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Rapid Killing of *Mycobacterium terrae* by *N*-Chlorotaurine in the Presence of Ammonium is Caused by the Reaction Product Monochloramine

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Abstract

We have studied the activity of the weak endogenous oxidant *N*-chlorotaurine against *Mycobacterium terrae*.

The study revealed slow killing of more than 2 h duration by 1% (55 mM) *N*-chlorotaurine. In the presence of ammonium chloride, however, killing times decreased to a few minutes, even by 0·1% *N*-chlorotaurine.

This phenomenon is explained by formation of the lipophilic and therefore more bactericidal monochloramine as a result of transhalogenation of ammonia by *N*-chlorotaurine.

N-Chlorotaurine (Cl-HN-CH_2-CH_2-SO_3H), the *N*-chloro derivative of the amino acid taurine, is a weak oxidant produced at high concentrations in man by granulocytes and monocytes (Lampert & Weiss 1983; Grisham et al 1984). *N*-Chlorotaurine has significant microbicidal efficacy against bacteria, yeasts (Nagl & Gottardi 1992, 1996) viruses (Nagl et al 1998a) and worms (Yazdanbakhsh et al 1987).

The bactericidal and fungicidal activity of *N*-chlorotaurine has been shown to be enhanced by the presence of low molecular-weight amino compounds and also in inflammation samples because of transformation to more bactericidal *N*-chloro derivatives (Nagl & Gottardi 1996). Rapid inactivation of omniresistant *Pseudomonas aeruginosa* by *N*-chlorotaurine in urine has been considered to be connected with the formation of monochloramine (NH_2Cl) by halogenation of ammonium (Nagl et al 1998c). The bactericidal activity of NH_2Cl has already been proved (LeChevallier et al 1988; Jacangelo et al 1991). Its mycobactericidal effect, however, has not yet been investigated.

This study was performed to examine whether the in-situ production of monochloramine by reaction of ammonium and *N*-chlorotaurine could induce sufficient activity against mycobacteria which are usually highly resistant to disinfectants.

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To verify that the bactericidal effect is caused solely by NH_2Cl and not by other *N*-chloro compounds of low molecular weight (amino acids and oligopeptides from the agar), the experiments were conducted with thoroughly washed bacteria.

Materials and Methods

Reagents

Pure *N*-chlorotaurine as the crystalline sodium salt (MW 181·52 g mol^{-1}) (Nagl & Gottardi 1996) was dissolved in 0·1 M phosphate buffer, pH 6·8–7·2. The identity of the *N*-chlorotaurine was proved by IR spectrometry, purity by iodometric titration (calculated 19·53% Cl^+, found 19·3% Cl^+, purity 99%). Reagent grade ammonium chloride, sodium thiosulphate and buffers (sodium dihydrogen phosphate and disodium hydrogen phosphate) were purchased from Merck (Darmstadt, Germany).

Bacteria, media and assay of antibacterial action

Mycobacterium terrae ATCC 15755 (van Klingeren Pullen 1987; Sattar et al 1995) was obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and grown on Löwenstein-Jensen slant agar (Becton Dickinson, USA) at 37°C for 21 days until a layer of non-countable colonies had formed. After addition of NaCl (0·9%, w/v; 10 mL) to the agar tube, bacteria were removed from the agar by sonication

for 2 min (Bandelin Sonorex RK 102 water bath, 35 kHz, 120/240 W) without loss of viability. Bacteria were centrifuged twice at 1800 *g* for 10 min and washed twice with NaCl (0·9%) to remove from the suspension organic compounds which could have reacted with *N*-chlorotaurine and falsified the results. The suspensions were vortex-mixed vigorously (3 × 5 s) before each centrifugation and sonicated again for 2 min to furnish a homogenous suspension of $2-9 \times 10^7$ colony-forming units mL^{-1}. This suspension (0·1 mL) was added to solutions (0·9 mL) of *N*-chlorotaurine or of *N*-chlorotaurine + NH_4Cl. Controls without *N*-chlorotaurine were treated the same way.

Subsequent to incubation for 1, 5, 10 and 20 min at room temperature, samples (0·1 mL) were mixed with sodium thiosulphate solution (3–9%, w/v; 0·1 mL) to inactivate 0·1%, 1% and 3% (w/v) *N*-chlorotaurine, respectively. This solution (50 μL) and similar volumes of serial tenfold dilutions in 0·9% NaCl were added to Löwenstein-Jensen slant agar which was carefully tilted five times to distribute the bacteria optimally on the agar surface and then stored at 37°C for 6 weeks. Colony-forming units were counted after 3 weeks of growth, later evaluations did not reveal any change in the results.

Results

Action of N-*chlorotaurine without additives*

In buffer solution, no killing by 1% *N*-chlorotaurine was observed within 120 min incubation time. Incubation times of 7 days and 14 days yielded a reduction in colony-forming units of 4·0 ± 1·2 and 5·9 ± 0·5 $\log_{10}$, respectively, for samples containing 1% *N*-chlorotaurine and of 1·0 ± 0·5 and 1·5 ± 0·4 $\log_{10}$, respectively, for controls (mean values ± standard deviation, n = 5, $P < 0.01$ by use of Student's paired *t*-test).

Action of N-*chlorotaurine in the presence of ammonium chloride*

In contrast with *N*-chlorotaurine without additives, addition of ammonium chloride resulted in significant bactericidal activity after incubation times of only a few minutes. Time-kill curves are shown in Figure 1. Killing proved to be more dependent on the concentration of ammonium chloride than on that of *N*-chlorotaurine.

As expected, no reduction in the number of colony-forming units could be observed in presence of 0·1–10% ammonium chloride without *N*-chlorotaurine. Inactivation of 10% ammonium chloride and 3% *N*-chlorotaurine by addition of 9% sodium thiosulphate before addition of the bacteria also removed any bactericidal effect.

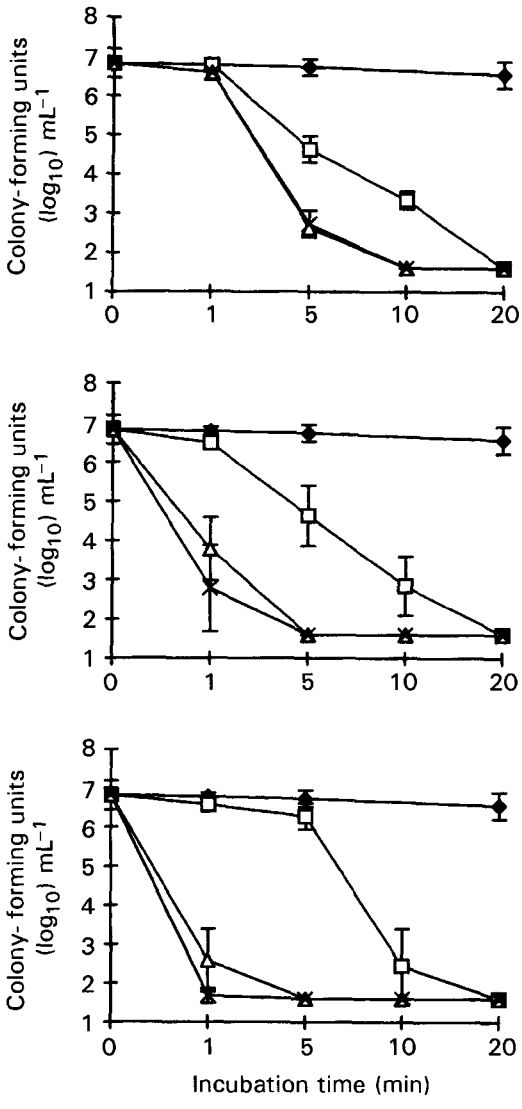

Figure 1. Killing of *M. terrae* by 0·1% (upper), 1·0% (middle) and 3·0% (lower) *N*-chlorotaurine in the presence of 0·1% (□), 1% (△) or 10% (×) NH_2Cl at room temperature and pH 6·8–7·2 (7·8 for 3% *N*-chlorotaurine + 0·1% NH_4Cl). Results from controls (◆) in phosphate buffer without any additives were no different from those containing 10% NH_4Cl only. Values are means ± standard deviations of colony-forming units ($\log_{10}$) mL^{-1}, n = 3–6; $P < 0.01$ for all cases with ≥0·5 $\log_{10}$ difference between samples and controls (Student's paired *t*-test).

The very large increase in killing power in the presence of ammonium chloride can, therefore, be attributed solely to the formation of NH_2Cl, which proved to be a highly mycobactericidal agent.

Discussion

In this study the mycobactericidal action of *N*-chlorotaurine has been investigated for the first time. Although, as expected, the action proved to be weaker than that against other Gram-positive and Gram-negative bacteria (Nagl & Gottardi 1992,

Table 1. Equilibrium concentrations* of NH_2Cl (mM) in aqueous solutions from different initial concentrations of ammonium chloride and *N*-chlorotaurine at pH 7·0.

Concentration of *N*-chlorotaurine (% (mM))	Concentration of ammonium chloride (% and (mM))		
	0·1 (18·7)	1·0 (187·0)	10·0 (1869·5)
3·0 (165·3)	4·7	14·7	34·6
1·0 (55·1)	3·1	8·7	18·5
0·1 (5·5)	1·1	2·4	3·9

* Calculated with $K_{NH2Cl} = 0{\cdot}02$.

1996), it was improved to a surprising extent by ammonium chloride, which produces monochloramine (NH_2Cl) by transhalogenation (Thomas 1979; Grisham et al 1984; Nagl & Gottardi 1996):

$$Cl{-}HN{-}CH_2{-}CH_2{-}SO_3^- + NH_4^+ \Leftrightarrow H_3N^+{-}CH_2{-}CH_2{-}SO_3^- + NH_2Cl \quad (1)$$

Increasing the molar ratio NH_4Cl/*N*-chlorotaurine significantly increased the speed of mycobactericidal action, indicating increased formation of NH_2Cl, in accordance with the law of mass action. With the known equilibrium constant of equation 1 ($K_{NH2Cl} = 0{\cdot}02$; Nagl & Gottardi 1996) the concentration of NH_2Cl in these experiments could be estimated (Table 1). It is apparent that there is a good correlation between calculated NH_2Cl concentrations and bactericidal activity denoted as $\log_{10}$ reduction in colony-forming units. From nine results with 1-min incubation time linear regression ($R = 0{\cdot}91$) gave an average of $0{\cdot}00497 \pm 0{\cdot}00080\,mol\,L^{-1}$ NH_2Cl necessary to effect a one $\log_{10}$ kill.

Because mycobacteria are, because of their special cell wall with high lipid content (Russell et al 1986), more resistant to chemical attack than other vegetative bacteria, NH_2Cl as a lipophilic compound (Grisham et al 1984) will be able to penetrate mycobacteria more easily than the hydrophilic *N*-chlorotaurine. Because the substantial difference between the action of *N*-chlorotaurine and NH_2Cl is unrelated to reactivity, but more to size and polarity (Thomas 1979; Nagl & Gottardi 1996), our findings accentuate the importance of penetration for rapid mycobactericidal action. This consideration is in keeping with the mechanism of inactivation of *Escherichia coli* by NH_2Cl, which is based on inhibition of respiration, substrate dehydrogenation and bacterial transport rather than on damage to the cell envelope (Jacangelo et al 1991).

This study reveals the importance of NH_2Cl as a highly effective bactericidal substance. However, because of its low stability it cannot be stored and has to be produced in-situ from ammonia by a chlorinating agent. For this reason *N*-chlorotaurine, which is well tolerated by living tissue (Nagl et al 1998b, c), must be preferred to other, more strongly oxidizing and therefore more irritating compounds such as chloramine T or hypochlorite.

Further studies will be necessary to evaluate the role of endogenous ammonium which obviously acts as an amplifier of the bactericidal properties of chlorine-based oxidants.

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