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Research Paper

Host-dependent Induction of Transient Antibiotic Resistance: A Prelude to Treatment Failure

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ABSTRACT

Current antibiotic testing does not include the potential influence of host cell environment on microbial susceptibility and antibiotic resistance, hindering appropriate therapeutic intervention. We devised a strategy to identify the presence of host–pathogen interactions that alter antibiotic efficacy *in vivo*. Our findings revealed a bacterial mechanism that promotes antibiotic resistance *in vivo* at concentrations of drug that far exceed dosages determined by standardized antimicrobial testing. This mechanism has escaped prior detection because it is reversible and operates within a subset of host tissues and cells. Bacterial pathogens are thereby protected while their survival promotes the emergence of permanent drug resistance. This host-dependent mechanism of transient antibiotic resistance is applicable to multiple pathogens and has implications for the development of more effective antimicrobial therapies.

1. Introduction

Recent CDC estimates indicate that one in five pathogens from hospital-acquired infections in the U.S. are multidrug-resistant (Kallen et al., 2010; Sievert et al., 2013), dramatically limiting therapeutic options to antibiotics that may be more toxic, less effective, or more expensive (Centers for Disease Control and Prevention, 2013). In such cases, patients often have longer hospital stays, delayed recuperation, long-term disability, and increased mortality. Deciphering the mechanisms that govern the emergence of multidrug-resistant pathogens is critical to the development of new approaches to control bacterial infections. Many mechanisms of antibiotic resistance have been established, including horizontal gene transfer; genomic mutation; and intrinsic bacterial mechanisms that pre-date antibiotics (Allen et al., 2010; Andersson and Hughes, 2010; Cox and Wright, 2013; D'Costa et al., 2011; Davies and Davies, 2010). Significant advances have been made regarding the generation of antibiotic resistant variants (phenotypic and genotypic) that emerge during infection; *e.g.*, *Staphylococcus aureus* small colony variants that promote persistent infections (Proctor et al., 2006); antibiotic resistance of *Pseudomonas aeruginosa* biofilms (Høiby et al., 2010); the evolution and spread of multidrug-resistant pneumococcal variants (Croucher et al., 2011), and heteroresistant subpopulations of vancomycin-susceptible *S. aureus* (El-Halfawy and Valvano, 2015). Despite this knowledge, the role of host–pathogen interactions in antibiotic resistance is poorly understood, and the use of host models as a primary approach to understanding resistance is not often considered or explored.

For the past several decades, drug development has followed a standard sequential procedure wherein: (i) efficacy is determined *in vitro*; (ii) pharmacokinetic/pharmacodynamic (PK/PD) parameters are measured *in vivo*; and (iii) dosing efficacy/toxicity *in vivo* is established for a limited number of model pathogens (Ambrose et al., 2007; Clinical and Laboratory Standards Institute, 2012; Food and Drug Administration, 2009). However, along with a limited amount of patient-dosing data, physicians rely on *in vitro* antimicrobial susceptibility testing (AST) of clinical isolates grown on the universal media standard Mueller–Hinton Broth (MHB) for therapeutic intervention (Clinical and Laboratory Standards Institute, 2012; European Committee on Antibiotic Suscepibility Testing, 2014). This standard procedure does not replicate mammalian biochemistry and may not correlate with patient outcome. To overcome these limitations, we investigated antibiotic resistance in the context of animal models of disease, and have identified a mechanism

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that stimulates bacterial resistance to multiple antibiotics during infection, while promoting the emergence of drug-resistant bacteria.

2. Materials and Methods

2.1. Bacterial Strains and Media

TIVAR+ *S.* Typhimurium ATCC 14028 (CDC 6516–60) and MT2057 (an isogenic KAN resistant derivative of 14028), or TIVAR− *S.* Typhimurium var. 5 (04)-9639 (a multidrug-resistant isolate) derived from chicken and cow, respectively, were used in these studies (Conner et al., 1998; Heithoff et al., 2008). These strains have identical oral and i.p. lethal dose 50s (LD_{50}) in BALB/c mice, 10^5 and <10 colony forming units (CFU), respectively (Heithoff et al., 2008). *Salmonella* human clinical isolates were obtained from fecal and blood samples derived from patients with gastroenteritis or bacteremia, respectively; animal isolates were derived from different disease outbreaks, individual cases, or surveillance submissions to diagnostic laboratories (Heithoff et al., 2008). Unless otherwise specified, *Salmonella* were derived from stationary phase cultures aerated at 37 °C containing the Mueller–Hinton broth (MHB) (Clinical and Laboratory Standards Institute, 2012); low phosphate, low magnesium medium (LPM) (Coombes et al., 2004) or N-minimal medium (Nelson and Kennedy, 1971) supplemented with 0.3% glycerol and 0.1% casamino acids; or the Luria–Bertani (LB) medium (Davis et al., 1980). *Yersinia pseudotuberculosis* IP32953 is a virulent human isolate (Chain et al., 2004), and was assayed from stationary phase cultures aerated at 28 °C containing MHB or LPM media.

2.2. MIC Assays

The minimum inhibitory concentration (MIC) was determined in MHB and LPM media (pH 7 and pH 5.5) according to Clinical and Laboratory Standards Institute (CLSI) guidelines (Clinical and Laboratory Standards Institute, 2012; Wiegand et al., 2008). MIC assays were performed on bacteria obtained from overnight culture, and from bacteria derived from macrophage lysates or host tissues. Bacteria were diluted in 0.15 M NaCl, and a 5 μl volume containing 10^4 CFU was spotted on agar plates of the media condition indicated, containing two-fold dilutions of each antibiotic. MIC values were derived following 20 h incubation at 37 °C (*Salmonella*) or 48 h incubation at 28 °C (*Y. pseudotuberculosis*), and were the result of at least three independent determinations.

2.3. Bacterial Cell Survival Assays

Bacterial cell survival was evaluated after cells derived from a given growth condition were exposed to antibiotics under the same or different growth condition (Groisman et al., 1997). *S.* Typhimurium 14028 was grown overnight in non-inducing medium for the TIVAR phenotype (N-minimal medium with 10 mM Mg^{2+} pH 7.7), diluted 1:100 in either inducing medium (N-minimal medium with 10 μM Mg^{2+} pH 5.8) or non-inducing medium (N-minimal medium with 10 mM Mg^{2+} pH 7.7), and incubated 4 h at 37 °C. Bacteria were diluted 1:200 and exposed to polymyxin B for 1 h in either inducing medium (N-minimal medium with 10 μM Mg^{2+} pH 5.8) or non-inducing medium (LB), and plated for CFU on LB medium. Percent survival was calculated as $CFU_{[polymyxin\ B]} / CFU_{[no\ drug]} \times 100$ at 1 h post drug exposure; values given are the REML model means ± SEM derived from at least 5 independent determinations.

2.4. Virulence Assays

Intraperitoneal (i.p.) infection. Salmonella grown overnight in LB medium were resuspended in 0.15 M NaCl and administered to mice via the i.p. route of infection (dose 10^2 or 10^3 CFU) (Heithoff et al., 1999). Five days post-infection, the bacterial cells were recovered from the spleen and other tissues/fluids of acutely infected animals. *Antibiotic treatment.* Mice infected i.p. with *Salmonella* (i.p. dose 10^2 CFU) were treated (or mock-treated) with a twice-daily polymyxin B or ciprofloxacin dosing regimen (30 mg/kg/day). Bacterial cells were recovered from the spleen of acutely infected animals and enumerated by direct colony count. Mouse survival was assessed for 10 days post-infection. Uninfected mice were also treated with polymyxin B to control for dosing toxicity. Six- to twelve-week old female BALB/c mice were used in all virulence studies. Institutional Animal Care and Use Committee of the University of California, Santa Barbara approved all mouse research protocols undertaken herein.

2.5. Cell Culture

The murine macrophage cell line RAW 264.7 was obtained from the American Type Culture Collection, Rockville, MD., and maintained in minimum essential medium (MEM) supplemented with L-glutamine and 10% heat-inactivated bovine growth-supplemented calf serum (HyClone Laboratories, Logan, UT). Cells were grown in a humidified atmosphere of 5% carbon dioxide and 95% air at 37 °C in 75-cm^2 plastic flasks (Corning Glass Works, Corning, NY). Cultured murine macrophages (RAW 264.7) were harvested by scraping with a rubber policeman and plated at a density of 2.5 to 5×10^5 cells/ml in 4 ml of culture medium in 35 mm-diameter, six-well dishes (Corning) and grown for 24 h to approximately 80 to 90% confluence (1 to 5×10^6 cells/well) (adapted from previous methods (Fleckenstein et al., 1996)).

2.6. Bacterial Infection of Cultured Murine Macrophages

Bacterial cells were used to infect cultured murine macrophage (RAW 264.7) monolayers grown in cell culture plates (Corning) at a multiplicity of infection (MOI) of 10:1. The bacteria were centrifuged onto cultured monolayers at 1000 ×*g* for 10 min at room temperature, after which they were incubated for 30 min at 37 °C in a 5% CO_2 incubator. The co-culture was washed once with cell culture medium and incubated for 45 min in the presence of gentamicin (100 μg/ml) to kill extracellular bacteria, washed once with pre-warmed cell culture medium, and incubated in 4 ml of culture medium containing polymyxin B at the concentration indicated or 10 μg/ml gentamicin ($t = 0$ time point) for 24 h (adapted from previous methods (Finlay and Falkow, 1988)).

2.7. Statistical Analyses

Log transformed intracellular CFU and PMB^r mutation frequency data were analyzed using ANOVA (ANOVA, GenStat, 15th Edition, VSN International, UK). Intracellular CFU and PMB^r mutation frequency data are presented as the means ± standard error of the mean (SEM). Cell survival was analyzed using residual (or restricted) maximum likelihood (REML) analysis (GenStat, 15th Edition, VSN International, UK). A single variate model was used to analyze percentage survival on a log scale. The fixed effects of the model were the factors group, drug concentration, and their interaction. The Wald chi-square test was used to determine significant main effects and/or significant interactions between factors. Any non-significant terms were dropped from the model and analysis repeated. Following analysis, data are presented as predicted model-based means, *i.e.*, predicted means are those obtained from the fitted model rather than the raw sample means. Differences between the individual means calculated using ANOVA and REML were determined by calculating an approximate least significant difference (LSD). A difference of means that exceeded the calculated LSD was considered significant. Statistical significance for difference in proportions of animal survival was calculated using Chi-square (Epi Info 7, CDC). For all statistical analyses, a significance level (P) of less than 0.05 was considered to be statistically significant. Degrees of statistical significance are presented as $^{***}P < 0.001$, $^{**}P < 0.01$, or $^{*}P < 0.05$.