Clinical significance of inhibition kinetics for Streptococcus pyogenes in response to penicillin

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Objectives: The antibiotic mode of action against clinical isolates of Streptococcus pyogenes and physiological factors involved in modifying the inhibitory response to the antibiotic were investigated.

Methods: We developed high-resolution respirometry for continuous monitoring of bacterial growth and inhibition kinetics. One hundred and ten clinical isolates from 90 paediatric patients were tested, including 48 isolates obtained from 28 patients with eradication failure. Respirometric inhibition curves were monitored at 4 mg/L penicillin G over a short 30 min period, corresponding to the drug’s serum half-life.

Results: None of the clinical isolates exhibited penicillin tolerance. Latency in the respirometric response of S. pyogenes to penicillin increased significantly with decreasing strain-specific respirometric growth rate. No difference in inhibition kinetics was found in vitro for isolates from patients with or without bacteriological treatment failure.

Conclusions: In streptococcal pharyngotonsillitis, tolerance is not a relevant concept to explain bacteriological treatment failure. Definitions of tolerance should be reconsidered in the framework of growth-dependent antibiotic susceptibility.

Introduction

Phenoxymethylpenicillin is the drug of choice for streptococcal pharyngotonsillitis because of its good tolerance, low side-effect profile and high in vitro activity against Streptococcus pyogenes.1,2 Bacteriological and clinical treatment failures, however, are well documented for S. pyogenes pharyngotonsillitis.3–7 A meta-analysis of therapeutic studies from 1953 to 1993 on streptococcal pharyngotonsillitis indicates an eradication failure rate of 12%.8 Only in a minority of the cases reported is failure attributed to poor patient compliance, re-infection or insufficient serum levels of penicillin.5,8 Hence, the discrepancy between lack of in vitro resistance to penicillin in wild-type S. pyogenes9 and failure of penicillin therapy requires explanation.

The role of penicillin tolerance as a cause for eradication failure remains controversial. ‘Tolerance occurs when, under defined conditions, a test substance that is usually bactericidal for the bacteria tested shows a diminished or absent bactericidal effect without loss of inhibitory action’.10 Rates of penicillin tolerance reported for S. pyogenes depend on the definition of tolerance as the ratio of MBC to MIC of ≥32.11 or ≥16.5,10 Amsterdam12 questioned this rationale, since ‘in view of the lack of reproducibility of conventional MBC methods, the definition [of tolerance] should be treated with caution’.

An unequivocally tolerant isolate of S. pyogenes cannot be obtained from the ATCC, National Reference Centre for Streptococci (Institute of Medical Microbiology, Aachen, Germany) or from authors who described tolerant S. pyogenes strains.13,14 Whereas some studies on S. pyogenes pharyngotonsillitis failed to isolate any penicillin-tolerant strain,15–17 Brett18 claimed penicillin tolerance in 29% (129 of 434) of clinical isolates of S. pyogenes tested in 1 year. Nevertheless, the clinical significance of tolerance has never been estab-
lished.3 Whereas one study observed that penicillin-tolerant strains were more frequently isolated from patients with initial penicillin treatment failure,2 another investigation reported a lower treatment failure rate in patients with penicillin-tolerant strains.3 In a prospective study on children with clinical symptoms of S. pyogenes-associated pharyngotonsillitis, we found an eradication failure rate of 20% (30 of 149 patients).1 In the present study, we present the penicillin-inhibition kinetics in these streptococcal isolates, to elucidate the clinical significance of penicillin tolerance of S. pyogenes.

One of our major concerns was the ill-defined antimicrobial activity during the long standard incubation time of 24 h for determination of MBC/MIC ratios, considering the short serum half-life of penicillin of 40 min.19 Using a new respirometric in vitro method, therefore, we analysed inhibition curves with high time resolution over periods of 30 min.

Materials and methods

Clinical isolates and reference strains

One hundred and ten clinical isolates of S. pyogenes were tested after collection from 90 paediatric patients with streptococcal pharyngotonsillitis. This prospective study included 48 isolates from 28 patients with eradication failure. The patients received penicillin V at a dose of 100 000 U/kg/day divided into three daily doses and given orally over a period of 10 days.1 Isolates were stored in tubes with Columbia agar (Becton Dickinson, Cockeysville, MD, USA) with 5% defibrinated sheep blood at 4°C. A penicillin-susceptible S. pyogenes (ATCC 19615; MIC = 0.03 mg/L, MBC = 0.06 mg/L) and a penicillin-tolerant Listeria monocytogenes (ATCC 19611; MIC = 0.125 mg/L, MBC > 128 mg/L) were used as reference strains. Listeria monocytogenes exerts intrinsic penicillin tolerance.20 A reportedly penicillin-tolerant streptococcal strain (L36K; MIC = 0.0156 mg/L, MBC = 0.5 mg/L) was kindly provided by Claas Schälen.14 These MIC and MBC values were determined with a macro broth dilution test.21 For respirometry, single colonies were incubated in 5 mL of culture medium 12 h before initiation of measurements. Todd–Hewitt and Columbia broth (Becton Dickinson) were used for S. pyogenes and L. monocytogenes, respectively. The NCCLS-recommended test medium for susceptibility testing of these organisms is Mueller–Hinton broth supplemented with 3–5% lysed horse blood.22 Preliminary test results yielded identical MICs for our quality control strains grown in Mueller–Hinton both supplemented with lysed horse blood and the culture media chosen for respirometry.

Antimicrobial agents

Screening for tolerance to penicillin G (Biochemie, Kundl, Austria) was performed at a final concentration of 4 mg/L. Since there is no NCCLS-recommended penicillin-resistant breakpoint for haemolytic streptococci (only susceptible: penicillin MIC ≤0.12 mg/L), the penicillin-resistant breakpoint for viridans streptococci was chosen in our study.22 Reference strains were additionally tested with clindamycin (Pharmacia & Upjohn, Puurs, Belgium; 3.3 mg/L final concentration), a drug with in vitro bactericidal action against S. pyogenes23 and with the bacteriostatic drug sulfadiazine (Sigma, St Louis, MO, USA; 120 mg/L final concentration). Sulfadiazine was dissolved in water (70°C) with 2.5 mol/L sodium hydroxide and added to Todd-Hewitt broth yielding a stock solution of 480 mg/L.

High-resolution respirometry

High-resolution respirometry was performed at 37°C in a two-chamber Oxygraph (OROBOROS Instruments, Innsbruck, Austria).24–26 The 2 mL glass chambers with titanium stoppers and PEEK-coated stirrers26 were sterilized before every experiment with 3% formaldehyde for 10 min. The chambers were washed three times with distilled water and filled with culture medium saturated with air at 37°C. After closing the chamber, the stirring rate was set at 280–310 rpm, and oxygen concentration was recorded every 5 s over experimental periods of 1.5–2 h (up to 8 h in specific cases).

Fifty micro litres of bacterial suspension was injected through a titanium cannula into the chamber, yielding an initial cell density of 10^6 cfu/mL. Respiration per volume is the negative time derivative of oxygen concentration, which was displayed online using the DatLab software.24,26 The increase of respiration over time (time derivative of respiration per volume) is the respiratory growth rate. Respiration reflects the total aerobic activity of the culture in the batch system, yielding an integrative measure of cell density and oxidative metabolic activity per cell. Oxygen concentration did not decline to <20% air saturation, thus preventing oxygen limitation of aerobic metabolism.24 Within 10–30 min of incubation, respiration increased to 47 ± 17 pmol/s/mL (range 15–90 pmol/s/mL). At this time, t_0, 50 µL of antibiotic stock solution was injected. Respiration was expressed as a percentage of the rate at t_0 (Figure 1). The rate of respiration at t_0 did not correlate with any index of the respiratory inhibition curves (P = 0.7–0.9).

Standard tests were performed for instrumental background and autoxidation of the culture media prior to every experiment in the sterilized chamber.26 Autoxidation was 2–4 pmol O_2/s/cm^3 for Todd-Hewitt broth, independent of oxygen concentration in the range from air saturation to 20 µM O_2. The sum of instrumental background and autoxidation never exceeded 5% of respiration recorded in the presence of cells and was subtracted from experimental records for analysis of respirometric growth curves.
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Cell count

Viable counts were performed by removing samples of 25 µL from the oxygraph chambers at 30 min intervals. To prevent antibiotic carryover, these samples were diluted 1:100 in Todd-Hewitt broth before logarithmic plating of 50 µL of these dilutions on Columbia agar plates (plus 5% defibrinated sheep blood) using a Whitley automatic spiral plater (Don Whitley Scientific, West Yorkshire, UK). Colony forming units were counted after incubation at 37°C for 24 h.

Data analysis

Continuous records of oxygen concentration and respiratory rate were analysed using DatLab (OROBOROS Instruments). Statistical analysis was carried out using Pearson’s correlation coefficient for continuous data. *P* < 0.05 was considered statistically significant. All statistical analyses were performed using the software SPSS 10.0 (SPSS Inc., Chicago, IL, USA).

Results

Growth curves of *S. pyogenes* ATCC 19615 based on respiration or viable cell count increased in the controls without the addition of antibiotics over test periods of 75 min (Figure 1a). Respiriometric growth rates over 15 min before drug addition served as internal baseline controls. After the addition of penicillin, respiration continued to increase over a latent period, followed by a decline of respiration corresponding to the decrease of the viable cell count. In contrast, immediate inhibition without a latent period was observed with clindamycin (Figure 1a). With sulfadiazine, respiratory growth continued over a latency time ≥ 2 h, followed by a constant respiration rate (data not shown), indicative of growth arrest due to bacteriostatic effects. During the latent period, respiratory growth was inhibited compared with controls without antibiotics (Figure 1).

Figure 1(b) shows respiration of *S. pyogenes* ATCC 19615 in comparison with *L. monocytogenes* ATCC 19611, an organism with intrinsic penicillin tolerance. Growth curves without antibiotics were identical in these organisms. The addition of penicillin resulted in a comparatively long latent period of 45 min in *L. monocytogenes*, when the baseline growth rate at the time of the addition of the drug was 68% per hour. Respiration increased up to a maximum of 150% and remained constant (at 140% for ≥ 8 h; data not shown), indicative of bacteriostasis due to penicillin tolerance. The MIC/MBC ratios of *S. pyogenes* ATCC 19615 and the reportedly penicillin-tolerant streptococcal strain L36K were 0.03/0.06 and 0.0156/0.5 mg/L, respectively. Their respiriometric inhibition curves were indistinguishable (Figure 1; L36K data not shown).

To simulate a bacteriostatic mode of action of penicillin in *S. pyogenes* ATCC 19615, the temperature was lowered from 37 to 20°C, resulting in growth arrest. Bacterial growth was monitored over 3 h at 37°C, to yield a high microbial density and high respiration per volume (Figure 2). After a sudden switch to 20°C, respiration was reduced by 90% to a constant level, representing a non-proliferative state with constant cell density. Neither respiration nor cell density declined after the addition of penicillin G to these dormant bacteria. The addition of formaldehyde (1% final concentration), however, killed the bacteria and respiration ceased immediately.

None of the 110 clinical isolates of *S. pyogenes* showed penicillin tolerance according to the traditional MIC/MBC testing. Applying short-term respiriometric inhibition analysis as an additional tool for the detection of penicillin tolerance,
penicillin inhibition curves of these 110 isolates resulted in average latencies of 10.4 min (±5.1 min S.D.; range from 1 to 25 min; Figure 3a). The duration of the latent period was inversely correlated with the baseline growth rate (increase of respiration over time before the addition of the drug), without any distinction between isolates from patients with treatment success or failure (Figure 3a; correlation coefficients –0.48 and –0.61, respectively).

Respiratory growth rates observed during latency increased in proportion to baseline growth rates measured before the addition of the drug (Figure 3b; correlation coefficients 0.48 and 0.51, for treatment success and failure). This indicates a continued increase of metabolic activity. Maximal respiration obtained at the end of latency did not correlate with growth rates before and during the latent period. Respiration increased to a maximum of 116% (±11 S.D.) compared with respiration at the time of drug addition. This relatively small variation of maximal respiration before the onset of inhibition reflects the correlation of high growth rates with short latencies and, vice versa, low growth rates with long latent periods (Figure 3a and b).

A positive correlation was obtained between the rate of inhibition after latency (measured over the time period from maximal respiration until 30 min past exposure to penicillin) and growth rate before the addition of the drug (Figure 3c; correlation coefficients 0.59 and 0.44, for treatment success and failure). In all parameters, the 48 strains obtained from 28 patients with eradication failure did not separate from the 62 strains acquired from patients with successful penicillin therapy (Figure 3).

**Discussion**

We investigated respiratory inhibition kinetics in *S. pyogenes* isolated from patients with and without bacteriological treat-
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Measurement failure under penicillin therapy. The extent of inhibition by penicillin was positively correlated with strain-specific growth rates observed under control culture conditions. Latent periods between the addition of the antibiotic and inhibition of bacterial respiration ranged from 1 to 25 min and decreased as a function of increasing growth rates before drug exposure. In the penicillin-tolerant \textit{L. monocytogenes} ATCC 19611, however, the latent period was as high as 45 min, clearly distinct from the respiratory pattern of bactericidal modes of action (Figure 3a).

The definition of tolerance has been criticized as arbitrary and purely dependent on methodology.\(^\text{11,12}\) MIC is measured over 18–24 h at various initially added drug concentrations. The half-life of penicillin G, however, is 40 min \textit{in vivo}.\(^\text{19}\) Respirometry requires a test window of merely 30 min for measurement of the inhibition kinetics and is thus superior to conventional MIC determination, since a continuous record is obtained of respiratory growth and inflection to respiratory inhibition (Figure 1). In addition, the MIC/MBC ratio does not take into account the relationship between the absolute MBC and the drug concentration clinically achieved in tissues. At a realistic penicillin concentration of 4 mg/L, killing curves were indistinguishable for the reportedly penicillin-tolerant \textit{S. pyogenes} L36K (MBC 0.5 mg/L) and fully susceptible isolates. This was confirmed by respiratory inhibition kinetics of \textit{S. pyogenes} L36K at a penicillin concentration of 4 mg/L, which reflected a bactericidal mode of action as seen in fully susceptible \textit{S. pyogenes} ATCC 19615 and the clinical isolates. Our results with the bacteriostatic drug sulfadiazine and the bactericidal drug clindamycin provide the basis for a distinction between respiratory responses to non-growth conditions and to cell death, under conditions of identical metabolic substrate availability. Only cell death led to an immediate decline in respiration, whereas bacteriostasis caused respiration to level off at a constant rate, indicative of non-growth conditions and futile energy turnover (Figure 1b).

Similarly, growth was completely arrested at 20°C. Under these conditions, a significant and constant level of respiration was maintained, and only killing by formaldehyde finally reduced the oxygen consumption rate to zero (Figure 2).

Continuous respirometric monitoring before and after application of the drug yielded information on the strain-specific growth rate, the lag time between exposure to the antibiotic and response (latency), the initial increase in respiration over this latent period and the subsequent rate of inhibition (Figure 3). High-resolution respirometry\(^\text{24}\) provides a prerequisite for the quantitative analysis of continuous changes of oxygen consumption rates (non-linear slopes of oxygen concentration over time) and online graphical display of inhibition curves.\(^\text{26}\) The identity of the simple patterns of these complex inhibition curves in isolates from patients with and without bacteriological eradication failure provides a strong argument against the clinical relevance of penicillin tolerance as previously defined in the isolates of \textit{S. pyogenes} tested. Respirometric inhibition kinetics allows sensitive and meaningful distinction between tolerance and bactericidal modes of action. Independent of their origin from patients with or without eradication failure, none of the clinical isolates of \textit{S. pyogenes} tested in our study was tolerant of penicillin at 4 mg/L. In agreement with our findings, various previous reports have questioned the significance of tolerance in streptococcal treatment failure.\(^\text{8,14,27}\) We propose to resolve the long-standing controversy on the implications of penicillin tolerance in bacteriological treatment failure.\(^\text{5,14}\) The dispute over definitions of tolerance should be replaced by the recognition of a continuity of quantitative relationships derived from respiratory inhibition kinetics.

The two phases of antimicrobial action observed by respirometry, i.e. minimal reduction in respiratory growth rate during latency followed by a rapid decline in oxygen consumption, may correspond to a sequence of two antimicrobial mechanisms exerted by penicillin: (i) interference with peptidoglycan synthesis, yielding a cell wall that cannot withstand osmotic forces, and (ii) activation of autolysins, which accelerates lysis by destroying the cell wall.

It is well known that β-lactam antibiotics exert their bactericidal effects during the logarithmic growth phase, whereas apparent tolerance is observed during stationary phases caused by density effects, nutrient starvation or low temperature.\(^\text{28,29}\) When \textit{S. pyogenes} is brought into a stationary growth phase, expression of penicillin binding proteins is reduced and the antimicrobial action of penicillin declines concomitantly.\(^\text{23}\) The unique finding in our study is the correlation between parameters derived from inhibition kinetics and the strain-specific growth rate. The paradigm of growth dependence of penicillin susceptibility is thus extended to the phenotypic variability of growth rate in a group of clinical isolates.

Bacteriological treatment failure in streptococcal pharyngotonsillitis may have multiple causes, including lack of compliance, re-infection and penicillin tolerance of the infecting streptococcal strain. Lack of compliance and re-infection are ruled out as the main factors for bacteriological treatment failure in our patient population.\(^\text{1}\) Our present results support the notion that tolerance is not a relevant factor to explain this clinical challenge. This suggests a physiological perspective, e.g. intracellular protection in human host cells\(^\text{12,30,31}\) or special growth conditions in affected tissue, to explain current eradication failures in \textit{S. pyogenes} pharyngotonsillitis. Inhibition kinetics by high-resolution respirometry presents a new, sensitive and practical surveillance technique, well suited to meet the increasing challenge of assessing the potential emergence of microbial tolerance or resistance to antibiotics.\(^\text{32}\)
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References


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