High-Dose Isoniazid Therapy for Isoniazid-Resistant Murine Mycobacterium tuberculosis Infection

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Received 3 September 1999/Returned for modification 14 July 1999/Accepted 2 October 1999

The use of isoniazid (INH) for the treatment of INH-resistant Mycobacterium tuberculosis infection has been controversial. The purpose of the present studies was to determine if there is a dose response with INH for INH-susceptible M. tuberculosis Erdman (ATCC 35801), and whether high-dose INH (100 mg/kg of body weight) was more effective than standard-dose INH (25 mg/kg) for therapy of tuberculosis infections caused by INH-resistant mutants of M. tuberculosis Erdman. Six-week-old CD-1 mice were infected with approximately 107 viable mycobacteria. Early control groups of infected but untreated mice were euthanized by CO2 inhalation 1 week later when treatment was initiated. INH (25, 50, 75, and 100 mg/kg) was given by gavage 5 days/week for 4 weeks. Late control groups of untreated mice and treated mice were sacrificed 2 days after the last dose of drug. Spleens and right lungs were removed aseptically and homogenized, and viable cell counts were determined by titration on 7H10 agar plates. In the next study, INH at 25 mg/kg was compared to INH at 25 mg/kg against an isogenic mutant of M. tuberculosis Erdman (INH MIC, 2 μg/ml) and the parent strain. This mutant was found to have a mutation in the KatG protein (Phe to Leu at position 183). In the first study, there was no dose response with increasing doses of INH. In the second study, there was no significant difference between the reduction of viable cell counts for mice treated with INH at 100 mg/kg and that for mice treated with INH at 25 mg/kg (parent or INH-resistant mutant). These preliminary results suggest that INH may be useful in combination therapy of M. tuberculosis infections caused by low-level INH-resistant organisms (INH MICs, 0.2 to 5 μg/ml) and that higher doses of INH are unlikely to be more efficacious than the standard 300-mg/day dose.

Isoniazid (INH) continues to be one of the primary antituberculosis agents. In a recently completed survey, the prevalence of primary INH resistance ranged from 0 to 16.9% and the prevalence of acquired INH resistance varied between 4.0 and 53.7% (16).

The mechanism of action of INH is complex. INH is a produg which is activated by a catalase-peroxidase enzyme (KatG) (17). The activated drug subsequently interacts with one or more targets: InhA (an NADH-dependent enoyl [acyl carrier protein] reductase [1]) and/or KaaA (a β-keto acyl carrier protein synthase) (9). Mutations in katG account for the majority of INH-resistant clinical isolates (12).

Victor et al. (13) reported that the MICs of INH for approximately 50% of the INH-resistant organisms from South Africa that they studied were between 0.2 and 5 μg/ml. If this is representative of INH-resistant organisms in other populations (particularly in developing countries), then INH may have a role in the treatment of those patients.

The use of INH for treatment of INH-resistant (defined as an INH MIC of >0.2 μg/ml) Mycobacterium tuberculosis infection has been controversial (11). We previously demonstrated that INH (25 mg/kg of body weight 5 days/week or 75 mg/kg 3 days/week) reduced the viable mycobacterial load by approximately 2 log units in lungs and spleens of mice infected with M. tuberculosis ATCC 49967 (strain CNL), a multiple-drug-resistant organism for which the INH MIC is 1 μg/ml (5). The activity of INH against M. tuberculosis H37Rv (ATCC 25618) and Erdman (ATCC 35801), MICs of 0.03 and 0.015 μg/ml, respectively, yields about a 2.5- to 3-log-unit reduction in organ cell counts (8). INH is usually dosed in humans at 5 mg/kg/day, up to 300 mg/day, yielding a peak level in serum of 3 to 5 μg/ml (14). In earlier studies with humans, INH was evaluated at doses of up to 20 mg/kg/day (2). The present study was undertaken to evaluate the activity of INH at doses of 25 to 100 mg/kg/day against INH-susceptible M. tuberculosis ATCC 35801. In addition, the efficacy of INH at a standard dose or a high dose against a low-level INH-resistant organisms (INH MICs, 0.2 to 5 μg/ml) and that higher doses of INH are unlikely to be more efficacious than the standard 300-mg/day dose.

MATERIALS AND METHODS

Drugs. INH and pyridoxine were purchased from Sigma Chemical Co., St. Louis, Mo. INH and pyridoxine were dissolved in water and freshly prepared each day prior to administration.

Isolates. M. tuberculosis Erdman ATCC 35801 was obtained from the American Type Culture Collection, Manassas, Va. This strain has been used previously in our laboratory for murine model studies (6, 7). The INH-resistant mutant was selected by growth on Middlebrook 7H10 agar plates (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC) enrichment (Difco Laboratories, Detroit, Mich.) containing 0.2 μg of INH per ml. Individual colonies were selected and regrown on 7H10 agar plates containing 2 μg of INH per ml. The MICs of INH for M. tuberculosis ATCC 35801 and for the INH-resistant mutant R3, as determined by a broth dilution method (16), were 0.015 and 2 μg/ml, respectively.

Medium. The organisms (ATCC 35801 and R3) were grown in modified Middlebrook 7H10 broth (7H10 agar formulation with agar and malachite green omitted), pH 6.6, supplemented with 10% OADC enrichment and 0.05% Tween 80 on a rotary shaker for 5 days at 37°C. The culture suspensions were diluted with modified 7H10 broth to yield 100 Klett units/ml (Klett-Summerson colorimeter; Klett Manufacturing, Brooklyn, N.Y.) or approximately 5 × 107 CFU/ml. The sizes of the inocula were determined by titration and counting from triplicate 7H10 agar plates supplemented with 10% OADC enrichment. The plates were incubated at 37°C in ambient air for 4 weeks prior to counting.

Sequencing of katG and inhA-orfl. katG, inhA, and the open reading frame, orfl, located in an operon immediately upstream of inhA from the INH-resistant M. tuberculosis strain were amplified by PCR with the oligonucleotide primers and conditions described by Kapur et al. (3). The PCR products were then
purified from an agarose gel and sequenced with an automatic DNA sequencer, model 377 (Applied Biosystems, Inc., Foster City, Calif.), at the Johns Hopkins Genetic Core Facility. Appropriate internal sequencing primers were synthesized by Genosys Inc. based on katG and inhA-sat1 DNA sequences in the database with accession no. MTCY180.10, MTCY277.05, and MTCY277.04, respectively.

The DNA sequences for katG and inhA-orf1 were compared with their wild-type sequences by the Clustal method to identify potential mutations.

**Infection studies.** Six-week-old female CD-1 mice (Charles River, Wilmington, Mass.) were infected intravenously through a caudal vein. Each mouse received approximately $10^7$ viable organisms suspended in 0.2 ml of modified TH10 broth. There were eight mice per group.

Treatment was started 1 week after infection. A group of untreated infected mice was sacrificed at the start of treatment (early controls). A second group of untreated infected mice was sacrificed at the conclusion of the treatment period (late controls). Treatment with INH was given by gavage (0.2 ml) 5 days per week for 4 weeks. Pyridoxine (10 mg/kg) was given along with INH. Mice were sacrificed (late controls). Treatment with INH was given by gavage (0.2 ml) 5 days per week for 4 weeks prior to reading. In the dose response study, INH was evaluated at 25, 50, 75, and 100 mg/kg against ATCC 35801-infected mice. In the next study, INH at 100 mg/kg was compared to INH at 25 mg/kg against an isogenic mutant of ATCC 35801 and the parent strain.

**Statistical evaluation.** Viable cell counts were converted to logarithms, which were then evaluated by one- or two-variable analyses of variance (ANOVA). Statistically significant effects from the ANOVA were further evaluated by Tukey honestly significant difference (HSD) tests (4) to make pairwise comparisons among means.

**RESULTS**

**Dose response study.** INH was evaluated at 25, 50, 75, and 100 mg/kg in mice which had been infected with $1.9 \times 10^7$ viable ATCC 35801 organisms. There was a significant difference ($P < 0.01$ [for all comparisons]) between results for INH-treated mice and for the early control group (Table 1). There was no significant difference between results for the various INH treatment groups ($P > 0.05$).

**High-dose INH against an isogenic INH-resistant mutant.** INH at 100 mg/kg was compared to INH at 25 mg/kg against an isogenic mutant of ATCC 35801 and R3 (INH MIC, 2 $\mu$g/ml) and the parent strain (INH MIC, 0.015 $\mu$g/ml). The mutant was found to have a mutation in the KatG protein (Phe to Leu at position 183). Mice were infected with $2.8 \times 10^7$ viable organisms. The inoculum for the parent strain was $2.2 \times 10^7$ viable organisms. INH at 25 or 100 mg/kg was active in spleens and lungs of mice infected with ATCC 35801 and the moderately INH-resistant mutant R3 (Table 2). There was no significant difference in the reduction of viable cell counts between mice treated with INH at 100 mg/kg and mice treated with INH at 25 mg/kg (parent or INH-resistant mutants). INH at 25 mg/kg was not significantly more active ($P < 0.01$) against the parent strain (Tukey HSD test following significant one-way ANOVA) in spleens or lungs than against the R3 mutant.

**DISCUSSION**

INH has been the most important agent for the treatment of tuberculosis since its introduction in the early 1950s. Its role in the treatment of tuberculosis caused by INH-resistant organisms is less clear. It has been suggested that when INH-resistant tuberculosis occurs, INH would be effective against those organisms that are still susceptible (i.e., that proportion of $<99\%$ that are susceptible). This rationale may be flawed, since a large number of organisms would be resistant and continued therapy with the INH-containing regimen would likely select for further resistance. In the clinical setting, selection for further resistance does not seem to occur if rifampin and/or pyrazinamide is included in a multidrug short-course regimen (10).

Mitchison and Nunn (10) reviewed the results of patients with pulmonary tuberculosis caused by drug-resistant organisms in 12 controlled trials of short-course chemotherapy. They found that the sterilizing activities of 6-month regimens containing four or five drugs, when these included rifampin or pyrazinamide, were influenced little by initial INH resistance. They attributed much of the success in these patients with INH-resistant *M. tuberculosis* infection to the strong sterilizing activity of rifampin and/or pyrazinamide. It is possible that the *M. tuberculosis* isolates from many of these patients actually had low-level resistance to INH and therefore benefited from the INH that was included in their regimens.

In the present study, there was no dose response to INH when the dose was increased from 25 to 100 mg/kg in mice infected with an INH-susceptible strain (ATCC 35801). Furthermore, INH at 100 mg/kg was not more active than INH at 25 mg/kg against a low-level INH-resistant mutant of *M. tuberculosis* Erdman in the murine tuberculosis model. It is noteworthy that INH was active against the low-level INH-resistant organism (2.5-log-unit reduction in spleens and 1.5-log-unit reduction in lungs).

Our preliminary results suggest that doses of INH greater than 300 mg/day are unlikely to be more effective for treatment.

<table>
<thead>
<tr>
<th>Organ and bacterial strain</th>
<th>Early controls</th>
<th>Late controls</th>
<th>INH (25 mg/kg)</th>
<th>INH (100 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spleen</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC 35801</td>
<td>$8.10 \pm 0.38$</td>
<td>$8.58 \pm 0.67$</td>
<td>$4.73 \pm 0.79$</td>
<td>$4.38 \pm 1.34$</td>
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<td>R3</td>
<td>$7.88 \pm 0.22$</td>
<td>$7.99 \pm 0.61$</td>
<td>$5.41 \pm 0.30$</td>
<td>$5.34 \pm 0.21$</td>
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<tr>
<td>Lung</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC 35801</td>
<td>$6.51 \pm 0.50$</td>
<td>$9.03 \pm 0.47$</td>
<td>$4.36 \pm 1.04$</td>
<td>$3.80 \pm 0.72$</td>
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<tr>
<td>R3</td>
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<td>$7.67 \pm 0.52$</td>
<td>$4.42 \pm 0.35$</td>
<td>$3.99 \pm 0.50$</td>
</tr>
</tbody>
</table>

* $n$, number of mice per group. Initially there were eight mice/group; lesser numbers indicate contamination of plates and/or technical problems.
of human pulmonary tuberculosis than is the standard dose. INH, when utilized in multiple drug combination regimens (particularly with rifampin and/or pyrazinamide), is likely to provide clinically useful activity for treatment of patients with low-level INH-resistant tuberculosis (INH MICs ≤5 μg/ml). It is not known whether INH would be useful (in mice or humans) if the MIC of INH was >5 μg/ml. Since low-level INH-resistance accounts for approximately 50% of INH-resistant organisms in some developing countries (13), it is likely that this agent should continue to be included in the treatment regimen when patients are found to have INH-resistant tuberculosis.

The study of an INH-monoresistant clinical isolate in parallel with the parent INH-susceptible strains would be particularly useful to better understand the potential activity of INH in the treatment of tuberculosis caused by INH-resistant organisms. In addition, it would be of interest to evaluate INH in the murine model by using INH-resistant clinical isolates with other mutations in \( \text{inhA} \) and \( \text{katG} \), or mutations in \( \text{inhA} \) and \( \text{kasA} \), to define the relationship between in vitro and in vivo drug activities.

ACKNOWLEDGMENT

This study was supported by NCDDG-OI program cooperative agreement U19-AI 40972 with NIAID.

REFERENCES