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Effect of chloramphenicol, erythromycin, moxifloxacin, penicillin and tetracycline concentration on the recovery of resistant mutants of *Mycobacterium smegmatis* and *Staphylococcus aureus*

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The effect of antimicrobial concentration on colony-forming ability of resistant mutant subpopulations of *Mycobacterium smegmatis* and *Staphylococcus aureus* was measured for chloramphenicol, erythromycin, moxifloxacin, penicillin and tetracycline. The relationship between drug concentration and the recovery of mutant colonies was distinct for each bacterium–antimicrobial combination; however, in each case application of large numbers of cells to drug-containing agar plates revealed a progressive reduction in mutant recovery as antimicrobial concentration increased. The minimal concentration that allowed no mutant recovery from more than 10^{10} input cells was measured to estimate the minimum inhibitory concentration (MIC) of the least susceptible, single-step mutant subpopulation, a parameter also called the mutant prevention concentration (MPC). These data expand the number of antimicrobial–bacterial combinations for which a mutant selection window can be measured.

Keywords: erythromycin, moxifloxacin, penicillin, tetracycline, chloramphenicol

Introduction

Surveillance studies with a variety of pathogens show that antimicrobial resistance can develop rapidly.[1–8] If this trend is allowed to continue without the introduction of new classes of agent, many microbial diseases are likely to become refractory to antimicrobial treatment. It may be possible to slow the development of *de novo*, step-wise resistance through more aggressive therapies that directly block the growth of the resistant mutant fraction of susceptible populations. In theory, antimicrobial therapy could be optimized such that failure due to the emergence of resistance during treatment occurs only rarely.[9] Empirical determination of such a dose is likely to require large numbers of patients to ensure that a particular regimen does not contribute to the rising prevalence of resistance or an increase in the frequency of other adverse events. As an alternative, we have proposed a conceptual strategy based on *in vitro* data.[10,11] In principle, antimicrobial concentrations that require a cell to attain two concurrent resistance mutations for growth will rarely allow selective enrichment of mutant subpopulations. That concentration is the minimum inhibitory concentration (MIC) of the least susceptible, single-step mutant, which is termed the mutant prevention concentration (MPC). Since the least susceptible, single-step mutant is not always available for testing and since mutant susceptibility might differ between pure cultures and small subpopulations, we have suggested that MPC can be estimated as the concentration that allows recovery of no mutant when a large, susceptible population (10^{10} cells) is applied to drug-containing agar plates.[12] This microbiological threshold can be readily measured *in vitro* for fluoroquinolones;[12,13] however, it is not clear how the concept applies to agents of other classes. For example, single-step mutations could lower susceptibility so much that MPC could not be measured, as is the case with rifampicin resistance.[13]

In the present work, we examined the recovery of resistant mutants of *Mycobacterium smegmatis* and *Staphylococcus aureus* from agar plates containing compounds representing five types of antimicrobial agent. In each case, the reduction in mutant recovery due to increasing drug concentration became progressively steeper when large numbers of cells (10^9–10^{10}) were applied to drug-containing agar. This is the result expected as antimicrobial concentration approaches the MIC for the least susceptible single-step mutant, suggesting that MPC can be measured for antimicrobial agents of many types.

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Materials and methods

Bacterial strains, growth conditions and antimicrobial agents

Wild-type *M. smegmatis* (strain mc^{2}155), obtained from Dr S. Cole (Institute Pasteur, Paris, France), was grown at 37°C in 7H9 liquid medium and on 7H10 agar, both supplemented with 10% albumen–dextrose complex (ADC).[14] *S. aureus* strain RN450, obtained from Dr B. Kreiswirth (Public Health Research Institute, Newark, NJ, USA), was grown at 37°C in CY broth (1% Casamino acids, 1% yeast extract, 0.1 M NaCl, 0.5% glucose and 0.05 M sodium glycerophosphate) and GL agar (0.3% Casamino acids, 0.3% yeast extract, 0.1 M NaCl, 0.2% sodium lactate, 0.1% glycerol and 1.5% agar, pH 7.8).[15] Bacteria were stored at –80°C in growth medium plus 15% glycerol. Chloramphenicol, erythromycin, penicillin and tetracycline were purchased from Sigma–Aldrich Corp. (St Louis, MO, USA). Moxifloxacin was obtained from Bayer Corp. (West Haven, CT, USA). Stock solutions (10 mg/mL) were prepared by dissolving penicillin in distilled water, moxifloxacin in 0.1 M NaOH, tetracycline in 50% ethanol, chloramphenicol in 95% ethanol, and erythromycin in 100% ethanol.

Measurement of antimicrobial susceptibility

The minimum concentration that inhibited growth of 99% of the input cells [$MIC_{(99)}$] was measured by applying serial dilutions of stationary phase cultures to agar plates containing various concentrations of antimicrobial agent. Colonies were counted after incubation (1–2 days for *S. aureus*; 3–4 days for *M. smegmatis*). Preliminary determinations using two-fold dilutions of drug provided an approximate value of $MIC_{(99)}$. This measurement was followed by a second determination, plus a replicate, that utilized linear drug concentration increments (about 20% per sequential increase). The fraction of colonies recovered was plotted against drug concentration to determine $MIC_{(99)}$ by interpolation.

MPC was defined as the concentration that blocked growth when at least 10^{10} cells were applied to agar plates.[11] To measure MPC, cells were grown with vigorous shaking to reach a concentration of about 10^9 cfu/mL for *M. smegmatis* and 10^{10} for *S. aureus*. Cells were applied to drug-containing agar plates and, at the same time, the cell density of the culture was determined retrospectively by applying serial dilutions to drug-free agar. The maximal bacterial inoculum applied to each agar plate was 300 µL of 10^{10} cfu/mL for *S. aureus* and 1 mL at 1 to 5×10^9 cfu/mL for *M. smegmatis*. Multiple plates at a given drug concentration were used so that the total number of cells tested exceeded 10^{10}. Agar plates were incubated at 37°C for various times depending on the species: 4 days with colony numbers recorded at 1-day intervals for *S. aureus* and 10 days with colony numbers recorded at 2-day intervals for *M. smegmatis*. Colonies were confirmed to contain mutant cells by regrowth on agar containing the selecting concentration of antimicrobial (control experiments in which mutant colonies were grown on drug-free agar before retesting on drug-containing agar showed that the mutants were stable).

Results

M. smegmatis or *S. aureus* were plated on agar containing various antimicrobial concentrations. After incubation, colonies were counted and the proportion of the initial inoculum recovered as colonies on the plates was calculated. As shown in Figure 1, raising the antimicrobial concentration gave a sharp drop in colony recovery. For some bacterium–antimicrobial combinations, a distinct shoulder or plateau was observed in the recovery curves at high drug concentration (moxifloxacin, erythromycin and tetracycline with *M. smegmatis*; erythromycin with *S. aureus*). For others only inflection points

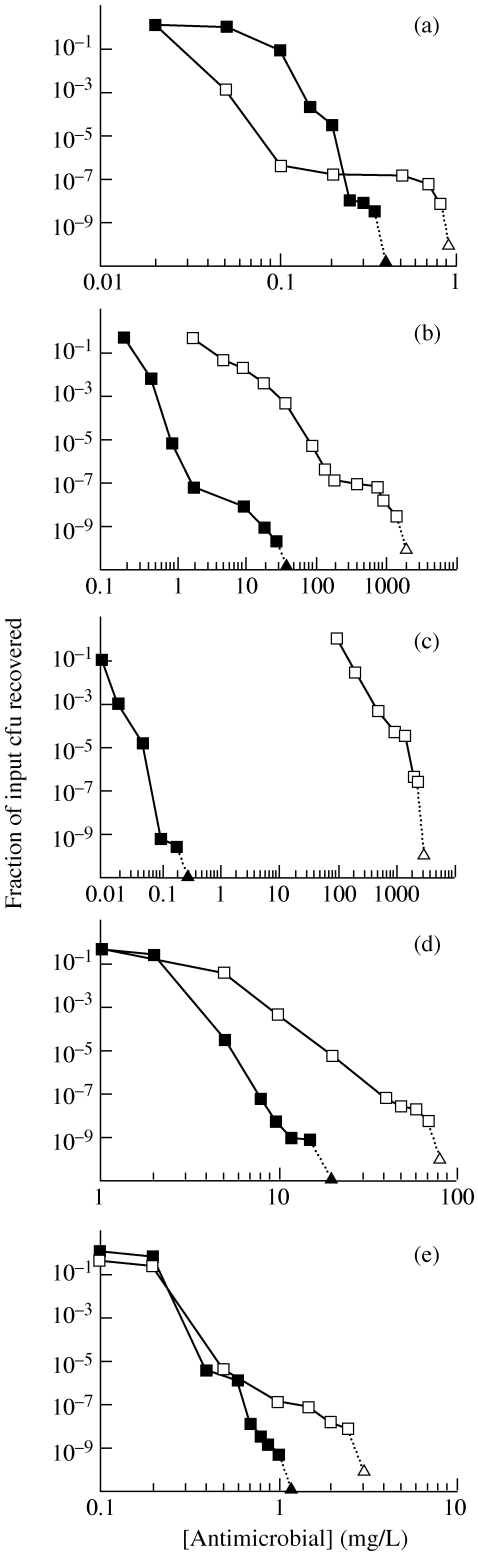

Figure 1. Effect of antimicrobial concentration on recovery of resistant mutants. *M. smegmatis* strain mc^{2}155 (open symbols) and *S. aureus* strain (RN450) (filled symbols) were applied to agar plates containing the indicated concentrations of (a) moxifloxacin, (b) erythromycin, (c) penicillin, (d) chloramphenicol or (e) tetracycline. Triangles indicate concentrations at which no colony was recovered when more than 10^{10} cells were applied to plates. Replicate experiments gave results similar to those shown.

Effect of antimicrobials on recovery of resistant mutants

Table 1. Mutant selection window for various antimicrobial agents with *M. smegmatis* and *S. aureus*

Organism	Compound	$MIC_{(99)}$ (mg/L)	MPC (mg/L)	$MPC/MIC_{(99)}$
M. smegmatis	moxifloxacin	0.04	0.9	22
	erythromycin	15	2000	130
	penicillin G[a]	230	2800	12
	chloramphenicol	6	80	13
	tetracycline	0.25	3	12
S. aureus	moxifloxacin	0.12	0.38	3.2
	erythromycin	0.5	32	64
	penicillin G[a]	0.014	0.22	16
	chloramphenicol	3	17	5.7
	tetracycline	0.24	1.2	5

[a]Specific activity 1600 U/μg.

were detected (penicillin with *M. smegmatis*; moxifloxacin, tetracycline, penicillin and chloramphenicol with *S. aureus*). In the case of chloramphenicol with *M. smegmatis*, no inflection point was obvious.

Although the shape of the mutant recovery–drug concentration curves varied among the bacterium–antimicrobial combinations, in each case the drop in mutant recovery became progressively steeper as antimicrobial concentration increased and high cell numbers were tested [the drop is probably steeper than shown, since the drug concentrations at which no colony was recovered (triangles) overestimate the true drug concentrations required to block colony growth]. Increasing steepness is the result expected as the MIC for the least susceptible mutant (MPC) is approached.[11]

It has been argued that resistant mutants are selectively enriched when antimicrobial concentrations fall between the minimal concentration that inhibits the growth of 99% of the cells [$MIC_{(99)}$] and MPC, a range called the mutant selection window.[10,13,16] [$MIC_{(99)}$ approximates the minimal concentration better than MIC because less selective pressure is present; however, for many antimicrobial–pathogen combinations, little absolute difference is likely to exist between $MIC_{(99)}$ and MIC due to the steep dependence of colony recovery on drug concentration.] Values of $MIC_{(99)}$ and MPC were therefore calculated and are listed in Table 1. When the size of the selection window was expressed as the ratio of MPC to $MIC_{(99)}$, it varied considerably among bacterial–antimicrobial combinations. For both bacterial species, the selection window was widest for erythromycin.

Discussion

Although the relationship between antimicrobial concentration and growth of bacterial mutants on drug-containing agar is characteristic of each antimicrobial–bacterium combination, two general categories can be identified. One, represented by rifampicin treatment of several organisms, exhibits a sharp drop in colony recovery followed by a broad plateau as drug concentration increases.[17] This result is most easily explained by resistance mutations reducing susceptibility so much that no achievable drug concentration can inhibit mutant growth. A similar result is expected from a bacterial population having a subpopulation of plasmid-containing cells that exhibit high-level resistance. In these situations no monotherapy regimen will keep drug concentrations above MPC and thereby block mutant growth. Restricting the development of this type of resistance requires combination therapy even if the fraction of mutant cells is low.

The second category of drug concentration dependence is illustrated by the combinations studied in the present work, by prior studies with fluoroquinolones,[12,18,19] and by treatment of *Candida albicans* and *C. glabrata* with miconazole (J.-Y. Wang *et al*., unpublished observations). Increasing drug concentration causes colony recovery to drop sharply at the MIC, pass through an inflection point, and then drop sharply a second time. The second drop occurs at the MPC. Fluoroquinolone studies show that resistant mutants are selectively enriched at drug concentrations between $MIC_{(99)}$ (or MIC) and MPC.[16,18] Whether monotherapy is appropriate for situations in this category depends on how long relevant tissue drug concentrations can be kept above MPC at each dosing interval. To address this issue, it is now necessary to measure MPC *in vivo* at the site of infection.

Confusion sometimes surrounds phenotypic or induced resistance. An example is the β-lactam resistance that arises from the induction of β-lactamases. When mutations are not responsible for this type of resistance, it is outside the scope of the present discussion. However, when β-lactamases are expressed from plasmid-borne genes, they behave as category-one resistance and require combination therapy even if the plasmid-containing cells are members of rare subpopulations. Thus the dosing strategies that derive from consideration of the mutant selection window hypothesis may be broadly applicable.

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