Susceptibility of *Mycobacterium tuberculosis* to weak acids

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Received 21 January 2003; returned 20 March 2003; revised 11 April 2003; accepted 16 April 2003

The susceptibility of *Mycobacterium tuberculosis* and *Mycobacterium smegmatis* to a range of weak acids and acid pH was investigated. *M. tuberculosis* was found to be more susceptible to acid pH and weak acids than *M. smegmatis*. The weak acids were more active against *M. tuberculosis* at acid pH than at neutral pH. *M. tuberculosis* was found to be less able to maintain its internal pH and membrane potential at acid pH than *M. smegmatis*. The antituberculous activity of weak acids correlated with their ability to disrupt the membrane potential but not the internal pH. The significance of these findings is discussed in relation to *M. tuberculosis* physiology and development of new antituberculous agents.

Keywords: weak acid, antimicrobial susceptibility, pH homeostasis, membrane potential, *Mycobacterium tuberculosis*

Introduction

Tuberculosis (TB) still remains a major infectious cause of morbidity and mortality world-wide, especially in the developing countries. Drug-resistant TB is becoming an increasing public health concern in recent years and poses a potential threat to the control of the disease. There is growing awareness that the current TB therapy is too long, taking a period of 6 months. Failure to adhere to the lengthy therapy is a frequent cause of drug-resistant TB. There is currently a great deal of interest in developing new drugs that are not only active against drug-resistant TB but can also shorten the duration of the therapy.

During our study of the mode of action of the frontline TB drug pyrazinamide, a drug that has shortened TB therapy from 9–12 months previously to 6 months, we have shown that *Mycobacterium tuberculosis* seems to be uniquely susceptible to the weak acid pyrazinoic acid ($K_a = 2.9$), the active form of pyrazinamide; whereas other mycobacteria (e.g. *Mycobacterium smegmatis* or bacteria (e.g. *Escherichia coli*) are more resistant to pyrazinoic acid. In addition, it is well known that during pyrazinamide susceptibility testing, which requires acid pH for activity, the growth of *M. tuberculosis* is inhibited if the medium pH is below 5.5. *M. tuberculosis* appears to be quite susceptible to acid pH compared with other mycobacteria. In Sauton’s simple salt medium, the growth of *M. tuberculosis* was restricted at pH 6.0, whereas other mycobacterial species grew quite well. In this study, we tested whether *M. tuberculosis* is also susceptible to other weak acids in addition to pyrazinoic acid and compared the susceptibility of *M. tuberculosis* to acidic pH and a range of weak acids with that of *M. smegmatis*. We have shown that *M. tuberculosis* is significantly more susceptible to acidic pH and weak acids in general than *M. smegmatis*. The antimycobacterial activity of the weak acids is enhanced at acid pH. The basis of the susceptibility of *M. tuberculosis* to acid pH and weak acids is investigated.

Materials and methods

*Mycobacterial growth and susceptibility to acid pH*

*M. tuberculosis* strain H37Ra was grown in 7H9 liquid medium (DIFCO) supplemented with 0.05% Tween 80 and 10% bovine serum albumin-dextrose-catalase enrichment (DIFCO) at 37°C for 3 weeks with occasional shaking. *M. smegmatis mc²6 (MC²)* was similarly cultivated in the 7H9 medium at 37°C for 4 days. To test the susceptibility of mycobacteria to different pH values, *M. tuberculosis* H37Ra or *M. smegmatis* cells were resuspended in sodium phosphate buffer adjusted to different pH levels (pH 3.0, 4.0, 5.0, 6.0, 7.0) in 1 mL to a cell density of 1.30 at OD₆₀₀ and incubated at 37°C. At 1, 3, 5 and 7 days, aliquots of the cell suspension were removed, washed and diluted before plating on 7H11 plates. The plates were then incubated at 37°C for 4 weeks for *M. tuberculosis* and for 5 days for *M. smegmatis* to determine the number of surviving bacteria.

*Susceptibility to weak acids and isolation of weak acid resistant mutants*

Various weak acids were obtained from Sigma Chemical Co., and were dissolved in DMSO at appropriate concentrations. The weak acids were incorporated into 7H11 agar at various concentrations. Three-week-old stationary phase *M. tuberculosis* H37Ra culture or 4-day-old *M. smegmatis* mc²6 culture were tested for susceptibility to weak acids on 7H11 plates at pH 6.8 and pH 5.5 as described. For the isolation of weak acid mutants, about 10⁸ colony forming units (cfu) of *M. tuberculosis* H37Ra

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were plated on acidic 7H11 agar plates (pH 5.5) containing various concentrations of weak acids such as salicylate, benzoic acid, nonyloxybenzoic acid and mfenamic acid. The plates were incubated at 37°C for 4 weeks before being examined for the emergence of spontaneous mutants.

Measurement of intracellular pH and membrane potential

The internal pH of mycobacteria was measured as described previously. Membrane potential was measured with [3H]tetraphenylphosphonium bromide (TPP+) using the method as described. Briefly, 3-week-old H37Ra or 4-day-old M. smegmatis cells were resuspended in Sauton's medium at different pH values to measure the change in the membrane potential in response to changes in external pH after incubating the cells at room temperature for 50 min. [3H]TPP+ (380 mCi/mmol) at 10 µM final concentration was then added to the cell suspension and the mixture was fully mixed before silicone oil was added and the mixture incubated for another 10 min. The mixture was spun at 12,000 rpm for 3 min, and 100 µL supernatant was taken for scintillation counting. The cell pellets were then snap-frozen in an alcohol/dry ice bath. The bottom of the tubes containing the cell pellets were cut off for scintillation counting. To determine the effect of weak acids on membrane potential and internal pH, various weak acids were incubated with mycobacterial cells suspended in pH 5.5 Sauton's medium for 1 h when the measurements were made as described above. Valinomycin (10 µM) and nigericin (10 µM) were used as controls for the membrane potential and internal pH measurements.

Results

Susceptibility of M. tuberculosis and M. smegmatis to acid pH

The acid sensitivity of M. tuberculosis and M. smegmatis was determined by exposing the bacilli to various acidic pH conditions using pH 7.0 as a control, and plated for survivors after exposure for different times. The relative sensitivity of the two mycobacterial species to acidic pH was expressed as the percentage of bacterial survival by dividing the cfu obtained after exposure to acid pH by that at neutral pH. At pH 3.0, there was relatively little difference between M. tuberculosis and M. smegmatis in terms of survival due to extreme acidity (Table 1). However, at pH 4.0 and 5.0, M. tuberculosis was significantly more sensitive to acid pH than M. smegmatis (Table 1).

Susceptibility of M. tuberculosis to weak acids

As shown in Table 2, M. tuberculosis was more susceptible than M. smegmatis to a range of weak acids. The antimycobacterial activity of the weak acids was more pronounced at acid pH than at close to neutral pH for both mycobacterial species. In addition, the activity of the weak acids appeared to correlate with their pKₐ values, i.e. the lower the pKₐ, the higher the antimycobacterial activity (Table 2). It is noteworthy that M. tuberculosis was susceptible to linoleic acid (MIC 37 mg/L at pH 5.5) but not to linoleic acid ethyl ester (MIC >1000 mg/L at pH 5.5), indicating that the acid form COOH is active and that M. tuberculosis does not have an appropriate esterase to convert linoleic acid ethyl ester to the active acid form.

Inability to isolate weak acid resistant mutants of M. tuberculosis

We have shown previously that no pyrazinoinic acid-resistant mutants of M. tuberculosis could be isolated. To determine whether this is a more generalized phenomenon, we attempted to isolate M. tuberculosis H37Ra mutants resistant to a range of weak acids such as salicylic acid, benzoic acid and 4-nonyloxybenzoic acid. However, we were unable to isolate any mutants resistant to the weak acids even at very high density of cells (10⁹ cfu/mL) on 7H11 plates (data not shown).

Inefficient maintenance of intracellular pH in M. tuberculosis

We compared the intracellular pH of M. tuberculosis and M. smegmatis in response to changes in external pH (Figure 1a). Between pH 5 and pH 7, the two organisms behaved similarly in terms of changes in internal pH. However, under more acidic conditions (pH 3–5), the internal pH of M. smegmatis remained fairly stable at values of 5.7–5.9; in contrast, the internal pH of M. tuberculosis became more acidic, reaching 5.2 at an external pH of 3.2 (Figure 1a). This indicates that M. tuberculosis is less efficient at maintaining the internal pH than M. smegmatis. In addition, valinomycin and nigericin had a more pronounced effect on lowering the internal pH of M. smegmatis.
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but had little effect on *M. tuberculosis* (Figure 1b and c). This finding lends further support to the idea that *M. smegmatis* has a more active apparatus to maintain its internal pH at acid pH conditions (pH 3–5) than *M. tuberculosis*.

Inefficient maintenance of membrane potential in *M. tuberculosis*

We compared the membrane potential of *M. tuberculosis* and *M. smegmatis* in response to changes in external pH. The membrane potential of *M. tuberculosis* was generally higher than that of *M. smegmatis* except at the very acidic pH of 3.5, at which there was little difference in the membrane potential between the two organisms. However, the membrane potential of *M. tuberculosis* was more sensitive to changes in external pH than *M. smegmatis* between pH 4 and pH 8.5 (Figure 2). The more responsive change in the membrane potential of *M. tuberculosis* compared with *M. smegmatis* is most likely due to a poor ability of *M. tuberculosis* to maintain its membrane potential under different external pH conditions.

Correlation between activity of weak acids and their ability to disrupt membrane potential or lower internal pH

The susceptibility of *M. tuberculosis* and *M. smegmatis* to weak acids was examined in the context of membrane potential and internal pH. It was found that the susceptibility of *M. tuberculosis* to weak acids appeared to correlate with their ability to disrupt membrane potential (Figure 3a). In contrast, weak acids had little effect on the disruption of membrane potential in the non-susceptible species *M. smegmatis* (Figure 3a). The antimycobacterial activity of the weak acids did not correlate well with their ability to decrease the internal pH (Figure 3b).

Discussion

In this study, we have shown that *M. tuberculosis* is more susceptible to acidic pH than the fast growing *M. smegmatis* (Table 1). The higher susceptibility of *M. tuberculosis* to acid pH is presumably a reflection of its poor ability to maintain pH homeostasis than the less susceptible *M. smegmatis*. Indeed, comparison of internal pH in response to changes in external pH indicated that *M. tuberculosis* has a lower ability to maintain internal pH at acid pH between pH 3 and pH 5 than *M. smegmatis* (Figure 1a). This is further strengthened by the finding that valinomycin and nigericin had a significant effect on lowering the internal pH in *M. smegmatis* but not in *M. tuberculosis* (Figure 1b and c). The deficiency of *M. tuberculosis* in maintaining the internal pH towards neutrality at very acidic pH conditions

Figure 1. Changes in internal pH of *M. tuberculosis* H37Ra (Ra) and *M. smegmatis* mc²⁶ (MC2) in response to external pH and valinomycin plus nigericin. Comparison of internal pH changes in response to external pH is shown in (a). The changes in internal pH of *M. tuberculosis* and *M. smegmatis* in response to valinomycin (V) plus nigericin (N) are shown in (b) and (c), respectively.

Figure 2. Comparison of the membrane potential of *M. tuberculosis* H37Ra and *M. smegmatis* MC2 in response to changes in external pH.

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M. smegmatis is uniquely susceptible to a range of weak acids compared with M. tuberculosis. The observation that various weak acids appeared to preferentially disrupt the membrane potential of M. tuberculosis over that of M. smegmatis (Figure 3a) supports this notion. This differential disruption of membrane potential in M. tuberculosis by the weak acids could result from the slow metabolism and consequently slow energy production in the slow growing M. tuberculosis and a defective efflux mechanism as shown for pyrazinamide.3

Whereas there is no difference in membrane potential between the two organisms at very acidic pH (pH 3), it is surprising that the membrane potential of M. smegmatis is generally lower than that of M. tuberculosis (Figure 2). This could indicate that the probe TPP+ used to measure the membrane potential is actively extruded by M. smegmatis but not by M. tuberculosis. The observation that valinomycin and nigericin did not affect the membrane potential in M. smegmatis but did so in M. tuberculosis (not shown) could be due to valinomycin and nigericin not getting into M. smegmatis cells or an active efflux mechanism for the membrane potential probe TPP+. Because valinomycin and nigericin were shown to affect the internal pH of M. smegmatis (Figure 1c), the first possibility of these agents not getting into the cells can be ruled out. Therefore, it is likely that M. smegmatis has an active efflux for TPP+, which is responsible for the measured lower membrane potential in this organism compared with M. tuberculosis.

That M. tuberculosis appears to be uniquely susceptible to weak acids may have implications for the design of new antituberculosis drugs. However, weak acids may not be easily absorbed through the gastrointestinal tract or bind to serum proteins. To circumvent this potential problem, it may be necessary to make precursors of weak acids such as ester or amide of weak acids for in vivo use. To show activity the weak acid precursors will have to be hydrolysed by gastrointestinal enzymes present in M. tuberculosis, which is known to contain a range of esterases and amidases in the genome.13 Future studies are needed to determine whether weak acid precursors can be developed into antituberculosis agents useful for the treatment of TB.

Acknowledgements

We thank Peter Maloney for helpful discussions. The research support from NIH (AI-44063) and the Potts Memorial Foundation to YZ is gratefully acknowledged.

References

4. Schaller, A., Guo, M., Gisanrin, O. A. et al. (2002). Escherichia coli genes involved in resistance to pyrazinamide, the active component of

(pH 3–5) could result from an increased proton permeability of the M. tuberculosis membrane or a decreased proton extrusion by the membrane-embedded ATPase compared with M. smegmatis. Further studies are needed to distinguish the two possibilities.

An important observation of this study is that M. tuberculosis is uniquely susceptible to a range of weak acids compared with M. smegmatis (Table 2) and indeed other bacteria such as E. coli (data not shown). The antituberculous activity of the weak acids appeared to inversely correlate with the pKₐ of the weak acid (Table 2), i.e. the lower the pKₐ (the stronger the weak acid), the stronger the antibacterial activity. For example, salicylic acid and nicotinic acid have pKₐ values of 3 and 4.8, respectively, and their MICs for M. tuberculosis were 10–20 and 200 mg/L at pH 5.5, respectively. In addition, the antimycobacterial activity of the weak acids was enhanced at acid pH (Table 2). This is consistent with the fact that, at acidic pH, weak acids become protonated and form uncharged species that permeates through the membrane easily compared with charged anion species.10 Enhanced activity of weak acids at acid pH is consistent with the observation that uptake and accumulation of weak acids are increased at acidic pH, as shown for pyrazinamide.3 The consequence of weak acid accumulation and recycling could lead to disruption of the proton motive force that is required for the transport of many nutrient substances into bacterial cells as a mechanism of action of weak acids.12


