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Persistence: a copacetic and parsimonious hypothesis for the existence of non-inherited resistance to antibiotics

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Abstract

We postulate that phenotypic resistance to antibiotics, persistence, is not an evolved (selected-for) character but rather like mutation, an inadvertent product of different kinds of errors and glitches. The rate of generation of these errors is augmented by exposure to these drugs. The genes that have been identified as contributing to the production of persisters are analogous to the so-called mutator genes; they modulate the rate at which these errors occur and/or are corrected. In theory, these phenotypically resistant bacteria can retard the rate of microbiological cure by antibiotic treatment.

Introduction

In the super-reductionist old days of bacterial genetics (between 1950 and 1970), words like “leaky” were used to describe, albeit not explain, phenotypic heterogeneity among cells of the same genotype. In these more enlightened and somewhat less reductionist times, the mechanisms responsible for this phenotypic variation have been worked out for a number of cases, e.g. [1,2].

What may well be the first description of phenotypic heterogeneity within monoclonal populations of bacteria was the observation in 1942 that some 1% of “*Staphylococcus pyogenes*” exposed to penicillin survive and produce colonies [3]. Two years later, Joseph Bigger presented evidence that this resistance to the bactericidal effects of penicillin was phenotypic rather than inherited; when recovered colonies were re-cultured, they were as susceptible to penicillin-mediated killing as their ancestors [4]. Bigger called these survivors “persisters”. Here we review evidence that supports the hypothesis that exposure to antibiotics contributes to the generation of persisters as well as reveals their existence. We postulate that persistence is analogous to mutation, an inadvertent consequence of different kinds of glitches and errors, rather than an evolved (selected-for) character. In this interpretation, the genes and processes that have been identified to contribute to the

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frequency of persister are analogous to those that modify the rate of mutation, so-called “mutator genes”. Using computer simulations, we explore the potential contribution of persistence to the microbiological course of antibiotic treatment.

Persistence can be attributed to a number of different mechanisms

Persistence appears to be a universal property of all bacterial species [5]. When exposed to cytotoxic drugs fungi and neoplastic cells exhibit an analogous phenomenon, minority populations of phenotypically and presumable genetically susceptible cells survive [6]. Genome-wide mutant screen-, and molecular genetic studies of persisters have identified two major classes of genes and mechanisms contributing to and/or modulating the frequency of persisters: one, changes in the expression of toxin or antitoxin genes; two, modifications and/or changes in effector protein concentrations that lead to changes in metabolism, for superb reviews see ([5,7-10]). Arguably, the genes responsible for competence for transformation in *Bacillus subtilis* also contribute to persistence; upon introduction to fresh media, replication is delayed for the small fraction of cells that are competent to acquire DNA [1].

What is clear and central to the following arguments is that multiple processes contribute to the generation and/or modulation of the fraction of genetically identical cells surviving exposure to antibiotics [11,12]. Stated another way, there are multiple reasons why viable bacteria in an otherwise replicating populations don't divide or divide at a lower rate or have longer lag periods than the majority population. During these periods of arrested or slowed growth, these bacteria can be refractory to antibiotics and thereby persisters.

Antibiotics promote the generation of persisters

Persistence is not a convenient phenotype to work with; it is only manifest in a minority of a population and, at least at the whole population level requires antibiotic exposure to be observed. Could it be that antibiotics not only reveal the presence of pre-existing persisters but also contribute to their generation? Three lines of evidence support a positive answer to this question.

1- Fluctuation experiments [13]—If the persisters in a given population are all generated before antibiotic exposure, as with random mutation, there would be considerable variation in the number of persisters among independent cultures of the same size. While this is the case for *S. aureus*, there is roughly the same amount of variation in the number of persisters among cultures derived from mixtures of these independent cultures [12]. The latter is what would be anticipated if the selective agent, the antibiotic, also contributes to the production of persisters.

2- Correlation between the number of persisters produced by exposure to different drugs—If persisters were a single class of non- or slowly- dividing cells produced during the course of growth, there would be a correlation in the number of cells surviving exposure to different antibiotics, even if these antibiotics differed in their ability to kill persisters. To test this hypothesis, we used methods described in [12] to estimate the number of cells surviving after 22 hours of exposure to 20X MIC ciprofloxacin and 20X

MIC gentamicin for 10 independent cultures of *S. aureus* Newman. The estimated correlation coefficient $r = 0.27$ for this experiment is not significantly different from 0, ($p \sim 0.44$).

3- SubMIC exposure increases the frequency of persisters—For both, *E. coli* and *S. aureus*, the fraction of persisters is greater when these bacteria are grown at low (subMIC) concentrations of antibiotics than antibiotic-free media [12,14].

We want to emphasize that while these observations support the hypothesis that antibiotics promote the generation of persisters, they do not reject the alternative. There may well be persister cells present before the population is exposed to antibiotics as well as those generated after exposure.

For *E. coli* and ciprofloxacin, a mechanism has been suggested to account for how exposure to this drug increases the level of persistence; the induction of an SOS response and thereby an increase in the TisB toxin [14]. Other than vague phrases like “increasing stress” it is not clear how exposure of *S. aureus* to ciprofloxacin or other antibiotics increases persistence levels [12]. Also requiring a mechanistic explanation is the great variance in the number of persisters produced by different cultures and in different experiments [11,12,15] and the associated frustrating problem of quantitatively replicating results. For example, although it is clear that exposure to subMIC concentrations of antibiotics increases the levels of persistence to multiple drugs, the magnitude of this contribution varies between experiments, at least in our experience.

An Evolutionary Perspective

Persistence has been described as a bet-hedging mechanism [16,17], a way for populations of bacteria to survive periodic confrontations with agents, like antibiotics, that kill growing cells. Consistent with this bet-hedging perspective are the results of mathematical modeling studies [12,16,18] showing that when periodically exposed to agents that kill growing cells, bacteria that produce higher frequencies of persisters have an advantage over those that produce lower frequencies. This was observed in experiments with competing populations of genetically competent *B. subtilis* (*com*+) and mutants that cannot produce competent cells (*com*–). In the absence of this episodes where the population is exposed to agents that kill growing cells the *com*– have an advantage over the *com*+. If, however, the cultures are periodically exposed to penicillin, the *com*– are favored [18].

Even if persisters provide a way for populations to survive episodes where they encounter agents that kill growing cells, this “episodic selection” [18] need not be the force responsible for the evolution and maintenance of persistence, no more than penicillin binding proteins evolved to bind to this antibiotic. Indeed, our models suggest that even if the generation of persisters could be attributed to single genes, those genes would only have an advantage when they are common [12]. When rare, the frequency of the phenotype generated by these genes, persisters, would be too low to provide the producing population an advantage and that subpopulation would be eliminated when the population at large is confronted with agents that kill growing cells.

Persisters and Biofilms

In addition to the persisters generated in planktonic populations of bacteria, which have been the main object of study of this phenotypic resistance since Bigger's 1944 paper, it has been suggested that the antibiotic refractory cells in biofilms should also be considered persisters [19,20]. Why not? After all, there are many mechanisms by which these phenotypically antibiotic resistant cells are generated. The fraction of non-replicating genetically susceptible bacteria in a biofilm with actively replicating cells would be phenotypically refractory to antibiotics and thereby persisters in the Bigger definition.

The PASH hypothesis

To us, the most parsimonious and copacetic explanation for the existence of persisters and the diversity of mechanisms responsible for their generation is that these phenotypically resistant cells are the product of different kinds of glitches and errors in cell division, Persistence As Stuff Happens (PASH) [12]. In accord with this hypothesis, persistence is analogous to mutation, an inadvertent product of errors rather than an evolved (selected for) character. What has evolved and has been elucidated and described in the recommended reviews [5,7-10] are genes that modify the rate at which persisters are generated and/or correct the errors responsible for their generation and are thereby analogous to mutator genes.

Potential clinical implications

As interesting as phenotypic variation may be academically, much of the motivation and justification for studying persistence are its potential clinical implications, the contribution of this non-inherited form of resistance to the microbiological course of antibiotic treatment and the generation of genetically resistant bacteria during treatment. Whether persistence evolved by natural selection or like mutation is an inadvertent product of errors, phenotypically resistant subpopulations are clearly not a good thing for either of these aspects of antibiotic treatment [21]. How important persistence is to antibiotic therapy, however, is not at all clear. The results of our theoretical studies of the pharmacokinetics and pharmaco- and population dynamics of antibiotic treatment suggest that the clinical consequences of persistence depend on the physical nature of these phenotypically resistant subpopulations; Whether the persisters are planktonic or reside in sub-habitats like biofilm, which are not only refractory to antibiotics but are removed at lower rates from the overall habitat than the planktonic cells from whence they are derived.

To illustrate this we use computer simulations of a model similar to that in [22] (Figure 1A). The bacteria are of three states, susceptible and persister planktonic cells, S and P, respectively, or B in sub-habitat that is flowing out at a lower rate as planktonic populations. S, P and B are the densities as well as the designations of these populations. Relative to S, the P and B populations are refractory to the antibiotic and replicate at a lower rate. Resources flow into a habitat of unit volume at a constant rate, w per hour, which is the same rate at which the planktonic cells, unused resources, R and antibiotics, A are removed. The B population is removed from habitat at lower rate than either S or P, $w_b < w$. The bacteria change states in the directions and rates noted in the figure, respectively $S \rightarrow P$, $P \rightarrow S$, $S \rightarrow B$ and $B \rightarrow S$, sp , ps , sb , and bs per cell per hour.

After the bacteria are at their equilibrium densities, antibiotics are introduced at a concentration, A_{MAX} at defined intervals, d hours. For the pharmacodynamics of the antibiotics and bacteria, we assume Hill functions [23]. More details about this model and the values of the parameters used can be seen in the Supplemental Material. The Berkeley Madonna™ program used for these simulations can be obtained from www.eclif.net.

While planktonic persisters will increase the time before clearance to an extent proportional to the rate at which they are generated, since these antibiotic refractory cells are washed out, they will not prevent clearance (Figure 1B). Unless it specifically kills planktonic persisters, increasing the dose of the antibiotic will have little effect on the course of treatment. If, however, the persisters are an antibiotic refractory subpopulation that is not turning over at a lower rate than the planktonic population, as in a biofilm, in the absence of other processes, like the host immune defenses [24] they can prevent clearance (Figure 1C). Because in this simulation the B population is replicating and is somewhat susceptible to the drug, increasing the concentration of the antibiotic can lead to a decline in the density of viable cells. This is particularly so, if as in [25] the drug is active against persisters and including those in biofilms.

Conclusion

While persisters may be products of errors and glitches rather than an evolved (selected for) character, at least in theory they can substantially retard the rate of microbiological cure by antibiotic treatment. Elucidating the mechanisms responsible for modulating the rates at which these phenotypically resistant cells are produced and why they are refractory to these drugs may lead to the identification and development of procedures to better treat infections with persistent subpopulations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Authors Highlights (sound bytes)

- Antibiotics contribute to the rate of production of persister as well as their identification.
- Multiple genes and processes contribute to the rate of production of persisters.
- In populations exposed to antibiotics, persister production will be favored when common, but not when rare.
- Errors and glitches in cell division and the initiation of replication provide a parsimonious explanation for the existence of persisters.
- Persistence is like mutation, an inadvertent rather than an evolved (selected for) character.

Figure 1.

Simulation of antibiotic treatment; (A) Diagram of the model (details and equations in the Supplemental Material). Changes in the viable cell densities and antibiotic concentrations. (B) Planktonic persisters: Black-persister-free control. Red- $sp=ps=10^{-6}$, Blue $sp=ps=10^{-4}$, Green $sp=ps=1E-3$, $sp=ps=10^{-3}$ with high antibiotic dose. (C) Refuge (biofilm) persister population, Black – persister-free control, Blue $sb=bs=10^{-6}$, Red, $sp=bs=10^{-4}$, Green $sp=bs=10^{-4}$ with high dose standard antibiotic. Purple $sp=bs=10^{-4}$ with a high dose antibiotic that's effective for treating “biofilms”. For other parameter values see Supplemental Material).