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Review

The Eagle Effect and Antibiotic-Induced Persistence: Two Sides of the Same Coin?

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The Eagle effect describes a phenomenon in which bacteria or fungi exposed to concentrations of antibiotic higher than an optimal bactericidal concentration (OBC) have paradoxically improved levels of survival than at the OBC due to a decreased net rate of cell death. Despite extensive observational reports of this effect in different microorganisms, its underlying mode of action is not well understood. Although aspects of the Eagle effect resemble persistence, there is strong evidence that these phenomena are substantially different phenotypic responses to antibiotic treatment. We present an overview of the microorganism and antimicrobial combinations in which the Eagle effect has been observed. Proposed underlying mechanism(s) are assessed, and the Eagle effect and microbial persistence are compared and contrasted. The clinical relevance of the Eagle effect is reviewed, incorporating evidence from experimental *in vitro* and *in vivo* studies, as well as clinical reports.

Highlights

It is the 70th anniversary of the first report of the Eagle effect, yet our understanding of the mechanisms driving the effect remains poor.

The Eagle effect, a paradoxical reduced killing activity at antibiotic concentrations above its MIC or MBC, has been extensively reported in Gram-positive and Gram-negative bacteria, mycobacteria, and fungi, with a diverse range of antibiotics from different classes.

The Eagle effect resembles some elements of bacterial persistence and tolerance, yet has a number of distinguishing attributes, most notably an increased number of surviving bacteria at supra-MIC antibiotic concentrations.

This phenomenon demonstrates that microorganisms have multiple means to evade antimicrobials. Further investigation could lead to clinical benefits, where high antibiotic exposures may compromise expedited clearance of infection.

The Eagle Effect

In 1948 Harry Eagle [1,2], who was studying the bactericidal activity of penicillin with a time–kill assay, described a puzzling phenomenon for some strains of bacteria, later called the **Eagle effect** (see Glossary). In time–kill assays, suspensions of bacteria are exposed to different concentrations of antibiotic, and the surviving organisms are enumerated at defined timepoints by transferring aliquots of culture to antibiotic-free agar and determining the number of CFUs that grow (Figure 1). Ordinarily, the **minimum bactericidal concentration (MBC)** of an antibiotic is defined as the minimum concentration of antibiotic required to give a 3-$\log_{10}$ ($\geq$99.9% killing) reduction in surviving bacteria (CFU/ml) compared to the initial inoculum [3]. The MBC may be the same concentration as the minimum inhibition concentration (MIC), which is the minimum concentration of antibiotic that inhibits visible growth (Figure 1). Alternatively, the MBC may be several twofold dilutions higher than the MIC (Figure 1). However, Eagle noted that, for some strains of bacteria, as the concentration of antibiotic increased above an **optimal bactericidal concentration (OBC)**, more bacteria paradoxically survived (Figure 1). By plotting surviving bacterial numbers over time, he determined that the rate of cell death in response to supra-MBC levels of penicillin, and by extension the bactericidal activity of penicillin, was reduced compared to lower concentrations closer to the MBC [1,2]. Three years earlier, Kirby and Garrod had also reported a similar observation: 1000-fold higher concentrations of penicillin resulted in increased surviving staphylococci [4,5]. However, at that time they attributed the result to contamination of the antibiotic preparation with penicillin-resistant bacteria [4,5].

Over the following 70 years the Eagle effect has been reported in a diverse range of microorganisms, becoming apparent in experiments examining the bactericidal activity of antibiotics at varying antibiotic concentrations [6–11]. This review provides the first comprehensive summary of literature reports of the Eagle effect for bacteria and fungi treated with different

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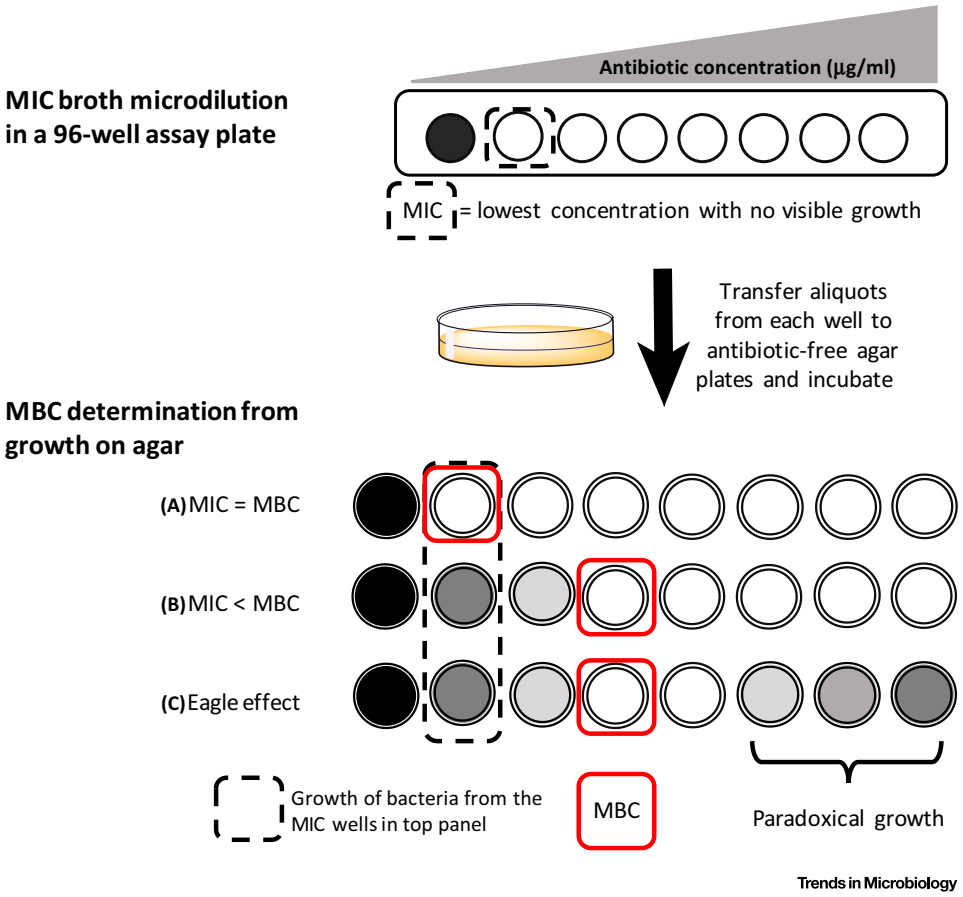

Figure 1. An Illustration of the Eagle Effect Detected in a Microdilution Minimum Bactericidal Concentration (MBC) Determination Assay. Top. A sample row from a minimum inhibition concentration (MIC) broth microdilution assay in a 96-well plate. Shading represents visible bacterial growth (turbidity) in the wells of the MIC assay plate. The MIC is the lowest concentration of antibiotic that prevents visible growth of bacteria (detected by visual observation or by measuring the OD600). The Eagle effect is not detectable by MIC assay alone. Bottom. Different outcomes for bactericidal antibiotics from a microdilution MBC assay. Shading represents the density of bacterial growth (colonies) on the agar plates, with increasing intensity illustrating higher bacterial density. (A) The MBC can be equal to the MIC or (B) the MBC can be higher than the MIC, but similar or fewer numbers of surviving bacteria should be observed at concentrations higher than the MBC. (C) The Eagle effect is said to occur when there is paradoxical growth of higher numbers of bacteria from samples exposed to antibiotic concentrations higher than the MBC.

antibiotic classes with varying modes of action [9–17], explores the mechanisms proposed to explain the phenomenon, and discusses the potential clinical relevance. Gaps in our understanding of the Eagle effect are highlighted, and it is compared and contrasted to the closely related phenomena of bacterial **persistence** and **tolerance** [18–21].

The Eagle Effect in Bacteria

The Eagle effect has been described for a remarkable range of Gram-positive bacteria, Gram-negative bacteria, and mycobacteria exposed to different antibiotic classes with diverse chemical structures, cellular targets, and sites of action. For example, the Eagle effect has been observed with inhibitors of cell-wall synthesis (β-lactams and glycopeptides), DNA synthesis (quinolones), RNA synthesis (ansamycin polyketide), and protein synthesis (aminoglycosides), and also with agents that permeabilise the cell membrane (polymyxin). The

Glossary

Eagle effect: a paradoxical reduced killing of microorganisms by antibiotics at concentrations higher than their optimum bactericidal concentration. The Eagle effect is typically observed following a time–kill or MBC experiment, where more microorganisms survive exposure to antibiotics at high concentrations than the number surviving at the lower, OBC. This effect varies from one antibiotic to another, and is only seen for some antibiotic/bacteria combinations.

Minimum bactericidal concentration (MBC): the lowest concentration of antibiotic that kills ≥99.9% bacteria at a defined endpoint, determined by a reduction of the initial inoculum viability (in CFU/ml) by ≥3-$\log_{10}$. As an example, an MBC value is the antibiotic concentration that reduces an initial inoculum of 10^6 CFU/ml to ≤10^3 CFU/ml. The MBC may be determined by transferring aliquots from wells of an MIC assay that do not have visible turbidity after 18–24 h incubation to antibiotic-free agar and counting the colonies after a 24–48 h incubation period (depending on the organism).

Optimal bactericidal concentration (OBC): the concentration of antibiotic needed to kill the maximal proportion of organisms in a defined time period.

Paradoxical growth: a term used interchangeably to describe the Eagle effect, along with the variants: paradoxical effect or paradoxical growth effect. This term refers to the larger numbers of surviving organisms on subculture to agar from media containing higher antimicrobial concentrations than the OBC, being contradictory to what one expects. This terminology is often used in literature describing the Eagle effect in fungi.

Persistence: the ability of a subpopulation of bacteria to survive an otherwise lethal concentration of antibiotics. The majority of a susceptible bacterial population will die following antibiotic treatment, but a small subpopulation remains viable in a dormant state. This ability to survive is not hereditary, and following regrowth the microorganism has the same

antibiotics and bacterial strains where the Eagle effect is reported are summarized in Table 1, with key details from these studies described below.

The paradoxical effect was first observed for Gram-positive bacteria, including *Staphylococcus aureus, Enterococcus faecalis*, and α- and β-hemolytic streptococci exposed to penicillin [1,2]. Not all strains of these bacterial species examined displayed the Eagle effect, indicating differences at a strain level that complicate attempts to study the effect. Nonetheless, the Eagle effect has also been reported for various next-generation β-lactam antibiotics, including carbapenem and cephalosporin derivatives, and for other bacteria, including *Corynebacterium diphtheriae* [22] and the Gram-negative bacterium *Proteus vulgaris* [23].

The Eagle effect has also been observed in several studies when *E. faecalis* [11,24] and *Clostridium difficile* bacteria [6,14,25,26] were exposed to glycopeptides. These studies describe a bactericidal concentration of antibiotic at one- to eightfold the MIC but reduced activity at concentrations 20- to 2000-fold the MIC [6,14,25,26]. This is concerning because vancomycin's bacteriostatic activity at high concentrations may contribute to clinical treatment failures for *C. difficile* infections, given that vancomycin reaches supra-MIC concentrations in the colon at the site of infection. The Eagle effect was recently reported for *C. difficile* exposed to the lipoglycopeptide telavancin as well as vancomycin, but not the lipoglycopeptides teicoplanin, dalbavancin, oritavancin, or ramoplanin, nor with other classes of *C. difficile* antibiotics (metronidazole or fidaxomicin) [26]. This study shows that analogues from the same antibiotic class do not necessarily all induce the Eagle effect. Antibiotics that do not exhibit an Eagle effect may have therapeutic advantages.

The Eagle effect has also been described for quinolone antibiotics, which primarily inhibit DNA synthesis but have also been shown to induce metabolic stress through reactive oxygen species. It is seen with clinically relevant Gram-positive, Gram-negative, and mycobacterial species exposed to multiple different quinolone antibiotics, including *S. aureus* treated with ciprofloxacin [8] and enoxacin [27], *Escherichia coli* treated with nalidixic acid, ciprofloxacin, and pefloxacin [28–30], and different *Mycobacterium* species treated with ciprofloxacin and moxifloxacin [13]. As a specific example, moxifloxacin had an optimum killing concentration of 0.5 μg/ml against a *Mycobacterium tuberculosis* strain, but at a 16-fold higher concentration (8 μg/ml) the bactericidal activity was decreased by 1.5 logs compared to the control [31]. Furthermore, a reduced bactericidal activity was again observed with high concentrations of moxifloxacin and ciprofloxacin against nonreplicating *Mycobacterium smegmatis*, though this Eagle effect was not observed in actively dividing cultures [12].

Given the diversity of antibiotic classes and bacterial species in which the Eagle effect has been reported, it is clearly a common survival mechanism of bacteria in response to antibiotics, with potential clinical implications. It is therefore imperative that this phenomenon receive greater attention with regard to its significance in the failure of antibiotic therapies.

Proposed Underlying Mechanisms of the Eagle Effect in Bacteria

Most reports describing the Eagle effect are observational, with few studies aimed at deciphering the underlying mechanisms driving the effect. One factor thought to contribute to the Eagle effect is the action of autolysins (murein hydrolases). These enzymes hydrolyze the cell wall components and contribute to bacterial killing by β-lactam antibiotics [37,46]. Multiple groups have studied the potential role of autolysins in the Eagle effect. Nishino and Nakazawa [32] found that more lysis occurred in *S. aureus* exposed to 0.5 μg/ml of cephalothin (41% lysed cells) than to 800 μg/ml (11%). They suggested that the antibiotic interfered with the functioning

susceptibility profile as the ancestral strain.

Tolerance: the ability of a whole population of bacteria to transiently remain viable upon exposure to a high antibiotic concentration. A tolerant bacterial population requires a longer time to be killed compared to a susceptible population. Tolerance can be induced under certain environmental stresses (phenotypic tolerance) or can be caused by a genetic mutation (genotypic tolerance), and in both forms there is no change in the MIC between the tolerant cells and their parental strain.

Table 1. Antibiotics That Have Shown Paradoxical Decreased Bactericidal Activity in High Concentrations against Bacteria

Antibiotic class	Antibiotic mode of action/target	Antibiotics	Microorganisms	Refs
β-lactams	Cell-wall synthesis inhibitor	Penicillin	*Staphylococcus aureus*	[1,2,32]
			Group A *Streptococcus*	[33]
			Enterococcus faecalis	[1,34]
			Group B *Streptococcus*	[1,2,35]
			Group C *Streptococcus*	[1,2]
		Cefmenoxime	*Proteus vulgaris*	[23]
		Benzylpenicillin and cloxacillin	*S. aureus*	[16]
		Amoxicillin; amoxicillin-clavulanate	*Enterococcus faecalis* clinical isolate	[36]
		Amoxicillin	*E. faecalis*	[37]
			Nontoxigenic *Corynebacterium diphtheriae*	[22]
		Men10700 (a new carbapenem) and imipenem	*E. faecalis*	[38]
		Carbapenems: Imipenem and meropenem	*S. aureus*	[7]
		BAL9141 (a type of cephalosporin)	*S. aureus*	[39]
Glycopeptides	Cell-wall synthesis inhibitor	Vancomycin	*Clostridium difficile*	[6,14,25,26]
			E. faecalis	[11]
		Telavancin	*C. difficile*	[26]
		Teicoplanin	*E. faecalis*	[24]
Quinolones	DNA synthesis inhibitor + metabolic stress through reactive oxygen species	Nalidixic acid	*Escherichia coli*	[28,29,40–42]
			Proteus mirabilis	[43]
		Ciprofloxacin	*E. coli*	[28]
			Streptococcus pneumoniae	[8]
			S. aureus	[8]
			Pseudomonas aeruginosa	[8]
			Mycobacterium bovis BCG	[13]
			Mycobacterium smegmatis (nonreplicating)	[12]
		Moxifloxacin	*Mycobacterium tuberculosis*	[31]
		Enoxacin	*S. aureus*	[27]
		Pefloxacin	*E. coli*	[30]
Aminoglycosides	Protein synthesis inhibitor	Amikacin	*E. coli*	[9]
		Gentamicin	*Klebsiella pneumoniae*	[9]
		Tobramycin	*E. coli* *P. aeruginosa*	[9]
Polyketide ansamycin	RNA synthesis inhibitor	Rifampin (Rifampicin)	*Streptococcus pneumoniae*	[44]
Polymyxins	Alters cell-membrane permeability	Polymyxin B	*Acinetobacter baumannii*	[45]

of autolytic enzymes that lead to cell-wall-deficient cells. A low antibiotic concentration resulted in less interference, leading to more antibiotic-induced lysis [32].

Defective autolysins were also implicated in the survival of a subset of *E. faecalis* cells exposed to high penicillin concentrations [34]. Fontana and colleagues suggested that *E. faecalis* strains had two autolysins, one with reduced activity following high concentration antibiotic exposure leading to reduced killing. Additionally, high penicillin concentrations would halt peptidoglycan synthesis and cell growth, resulting in less bactericidal activity [34]. They also showed that the Eagle effect was more likely during the lag phase than the exponential growth phase [34]. Puntorieri and colleagues also found that different strains of *E. faecalis* had varying responses depending on the antibiotic, and that autolysin enzymes were again implicated [36].

β-Lactamase enzymes have also been associated with **paradoxical growth**. High concentrations of cefmenoxime induced greater β-lactamase production in *P. vulgaris*, leading to reduced antibacterial activity [47]. No Eagle effect was observed with a β-lactamase-deficient mutant, or when a β-lactamase inhibitor was included in the bacterial culture [47]. It was suggested that the varying stability and affinity of different antibiotics to β-lactamases could explain their varying abilities to induce paradoxical growth [47], and why some antibiotics within the same class do not induce the Eagle effect in the same bacterial strain. This *in vitro* observation was subsequently tested *in vivo*, in a mouse *P. vulgaris* infection model. There was a 43% survival rate in mice with cefmenoxime treatment at 3.13 mg/kg, but only 20% at 50 mg/kg [48]. This variation was not evident with a non-β-lactamase-producing *P. vulgaris* strain, supporting their hypothesis that cefmenoxime-induced β-lactamase production contributes to the Eagle effect [48].

Postulated mechanisms behind paradoxical growth induced by quinolone antibiotics are related to inhibition of protein synthesis, although quinolones are primarily known for their antimicrobial action through inhibition of DNA synthesis. Luan and colleagues suggested that reduced reactive oxygen species (ROS) levels correlated with inhibition of protein synthesis were responsible for the paradoxical survival of *E. coli* [40]. Similarly, reduced protein synthesis was implicated as the cause for reduced killing of *M. tuberculosis* by moxifloxacin at high concentrations [31].

These proposed mechanisms highlight the multifactorial nature of the Eagle effect and may explain to some extent why this phenomenon occurs in a wide range of microorganisms with varying antibiotics.

The Eagle Effect I*n vivo*: Clinical Relevance for Bacterial Infections?

Most reports of the Eagle effect have been derived from *in vitro* observations during bacterial time–kill or single-time-point minimum bactericidal assays. Few studies have investigated the *in vivo* significance of the Eagle effect. However, several animal models support that the Eagle effect observed *in vitro* translates to *in vivo* consequences, including in human patients (Box 1). Studies with a mouse model of *P. vulgaris* peritoneal infection treated with cefmenoxime [48], a rat model of *S. aureus* bacterial endocarditis treated with cloxacillin [49], and a rabbit model of *C. diphtheriae* endocarditis treated with amoxicillin [22] all reported reduced bactericidal activity and animal survival when high concentrations of antibiotics were used. Furthermore, a rabbit model of *S. pneumoniae* pneumococcal meningitis demonstrated a less effective rate of killing and reduction in bacterial titers by rifampin dosed at 20 mg/kg/h compared to 10 mg/kg/h [44]. A gold-containing compound called auranofin (formerly approved for rheumatoid arthritis treatment) and a structural analogue (MH05) were tested in pneumococcal sepsis mouse models comparing two strains of *S. pneumoniae*. Both compounds exhibited reduced efficacy at 10 mg/kg compared to 1 mg/kg (strain 48) or 5 mg/kg (highly virulent strain 3498) [50]. There

Box 1. Case Reports of the Eagle Effect in Human Patients

In humans, there have been two case reports of bacterial endocarditis where significant reductions in penicillin dosage improved the drug's bactericidal activity in the patient's serum and ameliorated the clinical outcome [51,52]. In the first case, the patient responded well to penicillin, and an increased bactericidal activity of the serum was noted after the dosage was halved [51]. Another possible paradoxical effect with penicillin was found in an infant, whose condition improved following reduction of the penicillin dose, even though an aminoglycoside was also included in the regime [52]. These reports provide some evidence, albeit limited in scope, that the Eagle effect may be clinically significant and should be taken into consideration during antibiotic treatment.

were more animal survivors in the groups receiving lower doses, and the bacterial loads in the bloodstream correlated with the level of survival [50].

The Eagle Effect in Fungi

The Eagle effect in fungi is more commonly referred to as 'paradoxical growth' or a 'paradoxical growth effect'. This refers to the counterintuitive growth of higher numbers of colonies on agar arising from broth cultures treated with concentrations of antimicrobial higher than the OBC (Figure 1). The Eagle effect in fungi was first reported in 1988 with cilofungin, an antifungal active against *Candida albicans* and *Candida tropicalis* [53]. It has since been observed in a range of *Candida* species (including *C. albicans*, *C. dubliniensis*, *C. parapsilosis*, *C. tropicalis*, *C. glabrata,* and *C. orthopsilosis* [15,17,54–56]), as well as in several *Aspergillus* species [57–60], and in fungal biofilms [61–63]. Most antifungals associated with the Eagle effect are echinocandins, which inhibit the synthesis of β-1,3- D-glucan, a major structural cell wall component [58,64]. However, the azole fluconazole caused paradoxical growth in *C. albicans* [65]. It has been shown that paradoxical growth in fungi is both species- and echinocandin-specific, with echinocandins such as caspofungin, micafungin, anidulafungin, all differing in their capacity to induce the Eagle effect [15,17,55,66,67]. Assessment of the effect is complicated by cell growth at high antifungal concentrations being dependent on the growth medium used [54,66,68]. However, regardless of the method, paradoxical growth is consistently found for *Candida* species when treated with echinocandins [55,69].

Underlying Mechanisms of the Paradoxical Effect in Fungi

Fungal cells exhibiting paradoxical growth exhibit decreased virulence and undergo morphological changes in their cell wall structure involving cell enlargement and abnormal septa [70–72]. Elevated chitin content is observed and is considered to be a compensatory mechanism for markedly reduced β-1,3-glucan composition in the presence of high caspofungin concentrations. Regulation of chitin synthesis is therefore postulated to be a survival mechanism during fungal paradoxical growth [57,70,71]. Indeed, inhibition of chitin synthesis eliminated caspofungin-induced paradoxical growth [56]. Increased chitin content has also been associated with reduced echinocandin susceptibility *in vivo* [73].

Multiple pathways have been implicated in paradoxical growth in fungi [56,74], including protein kinase C (PKC), Ca^{2+}-calcineurin signalling, and the high-osmolarity glycerol (HOG) response pathways [56,75,76]. These pathways are involved in regulating chitin synthesis in *Candida* spp. [76,77], including upregulation of chitin synthesis as a survival mechanism upon exposure to high echinocandin concentrations [77], and therefore potentially contribute to caspofungin tolerance [78]. The PKC pathway was implicated due to upregulation of the MKC1 protein kinase within the PKC pathway, after cells were exposed to high caspofungin concentrations [75]. The Ca^{2+}-calcineurin signalling pathway in *Aspergillus fumigatus* was associated with a response to caspofungin, through upregulation of genes involved in chitin synthesis [57]. If this pathway was inhibited, the caspofungin-induced paradoxical effect was abolished [56,75].

Similarly, the HOG pathway is essential for the maintenance of cell wall integrity through its role in osmoregulation and responses to stress, being activated in the event of stress such as exposure to high concentrations of antifungals [79]. Heat-shock protein 90 (Hsp90) has also been implicated in paradoxical fungal growth: addition of the Hsp90 inhibitor radicicol attenuated a micafungin-induced paradoxical effect [80]. Overall, this evidence shows the complex interplay of different pathways involved in cell wall synthesis and how compensatory actions of these pathways occur to ensure the retention of cell wall integrity, meaning that cells that are supposed to be killed at high antifungal concentrations remain viable and have improved survival, consistent with the Eagle effect phenomenon. At a genetic level, paradoxical growth in fungi does not seem to implicate mutations conferring resistance to antifungals nor mutations within the *FKS1* gene encoding β-1,3-glucan synthase in *Candida* [81].

Clinical Relevance in Fungal Infections

As with bacteria and antibiotics, the clinical relevance of the Eagle effect in fungi remains debatable [74,82,83]. There are disparities concerning the concentration levels at which the paradoxical growth is likely to be observed in different species and whether the concentrations associated with paradoxical growth are relevant to those used in the clinic. Paradoxical growth in fungi has been observed to follow a quadriphasic pattern, where growth occurred below the MIC, followed by inhibition of growth at concentrations near the MIC, resumed growth at supra-MIC concentration, and finally returned to growth inhibition at even higher concentrations [66]. In other words, the antifungal displayed two optimal rates of bactericidal activity and a reduced bactericidal activity which resulted in the paradoxical growth of viable cells between 4 and 32 μg/ml for caspofungin against *Candida* spp. [66,75]. In *Candida* spp., some of the concentration ranges at which the paradoxical growth occurs are achieved in the serum during typical clinic treatment [64,66]. However, for *Aspergillus* spp., the current dosing concentration is higher than the concentrations shown to cause the Eagle effect [67] and corresponds to antifungal activity *in vitro*. Therefore, the paradoxical effect may not be considered clinically relevant in these species, even more so with evidence that the presence of serum may abolish the Eagle effect in certain fungal species (Box 2).

A number of studies seem to support the argument that the *in vitro* echinocandin paradoxical effect does not necessarily translate to compromised *in vivo* efficacy. For example, caspofungin treatment in a mouse model of invasive pulmonary aspergillosis resulted in a paradoxical higher fungal burden in the group receiving the highest dose (4 mg/kg), but the rate of survival was not significantly different from that in the group receiving a lower dose (1 mg/kg) [84]. No significant differences were found in the *in vivo* efficacy of echinocandins in a mouse infection model of *C.*

Box 2. Effects of Human Serum on Paradoxical Growth in Fungi

A study conducted by Shields and colleagues argued that paradoxical growth in *Candida* spp. may not be clinically relevant as it was abolished in the presence of 50% human serum [56]. In this study, they found that paradoxical growth was observed in eight *C. albicans* bloodstream isolates in the presence of caspofungin in clinically relevant concentrations in broth microdilution and time–kill assays. However, paradoxical growth no longer existed when the media were supplemented with 50% human serum at caspofungin concentrations of up to 64 μg/ml (eight times the typical peak serum concentration of caspofungin in the clinic; typical serum trough and peak concentrations of caspofungin in serum are 2 and 8 μg/ml, respectively) [56]. It is also interesting to note that, in the presence of 50% human serum, the MIC of caspofungin against these isolates was increased by twofold, and when 10% serum was added, the concentrations at which paradoxical growth occurred were higher than those with no serum [56]. This suggests that, to some extent, serum may have an effect on caspofungin activity. Echinocandins are highly protein-bound (>95%), so it would be expected that, in the presence of serum, *in vitro* activities would be reduced. However, it appears as though the changes in activity do not correlate with individual echinocandin's protein binding [90]. For example, within the echinocandin class, caspofungin was the least affected by serum in terms of its change in *in vitro* activity compared to micafungin and anidulafungin [90], and it is also the one most often associated with paradoxical growth in fungi [66,69,81].

dubliniensis, despite prior observation of paradoxical effects of these echinocandins *in vitro* [85]. In another mouse model of candidiasis, one *Candida* isolate exhibited paradoxical growth both *in vitro* and *in vivo*, but this occurrence was not reproducible in subsequent experiments [86]. Caspofungin and micafungin showed no statistically significant differences in reducing the fungal burden in a mouse model of invasive pulmonary aspergillosis, despite displaying paradoxical killing activity *in vitro* [87]. Furthermore, clinical trials of micafungin and caspofungin treatment of invasive candidiasis showed that the efficacy of treatment was similar among patients receiving low and high doses of these antifungals [88,89]. As such, paradoxical growth in fungi does not necessarily have a clinical relevance to the treatment of fungal infections as the main antifungal classes remain clinically effective.

Tolerance and Persistence in Bacteria

Bacteria are able to grow in the presence of antibiotics through the acquisition of resistance-conferring mutations, which results in a marked increase in MIC (Figure 2A). Some resistant bacteria also carry pre-existing mutations conferring resistance, or have resistance pathways that are upregulated (induced) in the presence of an antibiotic. However, bacteria can also develop tolerance towards antibiotics [21,91], when a whole bacterial population remains transiently viable under high antibiotic concentrations with no change in MIC. Tolerant cells are characterized by prolonged growth and lag phase [91,92], as well as an increased minimum duration of time required to kill 99% of the population (MDK_{99}) [21]. Tolerance may also be integrally associated with treatment failure, as acquisition of tolerance precedes the development of resistance towards an antibiotic [93]. Repeated cycles of antibiotic exposure led to *E. coli* cells acquiring mutations conferring tolerance and, in turn, cells harboring resistance mutations emerged and resulted in a resistant bacterial population [93]. This evidence further highlights the potential clinical relevance of tolerance, especially since antibiotic tolerance has been reported in a variety of clinically relevant pathogens, including *Acinetobacter baumannii* [94], *P. aeruginosa* [95,96], *E. coli* [97], *S. aureus* [98,99], *S. pneumoniae* [100,101], and *M. tuberculosis* [102].

Whereas tolerance is characteristic of an entire bacterial population, persistence refers to the survival ability of a subpopulation of bacteria following treatment with a lethal antibiotic

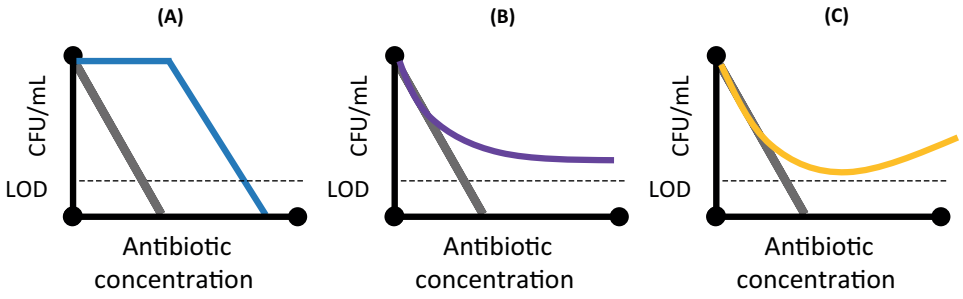

Figure 2. Antibiotic Concentration versus Live CFU/ml Representative of (A) Resistance, (B) Persistence, and (C) Cells Exhibiting the Eagle Effect. The gray line in each graph represents the typical decrease in CFU/ml concentration of cells of a susceptible bacterial strain with increasing antibiotic concentration. A resistant bacterial strain is represented by the blue curve (A), which illustrates that a greater concentration of antibiotic is required to achieve a given reduction in CFU/ml. The presence of persister cells is indicated by the purple curve (B), which illustrates survival of a subpopulation of cells at antibiotic concentrations above the concentrations needed to kill the majority of susceptible and resistant populations. In cells experiencing the Eagle effect, represented by the yellow curve (C), initially the bacterial population is reduced to a level similar to that seen with the persistence effect. However, at even higher concentrations a higher number of bacterial cells survive. Abbreviation: LOD, Limit of Detection.

concentration [18–21,91]. When antibiotics kill most of the population, this subpopulation, formed of persister cells, is able to live for an extensive time. Persister cells were first discovered by Bigger in 1944, when he was unable to completely sterilize flasks from staphylococci by penicillin treatment [103]. In time–kill assays the persistent subpopulation displays a typical biphasic killing curve; the majority of the population are killed at the same rate as susceptible cells, leaving a subpopulation of surviving cells at high antibiotic concentration (Figure 2B) [21,104]. When suitable growth conditions resume, for example when antibiotic treatment ceases, persister cells will grow into a population of cells having a similar susceptibility profile as the parental cells, highlighting that persistence is noninherited [105]. Due to their ability to survive antibiotic exposure, persister cells are attributed to be one cause of recalcitrant infections [106]. A mathematical model has also demonstrated that the presence of persister cells can prolong the length of therapy required to clear an infection and may promote antibiotic failure [107].

Underlying Mechanisms of Tolerance and Persistence

Dormancy, or a state of metabolic inactivity, has been associated with the onset of tolerance [108,109]. Cells entering stationary phase or progressing to dormancy reduce the rate of cell metabolic functions such as cell growth and protein synthesis. Consequently, bacterial cells can 'survive' antibiotic exposure if the antibiotic target is reduced or quiescent due to progression to dormancy [108]. Antibiotics such as β-lactams require actively dividing cells to exert their bactericidal action. When bacteria slow down their metabolism, there is less reliance on the target for survival, hence the bacteria develop antibiotic tolerance [21]. Certain gene mutations and differential expressions of specific proteins have also been associated with development of tolerance (Box 3).

As for persistence, an early theory highlighted the role of toxin–antitoxin (TA) systems in the rise of persister cells [109]. The *hip* gene has been implicated in the persister phenotype, with *hipA7* mutants associated with increased persistence. The mutant HipA7 toxin may not interact as strongly with its antitoxin, thus increasing its toxin activity and leading to increased bacterial persistence [108]. However, various environmental and cellular signaling are also responsible for persister cell formation [20]. The earliest study postulating persister formation proposed that staphylococcal cells that survived penicillin exposure were dormant and nondividing cells [103], characteristics of stationary-phase bacteria. Since then, it is understood that there is a higher likelihood of persister formation when a culture enters stationary phase compared to exponential growth phase [106]. More specifically, it has been proposed that stationary-phase cultures could have up to 100-fold more persister cells than growing cultures [110], reaching up to 1% of the total population in stationary phase [109]. Studies to understand persister

Box 3. Gene Mutations and Differential Gene Expression Implicated in the Development of Tolerance

Tolerance can arise from different mechanisms in different bacteria, and these mechanisms are likely to depend on the exact nature of the environmental stress. During development of tolerance, therefore, gene expression changes and certain proteins are expressed differently. For example, mutations in the *ileS* gene region were implicated in induced vancomycin tolerance in methicillin-sensitive *S. aureus* FDA209P [114]. It was postulated that mutations in the *ileS* gene led to activation of the global stress regulator, (p)ppGpp, which then led to slowing of growth and reduced cell-wall synthesis [114], a hallmark of tolerance. Furthermore, the mutated putative inorganic phosphate transporter gene *pitA* was found to be responsible for tolerance towards daptomycin in *S. aureus* as impairment in the *pitA* gene caused elevation of inorganic phosphate and polyphosphate, which play a role in the bacterial stress response [115]. Changes in gene expression involved in protein translation in response to quorum sensing signalling with the small molecule 2′-amino acetophenone (2-AA) have also been postulated to promote tolerance in *P. aeruginosa* [116]. Finally, the Obg protein involved in DNA replication in *E. coli* was demonstrated to regulate persistence, with overexpression of Obg resulting in a substantial increase in persisters [117].

formation have shown that progression into stationary phase coupled with intracellular ATP depletion was associated with persister formation [111], and so was halted protein synthesis [112,113].

The Eagle Effect Versus Persistence

Similarities Versus Differences

Considering the experimental conditions under which the Eagle effect and persistence are observed, one may argue that the Eagle effect falls under the same umbrella as persistence. In both cases (i) phenotypic changes in the cell wall structure have been observed in some cases, (ii) survival is enhanced with high bacterial loads, and (iii) a subpopulation of bacteria survives exposure to antibiotic concentrations that kill the majority of the population. On the other hand, there is evidence to argue that the Eagle effect and persistence as it is classically defined are two distinct phenomena. These similarities and differences are described below.

Certain phenotypic changes have been observed to occur in both persisters and some bacteria displaying the Eagle effect. An example of this is a change to the cell wall structure upon exposure to cell wall synthesis inhibitors, and potentially this change may play a role in the organism's survival. It has been shown that *M. tuberculosis* cells that are cell-wall-deficient could tolerate ethambutol, a cell-wall-synthesis inhibitor [118]. Likewise, a compromised cell wall structure was suggested to contribute to survival of cephalothin-treated *S. aureus* cells treated with a high concentration of cephalothin [32].

There are also similarities when comparing the impact of high inoculum loads on the Eagle effect and persistence. Eagle observed that the paradoxical reduced bactericidal activity of penicillin occurred under high inoculum/bacterial load, with this condition representative of an established infection [119]. Similarly, high bacterial load and low metabolic activity, consistent with dormancy during stationary phase, has been implicated in persistence [108,109], even though actively growing bacteria in exponential phase can also give rise to persisters [120].

A reduction in the levels of an antibiotic's target has been postulated to contribute to both the Eagle effect and persistence. Eagle hypothesized that the reduced effectiveness of penicillin was due to decreased bacterial growth, resulting in less available target for the cell-wall inhibitor to act on [119]. Furthermore, reduced antibiotic targets have been postulated to contribute to the Eagle effect in both β-lactams and quinolones [31,34]. This feature is also reminiscent of persistence. Bacterial persistence can be either time-dependent or dose-dependent [21]. If a bacterial subpopulation exhibits time-dependent persistence, the bacteria are in a state of tolerance to antibiotics and grow slowly. This occurrence of time-dependent persistence is more likely to be responsible for the prolonged survival of persisters to antibiotics that target mechanisms of cell growth, such as cell-wall or protein-synthesis inhibitors [21]. In other words, these persisters occur and survive because they 'reduce' the antibiotic target by reducing their growth rate. Eagle also reported [1] that the *S. aureus* strain tested had a longer $MDK_{99.9}$ at a high concentration of penicillin compared to a lower concentration. This is consistent with what is known to be a characteristic of bacterial persistence [21].

However, it has been shown that, in *E. coli,* persister cells also experience antibiotic damage to the same extent as nonpersisters, but the availability of a DNA repair mechanism following antibiotic cessation was a critical factor that enabled persister survival [110]. As such, a lack of antibiotic target may be only one contributing factor in persister formation and survival, indicating that different mechanisms of persister induction are potentially in play compared to what is currently known and proposed to underlie the Eagle effect. Having said that, no

studies have characterized surviving cells from the Eagle effect to the same extent as persisters in this respect.

The key distinguishing feature between the Eagle effect and bacterial persistence is that persisters exhibiting dose-dependent persistence are more effectively killed when exposed to higher concentrations of antibiotics [21]. This is because a subpopulation of dose-dependent persisters transiently overexpress resistance factors that enable them to survive in the first place [21]. When exposed to the same antibiotic at a much higher concentration, their survival decreases. In contrast, in the Eagle effect, more bacteria survive exposure to higher antibiotic concentrations than to OBCs (Figure 2C). This is because the rate of cell death is dependent on the concentration of the antibiotic and is reduced with concentrations of antibiotic higher than the OBC.

Bacterial persistence and the Eagle effect may share similarities with regard to the conditions in which they occur, but there are fundamental differences that make them two distinct phenomena. More studies that characterize cells surviving the Eagle effect will provide a better understanding of the other similarities and differences between these two phenomena.

Experimental Approaches to Study Persistence Versus the Eagle Effect

To date, a variety of novel experimental approaches, including fluorescence-activated cell sorting (FACS), DNA sequencing, microfluidic devices, and live cell microscopy-based approaches, have been developed to detect and characterize persisters and tolerance [92,106,121–125]. This has expanded our knowledge of persisters, but these techniques have not yet been applied to characterize the Eagle effect. No studies have isolated cells from bacterial cultures displaying paradoxical growth to test potential similarities and to assess differences with persisters with regard to their phenotypic and genotypic characteristics. It is intriguing to hypothesize about whether there are any shared mechanisms between cells displaying the Eagle effect versus persisters, and whether studies of persisters can inform the study of Eagle effect cells. We believe that techniques that have been utilized to characterize persister cells should be applied to gain a better understanding of Eagle effect cells.

An important factor to consider before any other experimental testing to characterize the Eagle effect cells is reproducibility. There are a myriad of antibiotic–bacteria combinations that have been shown to exhibit the Eagle effect, but at the same time similar types of antibiotics or different strains from the same species do not exhibit the effect. There are many observational reports in the literature of specific antibiotic–strain combinations that have not been independently reproduced, which is critical to enable detailed studies of the effect. Furthermore, it is likely that there will be different underlying factors contributing to the Eagle effect, depending on which strain–antibiotic combination is examined. To advance our knowledge of the Eagle effect it will be crucial to identify particular strain and antibiotic combinations that consistently and reproducibly exhibit the Eagle effect.

Persister cells' ability to survive antibiotic exposure is transient, and they revert to the same susceptibility profile as their original population upon regrowth in the presence of the same antibiotic [107,109]. Eagle has shown in his early reports that, in the *E. faecalis* strain exhibiting paradoxical growth, the surviving population had the same susceptibility profile as the original inoculum [1]. Survivors of an *S. aureus* strain tested did have an increase in MIC but did not differ in their rate of penicillin-induced death [1]. Therefore, it would be interesting to dissect the susceptibility profiles of surviving cells for other bacterial strains that have been reported to display the Eagle effect compared to the original inoculum/population, as well as to investigate

whether or not there is a consistent pattern across different bacteria–antibiotic combinations. Furthermore, bacterial strains that have been reported to exhibit the Eagle effect should be investigated for the presence of mutations that have been associated with persistence, such as the *hipA7* gene identified in *E. coli* [106]. Conversely, bacterial strains with mutations associated with persistence can be examined for their propensity to exhibit the Eagle effect.

Persister cells are heterogeneous in nature, and they exhibit a range of metabolic activities [120,126]. Determining whether or not Eagle effect cells are similar or different from persisters, therefore, could potentially be achieved by measuring the level of metabolic activity of cells surviving at high antibiotic concentrations. FACS has been used in combination with Redox Sensor Green to measure the growth rate and metabolic activity at single-cell level of *E. coli* persisters [120]. An aminoglycosides potentiation assay has also been established to characterize persisters' metabolism, and it found that persisters metabolize mainly glycerol and glucose [127]. These assays can serve as a starting point to investigate Eagle effect surviving cells in more detail and potentially ascertain that they are different from persisters.

Clinical Significance

There is increasing appreciation of the clinical significance of tolerance and persistence, yet little acknowledgment of the potential clinical significance of the Eagle effect, despite the first reports of persisters and the Eagle effect occurring within a few years of each other in the mid to late 1940s. Some evidence suggests that combination therapy could improve treatment outcomes in cases where the Eagle effect is suspected, and a number of *in vitro* studies provide evidence for benefits of combination therapy to combat persisters (Box 4). Therefore, further studies regarding the Eagle effect are necessary to gain a greater understanding about its underlying mechanisms and, for clinically relevant examples, how it can be overcome in the clinical setting (see Outstanding Questions).

Concluding Remarks

Persister cells and antibiotic tolerance have been the subject of increasing scrutiny as more evidence emerges confirming their role in treatment failures and antibiotic resistance. A growing number of studies have focused on characterizing persister cells, and the underlying mechanisms leading to their formation, in the hope of finding ways to eradicate them and improve clinical outcomes for patients. In comparison, while there are many observational reports of the Eagle effect, comparatively few studies have attempted to dissect the Eagle effect and its potential clinical implications. Current evidence suggests that the Eagle effect and persistence

Outstanding Questions

Does the Eagle effect differ from persistence at the molecular level?

What type of experiments will provide the most insight into the mechanism(s) driving the Eagle effect?

Is there an underlying conserved mechanism of the Eagle effect amongst different classes of antimicrobials?

Given that persistence and the Eagle effect share some similar characteristics, can the methods used to characterize persisters be utilized to study Eagle effect cells?

Why are some species of microorganisms more prone to the Eagle effect than others?

How should we best evaluate the potential clinical relevance of the Eagle effect, given that not all bacteria (or antibiotics) exhibit this phenomenon?

Box 4. Can Combination Therapy Be a Potential Solution?

In light of the Eagle effect potentially accounting for some treatment failures with β-lactams, a number of studies have explored the possibility of improving clinical outcomes with combination therapy. A case report in 1986 described a successful combination treatment of penicillin and gentamicin in a case of endocarditis [52] where an Eagle effect was suspected to be hindering recovery. This was one of the first studies that provided some evidence that combining β-lactams with an aminoglycoside could overcome the Eagle effect in the clinic. Since then, several studies have shown that combining β-lactams with a protein-synthesis inhibitor can result in successful treatments [128,129]. As for persisters, there have been a number of *in vitro* studies that combined multiple antibiotics or antibiotics with other compounds to inhibit persisters and/or enhance persister killing. Examples of these include a combination of daptomycin, cefoperazone, and doxycycline to eradicate *Borrelia burgdorferi* persisters [130], addition of nitric oxide to prevent persister formation [131], and glucose supplementation to enhance persister killing by daptomycin [132]. Recently, a new class of synthetic retinoids was tested either alone or in combination with gentamicin, where they were found to be efficacious in killing persisters *in vitro* and in an *in vivo* model of *S. aureus* infection [133]. Similarly, a combination of mannitol with gentamicin was found to be effective in treating a chronic biofilm infection in a mouse model [134]. However, to the authors' knowledge there has not been any evidence in a clinical setting that combination therapy can improve the clinical outcome of recalcitrant infections caused by persisters based on the same rationale.

are two distinct mechanisms of microorganism survival. Hence, the Eagle effect represents another fascinating example of how bacteria can circumvent antimicrobial treatment to ensure their survival through an intense environmental pressure. A greater understanding of the different mechanisms employed by microorganisms to evade antimicrobials could ultimately result in improved therapeutic outcomes for patients by promoting changes in policy, practice, and new drug development.

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Disclaimer Statement

M.A.C. and M.A.T.B. are inventors on patents describing new antibiotics, which may lead to commercial returns.

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