Does Pyrazinoic Acid as an Active Moiety of Pyrazinamidate Have Specific Activity against Mycobacterium tuberculosis?

LEONID B. HEIFETS,1,2* MARCELLA A. FLORY,1 AND PAMELA J. LINDHOLM-LEVY1

National Jewish Center for Immunology and Respiratory Medicine, Denver, Colorado 80206,1,2 and Department of Microbiology and Immunology and Department of Medicine, University of Colorado Health Sciences Center, Denver, Colorado 802622

Received 17 February 1989/ Accepted 26 May 1989

The commonly accepted hypothesis explaining the mechanism of action of pyrazinamide (PZA) is based on the assumption that PZA-susceptible Mycobacterium tuberculosis strains produce pyrazinamidase, which hydrolyzes PZA to the antibacterial moiety pyrazinoic acid (POA). It is not clear whether POA has specific antimicrobial activity or the inhibition of growth caused by POA is due to its ability to lower the pH of the environment below the limits of tolerance of M. tuberculosis growth. We confirmed in this study that POA, depending on the concentration, lowered the pH of 7H112 broth (pH 6.0), which ranged from 5.8 at 120.0 μg/ml to 4.6 at 960.0 μg/ml. Therefore, we tested the inhibitory effects of different concentrations of POA in broth in which the final pH was adjusted to 5.6 by adding appropriate amounts of phosphoric acid and diethylammonium phosphate. Under these conditions, we found a clear dose-response correlation, proving that POA does have specific antimicrobial activity. The MIC of POA at pH 5.6 was 240 to 480 μg/ml, 8- to 16-fold higher than the MIC of PZA under the same conditions and much higher than the concentrations achievable in humans. This suggests that the action of POA in an acid environment is a combined effect of its specific activity and its ability to lower the pH below the limits of tolerance of the target organism.

The results of clinical trials (2, 6, 11, 12) indicating the unique role of pyrazinamide (PZA), when used in a combination with isoniazid and rifampin, in reduction of the duration of chemotherapy from 9 to 6 months renewed interest in PZA, which is now considered the third most important drug in modern tuberculosis therapy (R. J. O'Brien and D. E. Snider, Editorial, Am. Rev. Respir. Dis. 131:309–311, 1985). However, there is less knowledge and a poorer understanding of the mechanisms of action and limitations of PZA than of any other contemporary antimycobacterial agent.

It is well known that an acid environment (pH ≤5.6) is necessary for activity of PZA against Mycobacterium tuberculosis in vitro (8). The following mechanism of action of PZA was proposed to account for the need for an acid environment (7). Susceptible M. tuberculosis strains are known to produce the enzyme pyrazinamidase, which converts PZA into pyrazinoic acid (POA). It was suggested that it is this enzyme-generated product, POA, that has high antibacterial activity in an acid environment, whereas PZA itself has no activity at all. Strains resistant to PZA do not produce pyrazinamidase, do not convert PZA into POA, and therefore are not vulnerable to the former. A contradiction to this theory can be derived from a study of the activity of PZA with different inoculum sizes in low-pH (5.6) liquid medium (3). PZA was active when a relatively small inoculum of M. tuberculosis was used, with an initial concentration of 5.34 to 5.41 log10 viable U/ml. Lack of PZA activity in the presence of a larger inoculum, 6.5 log10 viable U/ml, was thought to be the result of either neutralization of the medium or the activity of bacterial pyrazinamidase, which deaminates PZA to the less active POA (1). One of the early Russian studies (9) indicated that exposure to PZA increased the acidity in small areas surrounding phagocytized M. tuberculosis organisms, lowering the usual intracellular pH range from 5.0 to 5.3 to 4.5 to 4.7, which is probably the effect of the transformation of PZA to POA. A question raised by this finding is whether POA has any specific antimicrobial activity or whether it affects the growth of M. tuberculosis simply by lowering the pH below the limits of tolerance.

The aims of this study were (i) to quantitate the acidifying effect of POA, (ii) to determine the existence and degree of specific antimycobacterial activity of POA, and (iii) to compare the MICs of POA and PZA under identical experimental conditions.

MATERIALS AND METHODS

Strains. M. tuberculosis H37Rv and nine drug-susceptible clinical isolates were the test organisms in this study. They were cultured in 7H9 broth until growth reached the equivalent turbidity of a no. 1 McFarland standard. The broth cultures were divided into 1-ml aliquots that were frozen at −70°C until needed.

Antimicrobial agent. PZA was obtained from Sigma Chemical Co. (St. Louis, Mo.), and POA was obtained from Aldrich Chemical Co., Inc. (Milwaukee, Wis.). POA was dissolved in hot water and filter sterilized through a prewarmed apparatus (Nalge/Sybron Corp., Rochester, N.Y.). POA was dissolved in bottled water for irrigation (Abbott Laboratories, North Chicago, Ill.) and filter sterilized. Dilutions of both drugs were made in sterile bottled water, and aliquots were made and stored at −70°C until needed.

POA solutions. To quantitate the acidifying effect of POA on 7H112 broth, doubling concentrations (to make 60, 120, 240, 480, and 960 μg/ml) were added to vials each containing 4.0 ml of specially prepared broth (Johnston Laboratories, Inc., Towson, Md.) that had a pH of 6.0. The same quantities of POA were added to two other sets of vials after the pHs of their broths were adjusted from 6.0 to 5.3 and 5.0 by adding an appropriate phosphoric acid solution. The pHs of all of the POA-containing broths were determined on an

* Corresponding author.
TABLE 1. Acidifying effect of POA

<table>
<thead>
<tr>
<th>Initial pH of 7H12 broth</th>
<th>pH of 7H12 broth after addition of the following final concn of POA (µg/ml):</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30.0</td>
</tr>
<tr>
<td>6.00</td>
<td>5.96</td>
</tr>
<tr>
<td>5.50</td>
<td>5.22</td>
</tr>
<tr>
<td>5.00</td>
<td>5.00</td>
</tr>
</tbody>
</table>

Accumet 815 MP pH meter (Fisher Scientific Co., Pittsburgh, Pa.).

**MIC determination.** The MICs of PZA were determined in 7H12 broth radiometrically (5, 10) at pH 5.6. Each isolate was tested with the following concentrations of PZA: 7.5, 15.0, 30.0, and 60.0 µg/ml. The MICs of POA were determined under identical experimental conditions. For comparison, the MICs of both PZA and POA were defined as the lowest concentrations that inhibited more than 90% of a bacterial population. For each test, a frozen aliquot of *M. tuberculosis* was thawed and 0.1 ml was added to a vial containing standard pH 6.8 7H12 broth. The vial was incubated at 37°C, and the radiometric growth index (GI) was read and recorded daily on a BACTEC TB-460 instrument. When the daily GI reached approximately 500, the broth was mixed thoroughly through an allergist syringe (Becton Dickinson and Co., Rutherford, N.J.) to break up any clumped bacilli. A 0.1 ml volume of the bacterial suspension was added to each of six vials of pH 6.0 7H12 broth. Another vial was inoculated with 0.1 ml of a 1:10 dilution of the suspension, to represent 10% of the bacterial population. The seven vials were incubated at 37°C, and the GIs were read and recorded on the BACTEC instrument until the GIs in all but the 1:10 vial were approximately 50. At this time, aliquots of POA were thawed and placed in a hot water bath to dissolve the crystals. Final concentrations in 7H12 broth were 960, 480, 240, 120, and 60 µg/ml. One vial besides the 1:10 control was left as an undiluted, drug-free control. The desired final pH was 5.6. To back titrate the effect of POA, an appropriate amount of a 1 N solution of K₂HPO₄ was added to the vial containing 960 µg of PZA per ml. The vials with pHs of >5.6 were inoculated with a solution of phosphoric acid in quantities that brought them to the desired level. The vials were incubated at 37°C with daily reading on the BACTEC instrument until the GI of the 1:10 control was between 150 and 200. The drug concentration producing a daily GI increase and final reading lower than those in the 1:10 control was considered to be the concentration that inhibited more than 90% of the bacterial population. Two strains were used for sampling and plating to compare MICs determined by this method and radiometrically. Samples were taken from each drug-containing and drug-free culture at various times. The samples were diluted on the basis of the GI and plated on 7H10 agar. After 2 weeks of incubation at 37°C in a 5% CO₂ atmosphere, the colonies were counted and the number of CFU per milliliter was calculated. The lowest concentration with fewer CFU per milliliter than in the 1:10 control was considered to have inhibited 90% of the bacterial population.

**RESULTS**

POA, when added to 7H12 broth with an initial pH of 6.0, 5.3, or 5.0, did bring about a further acidifying effect, with a good correlation between the POA concentration and the pH achieved (Table 1). A concentration of 60 µg or less per ml did not produce a detectable decrease in pH, probably because of the buffering capacity of this medium.

To determine the MIC of POA at pH 5.6, a range of POA concentrations, 60, 120, 240, 480, and 960 µg/ml, was made in 7H12 broth and appropriate solutions of phosphoric acid were added to the vials containing 60, 120, and 240 µg of POA per ml; and a 1 N solution of K₂HPO₄ was added to the vial containing 960.0 µg of PZA per ml to provide the same pH 5.6 in all of the vials. These conditions provided an opportunity to separate the evaluation of the specific antimicrobial activity of POA from possible growth inhibition due to lowering of the pH to create an environment unfavorable for *M. tuberculosis*. For all 10 strains, the MICs of POA were 240 to 480 µg/ml at pH 5.6 (Table 2). The MICs of PZA for the same strains were 15 to 30 µg/ml (60 µg/ml for one strain), about 8 to 16 times lower than the MICs of POA.

**DISCUSSION**

Evaluation of the inhibitory activities of different concentrations of POA under identical pH conditions (5.6) in 7H12 liquid medium showed a clear dose-effect correlation and indicated that POA does have specific activity against *M. tuberculosis*. The MICs of POA found in these conditions were 8 to 16 times higher than the MICs of PZA. It was suggested previously that POA is less active in vitro than PZA (1, 3, 4), with only a twofold difference between the MICs of POA and PZA (4). Even with the greater difference found in our study, in which the MIC of POA was 240 to 480 µg/ml, the ultimate question concerns the validity of the theory (7) that suggests that PZA acts by its conversion to POA in vivo, since the concentration of POA in human serum does not exceed 10 µg/ml (4). Our studies support the part of this theory (7) that states that POA is an antimicrobial agent, but they seem to contradict (owing to the high MICs found) the suggestion that it is the only antimicrobial moiety of PZA. A possible explanation for this controversy is that the assumption that high concentrations of POA, close to the MIC and much higher than the concentrations of both PZA and POA found in serum, might be achieved in the immediate surroundings of mycobacterial cells. Such speculations are supported by the finding (9) that the pH in small areas around phagocytized mycobacteria can drop from the usual intracellular 5.0 to 4.7 when the organisms are exposed to PZA. We found (Table 1) that such a decrease in the pH of the medium is possible in the presence of 240.0 µg of POA or more per ml. The assumption based on these data is that mycobacterial cells act like pumps consuming PZA from the surrounding environment (25 to 50 µg/ml) and transforming
it into POA, which accumulates at the surface of each cell and is delayed in diffusing into the medium. To confirm such a hypothesis, a mechanism responsible for such a delay of the POA diffusion and/or its binding to the phagocytized mycobacteria should be identified. The mode of action of PZA against M. tuberculosis remains unclear, but the fact that POA does have specific antimicrobial activity is an important step toward understanding the mechanism of action of PZA. Our findings suggest that the action of POA in an acid environment is more likely a combined effect of its specific activity and its ability to lower the pH below the limits of tolerance of the target organism.

ACKNOWLEDGMENT

This work was supported by biomedical research support grant SO7RR 05842-08 from the National Jewish Center for Immunology and Respiratory Medicine, Denver, Colo.

LITERATURE CITED