterial *ahpC* genes suggested that increased production of alkyl hydroperoxide reductase conferred resistance to higher levels of INH (33), but this effect was not found in *M. bovis*. The findings of the present study, and those of Sherman et al. (27), indicate that AhpC may not be directly involved in INH resistance in *M. tuberculosis*, as no significant increase in the MIC was observed. The apparent protective effect, seen in *M. smegmatis*, of multicopy plasmids bearing *ahpC* is a reflection of decreased KatG activity, as evidenced by the increased susceptibility of the transformants to H_2O_2 (Table 2). Since increased levels of catalase-peroxidase protect against the effects of cumene peroxide, a substrate of AhpC, it seems probable that both enzymes act on a common intracellular organic peroxide (27). This provides a physiological explanation for why mutations leading to the overexpression of *ahpC* seem to arise predominantly in the absence of KatG activity and may indicate that mycobacteria try to maintain a constant level of total peroxidase activity (i.e., KatG and AhpC plus any others) for metabolic purposes. Introduction of multiple copies of *ahpC*, and the resultant increase in the level of the enzyme, could perturb this peroxidative homeostasis and might reduce the requirement for KatG activity.

It has been implied that the emergence of *ahpC* overexpression not only compensates for the loss of catalase-peroxidase but also may confer a selective advantage on the corresponding strain in vivo (27). The studies presented here do not support this hypothesis, at least in the case of BH3 in mice, as no significant difference in the level of virulence, reflected by viable counts, was seen compared to a strain producing greatly reduced levels of catalase-peroxidase. Furthermore, after an experimental infection of 80 days, mutations to the *ahpC* regulatory region were not detected in the sample examined, although a much longer time course may be required for such mutants to emerge.

Unfortunately, owing to the vagaries of the genetic selection used, BH3 and BH8 were not perfectly matched, once again underscoring the need for an efficient gene replacement system for working with the tubercle bacilli. The principal conclusion about the role of *ahpC* that could be drawn from the virulence experiments in mice was that overexpression did not compensate for loss of catalase-peroxidase in infection of the immunocompetent host. However, although small effects of AhpC overproduction might have been concealed in comparisons with BH8, which retained some KatG activity, this seems unlikely as the results obtained with these *M. tuberculosis* strains are fully consistent with those of more recent work using isogenic *M. bovis* strains in guinea pigs. In these latter experiments, a fully virulent, INH-sensitive *M. bovis* strain was exposed to increasing concentrations of INH to produce a mutant that was highly resistant to INH (34). This mutant was shown to have lost all catalase activity and to have become avirulent for guinea pigs. Reintroduction of a functional *katG* gene into this mutant restored catalase activity, INH susceptibility, and virulence for guinea pigs (34). When, instead of a *katG* gene, an *ahpC* gene with an upregulated promoter was incorporated into the mutant, the resulting recombinant was more resistant to hydrogen peroxide but remained avirulent for guinea pigs (unpublished results).

While alkyl hydroperoxide reductase, unlike KatG, does not appear to be a virulence factor in the experimental conditions used here, its proposed compensatory role for loss of KatG activity presumably enhances the survival of the organisms under some conditions. The data presented in Fig. 4 show quite clearly that *M. tuberculosis* strains which produce catalase-peroxidase have a competitive advantage in immunocompetent hosts once the T-cell-mediated immune response (23), leading to the activation of macrophages and the release of large amounts of reactive O_2 species, has been induced. It seems likely that it is at this stage of the infection that an active catalase-peroxidase would be particularly important. This is in contrast to the situation in mice that are partially (Fig. 5) or fully (Fig. 6) immunocompromised and unable to mount an effective immune response leading to macrophage activation

and could explain why INH-resistant strains of *M. tuberculosis* are recovered more frequently from human immunodeficiency virus-infected individuals than from immunocompetent persons in industrialized countries (8).

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