Pyrazinamide (PZA) is an unconventional front line tuberculosis drug characterized by high in vivo sterilizing activity, but poor in vitro activity. This disparity in PZA activity may reflect differences between the in vivo tissue environment and in vitro culture conditions. This study examined the effect of anaerobic conditions, which exist in granulomatous lesions in vivo, on PZA activity in vitro. Low oxygen enhanced the activity of PZA against Mycobacterium tuberculosis, with anaerobic conditions resulting in greater enhancement than microaerobic conditions. ATPase and respiratory chain enzyme inhibitors enhanced PZA activity under normal atmospheric conditions, but not under anaerobic conditions. Furthermore, the inhibitors did not enhance isoniazid or rifampicin activity. Nitrate as an alternative electron acceptor antagonized PZA activity under anaerobic conditions. These findings provide further support for a proposed mechanism of action of PZA in which the active form of PZA (pyrazinoic acid) depletes the membrane energy reserve. They also provide another explanation for the higher sterilizing activity of PZA within in vivo lesions with low oxygen than under in vitro drug susceptibility testing conditions with ambient oxygen.

INTRODUCTION

Pyrazinamide (PZA) is an important front line tuberculosis (TB) drug responsible for the shortening of TB therapy from 9–12 months to the current 6 months, presumably due to its ability to kill ‘semi-dormant’ bacilli that are not killed by other drugs (Mitchison, 1985; Heifets & Lindholm-Levy, 1992). Interestingly, PZA is not active in vitro under normal culture conditions near neutral pH (Tarshis & Weed, 1953) but is only active under acidic conditions (e.g. pH 5-5) (McDermott & Tompsett, 1954), with an MIC of 50–100 µg ml⁻¹ at pH 5-5-6-0 (Zhang & Mitchison, 2003). This may well be a reflection of the in vivo conditions present during active inflammation. However, acid pH alone cannot be the only reason for the difference in activity of PZA observed in vivo versus in vitro, as the serum concentration of PZA is somewhat lower (30–60 µg ml⁻¹) than the MIC in vitro (Zhang & Mitchison, 2003). Although various factors could affect PZA activity (Zhang et al., 2002), exactly what accounts for the overall high sterilizing activity in vivo remains unknown. Recently our laboratory has shown that iron, the level of which could be elevated in local inflammatory lesions (Aisen, 1980), can enhance the activity of PZA in vitro (Somoskovi et al., 2004). However, other potential mechanisms that contribute to PZA activity need to be investigated. One such factor could be the difference in the level of oxygen present in vivo versus in vitro. Tubercle bacilli in granulomatous lesions in vivo are believed to reside in a low oxygen environment (Canetti, 1955). We wondered whether a hypoxic environment in vivo could enhance the activity of PZA, thereby contributing to the differences observed in the activity of PZA in vivo and in vitro. In the current study, we have examined the effect of aeration on PZA activity and shown that low oxygen and anaerobic conditions enhance the activity of PZA.

METHODS

Drugs and chemicals. PZA, isoniazid (INH), rifampicin (RIF), 1,3-dicyclohexylcarbodiimide (DCCD), sodium azide, rotenone and sodium nitrate were obtained from Sigma-Aldrich. PZA, INH, sodium azide and sodium nitrate were dissolved in deionized water at stock concentrations of 10 mg ml⁻¹, 50 µg ml⁻¹, 20 mM and 1 M, respectively, and filter-sterilized. RIF was dissolved in DMSO at a stock concentration of 10 mg ml⁻¹. DCCD and rotenone were dissolved in 95% ethanol at stock concentrations of 200 mM and 800 µM, respectively. All drugs were freshly prepared each day before use.

Mycobacterial growth. Mycobacterium tuberculosis strain H37Ra was grown in 7H9 liquid medium (Difco) supplemented with 0.05% Tween 80 and 10% bovine serum albumin-dextrose-catalase (ADC) enrichment (Difco) at 37°C for approximately 7 days (mid- to late-exponential phase) with occasional agitation.
Effect of aeration on PZA activity. Cells from a 7-day-old *M. tuberculosis* H37Ra culture were harvested by centrifugation at 2800 g for 10 min at 4 °C, washed in PBS (pH 7-0) and then resuspended in 7H9 medium, adjusted to pH 5-5 using HCl to a cell density of about $10^6 - 10^7$ bacilli ml$^{-1}$ based on a standard curve. The cell suspension was then divided into 5 or 15 ml aliquots that received 100 μg PZA ml$^{-1}$, along with controls that received no PZA, followed by incubation at 37 °C for 5 days under aerobic, microaerobic and anaerobic conditions. For aerobic conditions 5 ml aliquots were placed in 50 ml tubes and incubated with rotary aeration at 220 r.p.m., while for microaerobic conditions 15 ml aliquots were placed in 15 ml standing tubes with limited headspace (less than 2 ml). A BBL GasPak 100 anaerobic system (Becton Dickinson) was used for anaerobic conditions, where 5 ml aliquots in 15 ml tubes were incubated in a 2.5-litre jar with a BBL GasPak Plus anaerobic system envelope with palladium catalyst. Methylene blue indicator strips were used to verify anaerobic conditions, where the indicator strip remained colourless during the experiment. After 5 days of incubation, the cells from 5 ml aliquots were harvested by centrifugation as described above, washed with PBS (pH 7-0), serially diluted and plated in triplicate on 7H11 agar plates supplemented with ADC. Plates were incubated at 37 °C for 3–4 weeks prior to determining the number of c.f.u. ml$^{-1}$.

Effect of sodium nitrate on PZA activity under anaerobic conditions. Cells from a 7-day-old *M. tuberculosis* H37Ra culture were harvested as described above, washed in one volume of PBS (pH 7-0) and resuspended in citrate buffer (pH 5-0 or 7-0). The cells were treated with rotenone (4 μM), 1,3-DCCD (1 mM), sodium azide (0.1 mM) or PZA (100 μg ml$^{-1}$), or rotenone, DCCD or sodium azide in combination with PZA, followed by incubation for 5 days at 37 °C under aerobic or anaerobic conditions as described above. After 5 days the cells were washed with PBS (pH 7-0) and the number of c.f.u. ml$^{-1}$ for each sample was determined as described above. Cells were also treated with INH (0.5 μg ml$^{-1}$) or RIF (4 μg ml$^{-1}$) alone in combination with rotenone, DCCD or sodium azide in order to determine if synergy with inhibitors was specific to PZA.

Effect of sodium nitrate on PZA activity under anaerobic conditions. Cells from a 7-day-old *M. tuberculosis* H37Ra culture were harvested by centrifugation as described above, washed in one volume of PBS (pH 7-0) and resuspended in citrate buffer (pH 5-0 or 7-0). The cells were treated with sodium nitrate (10 mM) or PZA (100 μg ml$^{-1}$), or sodium nitrate in combination with PZA, followed by incubation for 5 days at 37 °C under anaerobic conditions in GasPak jars as described above. After 5 days the cells were washed with PBS (pH 7-0) and the number of c.f.u. ml$^{-1}$ was determined.

**RESULTS AND DISCUSSION**

In order to assess the effect of aeration on PZA activity, tubercle bacilli were treated with PZA under aerobic, microaerobic or anaerobic conditions followed by c.f.u. ml$^{-1}$ determinations. As can be seen in Fig. 1, compared with their respective controls, the c.f.u. decreased 2-2-, 13-3- and 386-fold when cells were treated with PZA under aerobic, microaerobic and anaerobic conditions, respectively. The reduction in c.f.u. ml$^{-1}$ under microaerobic and anaerobic conditions was statistically significant when compared to controls ($P < 0.05$; determined by Student’s t-test). However, there was no significant ($P > 0.05$) reduction in c.f.u. ml$^{-1}$ when cells were treated with PZA under aerobic conditions when compared to control. This was expected, as PZA has less activity on young, growing cultures at acid pH (5-5) than on old cultures (Zhang et al., 2002). These results suggest that the level of oxygen has an effect on PZA activity such that the lower the oxygen level, the greater the PZA activity. Our laboratory recently proposed a model for the mechanism of action of PZA (Zhang et al., 1999, 2003; Zhang & Telenti, 2000), whereby pyrazinoin acid, the active form of PZA, functions much like a weak acid, bringing protons into the cell and thereby disrupting the proton motive force of the bacterium and inhibiting transport of nutrients into tubercle bacilli. When *M. tuberculosis*, an obligate aerobe, is subjected to an anaerobic environment, it would produce little or no energy due to inactivity of the electron transport chain. This reduced, or lack of, energy production under anaerobic conditions could increase the susceptibility of *M. tuberculosis* to PZA, as PZA would disrupt the already low energy status of the cell under low oxygen conditions. This could therefore explain the enhancement of PZA activity observed under low oxygen conditions (Fig. 1).

We used a sudden shift of the oxygen conditions in this study. This was because we wanted to test the effect of oxygen concentration on PZA activity in a more controllable fashion. We started with the same inoculum culture and the same number of cells for aerobic, microaerobic and anaerobic conditions for the purpose of easy comparison, whereas if the Wayne model of ‘dormancy’ (Wayne & Hayes, 1996) had been used, we would not have been able to easily compare the effect of oxygen on PZA activity. While we expect the finding of enhancement of PZA activity by sudden exposure to low oxygen in this study to be applicable to the slow adaptation to microaerobic or anaerobic conditions in the Wayne model, this remains to be confirmed in future studies.

Previously, our laboratory has reported an enhancement of PZA activity by various inhibitors of the electron transport chain (Zhang et al., 2003). This synergy of PZA with various inhibitors is likely to be due to the decrease in energy production caused by the inhibitors, which is exacerbated

![Fig. 1. Effect of aeration on PZA activity against *M. tuberculosis*. *M. tuberculosis* H37Ra cells were treated with 100 μg PZA ml$^{-1}$ (filled bars) or not (open bars), under aerobic, microaerobic or anaerobic conditions for 5 days prior to c.f.u. determination on 7H11 plates. Error bars represent SD.](image-url)
by PZA, based on the above model of PZA mode of action. In the present study, the effect of anaerobiosis on that synergy was examined. DCCD, an ATPase inhibitor, rotenone, a Complex 1 inhibitor and sodium azide, a cytochrome c oxidase inhibitor, were included because of a prior observation of their enhancement of PZA activity. Fig. 2 shows the results of treating *M. tuberculosis* with PZA or various ATPase or respiratory chain enzyme inhibitors or a combination thereof under aerobic and anaerobic conditions at pH 5.0 and 7.0. Under aerobic conditions at pH 5.0 an enhanced effect of PZA activity was observed when PZA was administered in combination with either DCCD, azide or rotenone (Fig. 2a) as reported previously (Zhang et al., 2003). A greater than 10-fold reduction in c.f.u. ml⁻¹ was observed for PZA plus DCCD, azide or rotenone compared with PZA alone. At pH 7.0 no enhancement of PZA activity by enzyme inhibitors was observed (Fig. 2b, d), as was expected, considering PZA is only active at acidic pH (McDermott & Tompsett, 1954). The presence of enzyme inhibitors did not result in additional enhancement of PZA activity under anaerobic conditions (Fig. 2c). This is probably because under anaerobic conditions there is no significant electron transport activity in tubercle bacilli such that the electron transport inhibitors have no effect and therefore cannot provide additional enhancement of PZA activity (Fig. 1).

We also examined the effect of proton motive force inhibitors administered in conjunction with the alternative drugs INH and RIF to determine whether the enhancement effect caused by the inhibitors was specific to PZA. We detected no enhanced effect of INH or RIF when given in combination with DCCD, azide or rotenone under either aerobic or anaerobic conditions at pH 5.0 or 7.0 (Fig. 3). Therefore, we conclude that the increased sterilizing activity observed for PZA when combined with energy inhibitors is specific to PZA.

Lastly, we tested the effect of alternative electron acceptors on PZA activity under anaerobic conditions. Although *M. tuberculosis* is generally thought to be an obligate aerobe, the presence of genes encoding enzymes required for anaerobic metabolism, such as nitrate reductases (Cole et al., 1998), and the slow adaptation of *M. tuberculosis* to microaerobic and anaerobic conditions as shown in the Wayne model (Wayne & Hayes, 1996) suggest that *M. tuberculosis* can at least survive under such conditions for some time. Based on our current model of PZA mode of action (Zhang et al., 1999, 2003; Zhang & Telenti, 2000), we hypothesized that if tubercle bacilli were given nitrate as an alternative electron acceptor under anaerobic conditions, allowing for energy production to compensate for the depletion of energy caused by PZA, the activity of PZA might be reduced. Indeed antagonism of PZA activity was

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**Fig. 2.** Effect of aeration on synergy between ATPase and respiratory chain enzyme inhibitors and PZA. *M. tuberculosis* H37Ra cells were treated with 100 µg PZA ml⁻¹, 1 mM DCCD, 0.1 mM azide and 4 µM rotenone under aerobic (a, b) or anaerobic conditions (c, d) for 5 days at pH 5.0 (a, c) or pH 7.0 (b, d) prior to c.f.u. ml⁻¹ determination on 7H11 plates. Error bars represent SD.
observed when cells were treated with PZA in the presence of nitrate (Fig. 4) such that the c.f.u. ml\(^{-1}\) of samples treated with PZA plus nitrate was the same as the untreated control, whereas PZA-treated bacilli under anaerobic conditions in the absence of nitrate had a lower c.f.u. ml\(^{-1}\) as expected (Fig. 4).

It is worth noting that the susceptibility of tubercle bacilli to PZA has never been tested under anaerobic or hypoxic conditions, since \textit{M. tuberculosis} is a strict aerobe and would not grow in the absence of oxygen in drug susceptibility test settings. The current PZA susceptibility testing was performed in the presence of atmospheric oxygen (approx. 20\%) where tubercle bacilli grow well. PZA is a peculiar and unconventional drug that does not kill growing cultures very well, but kills old, non-growing cultures with less energy reserves more effectively (Zhang \textit{et al.}, 2002). In this study we have shown that anaerobic or hypoxic conditions, which do not allow tubercle bacilli to grow, enhanced the activity of PZA (Fig. 1). In addition to acid pH (McDermott & Tomsett, 1954) and iron (Somoskovi \textit{et al.}, 2004) that enhance the activity of PZA, this study provides another explanation for the high sterilizing activity of PZA against tubercle bacilli \textit{in vivo} in granulomatous lesions with low oxygen. Additionally, we have shown that PZA activity is decreased when tubercle bacilli are supplied with the alternative electron acceptor nitrate for energy production under anaerobic conditions. These observations provide further support for our model of the mode of action of PZA, whereby PZA disrupts the membrane potential and energy store of old, non-growing tubercle bacilli with little metabolic activity or energy reserves, which leads to a slow death of the bacilli (Zhang \textit{et al.}, 2003).

**ACKNOWLEDGEMENTS**

The work was supported by research funding from NIH (AI4063 and AI4985). M. M. W. was supported by NIH training grant T32 AI07608.
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