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Adaptive resistance to cationic compounds in *Pseudomonas aeruginosa*

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ABSTRACT

Adaptive resistance is an autoregulated phenomenon characterised by induction of resistance in the presence of drug and reversal to the sensitive phenotype in its absence. This type of resistance is well documented for polycationic antibiotics, including aminoglycosides and polymyxins, in *Pseudomonas aeruginosa* and other aerobic Gram-negative bacilli. It is not caused by selection of resistant mutants but rather by phenotypic alterations in order to survive the lethal drug effect. Adaptive resistance to aminoglycosides is mainly mediated by the MexXY–OprM efflux pump that is rapidly upregulated in bacteria surviving the first exposure to aminoglycosides and is downregulated when bacteria are no longer in contact with the drug. A two-component regulatory system designated ParR–ParS plays a major role in adaptive resistance induced by cationic peptides. In the presence of cationic peptides, ParR–ParS activates the lipopolysaccharide modification operon (*arnBCADTEF*) leading to increased resistance in polymyxins and aminoglycosides. The bactericidal kinetics related to adaptive resistance have important clinical implications and provide a rationale for administering cationic antibiotics in larger initial and longer interval bolus dosing. A better understanding of this phenomenon and the molecular mechanisms responsible will be essential not only for optimum use of cationic antibiotics but also for developing new agents with ability to counteract the detrimental effects of adaptive resistance and thus enhance the therapeutic efficacy of polycationic compounds.

1. Introduction

The term adaptive resistance was introduced in the English literature in 1971 by A.C.R. Dean in an article entitled 'Adaptive drug resistance in Gram-negative bacteria' [1]. It was known that living cells are adaptable to changes in the environment; if conditions change, the proportions of the various cell components adjust to those ratios compatible with the optimum rate of growth in the new environment. The author of the aforementioned article expressed the view that antimicrobial resistance could arise in a somewhat analogous manner; when bacteria grow in the presence of drug they adapt to the new environment and may develop resistance to that agent. Since these pioneering observations, the phenomenon of adaptive resistance has been well documented for aminoglycosides against Gram-negative bacilli and in particular *Pseudomonas aeruginosa* [2,3], but it has also been observed for other cationic compounds such as the polymyxins [4–7]. Over the last two decades, in vitro [2,3,8] and in vivo [9–11] studies have examined the characteristics of adaptive resistance and, more recently, different groups of investigators have elucidated the molecular mechanisms involved in adaptive resistance both to aminoglycosides and polymyxins [12,13].

Although adaptive resistance has important pharmacodynamic features, it has been overlooked or not adequately appreciated by many physicians. Understanding the unique bactericidal kinetics related to adaptive resistance should improve opportunities to use cationic compounds more effectively in the treatment of infections caused by Gram-negative bacilli, particularly in the era of multidrug resistance where the use of aminoglycosides and polymyxins is increasing. In this

report, we review the characteristics of adaptive resistance, the responsible molecular mechanisms, and have made an attempt to give a clinical perspective on this phenomenon.

2. Definition

The term adaptive resistance describes an autoregulated phenomenon characterised by rapid induction of resistance in the presence of drug and reversal to the sensitive phenotype in its absence. Without the drug-sustaining effect the resistance is unstable; in continued presence of the drug resistance is enhanced and prolonged. Adaptive resistance is distinct from genetic resistance, which is stable and arises after chromosomal mutation or acquisition of a genetic element.

3. Adaptive resistance to aminoglycosides

3.1. *In vitro* studies

Adaptive resistance to aminoglycosides was obscured from clinical recognition for many years as these agents are almost always combined with other antibiotics and conventional susceptibility tests are not able to detect this type of resistance [2]. Several *in vitro* and *in vivo* studies have shown that the first exposure of *P. aeruginosa* to an aminoglycoside antibiotic induces a drug refractory state during which the bactericidal effect of subsequent doses is greatly reduced or even absent [2,3,8,9,11,14].

In the first in vitro studies, as shown in Fig. 1, the kinetics of the process was demonstrated using an aminoglycoside and strains of *P. aeruginosa*. Throughout the experiments taking hourly measurements of bacterial killing, bacteria that had no prior exposure to the drug showed the same rapid bactericidal rate characteristic of first drug exposure. In the test culture, where the bacteria had been first exposed to 8× the minimum inhibitory concentration (MIC) for 1 h at 37 °C, the results were strikingly different. Within 2–6 h after removal of the drug and cultivation of the bacteria in drug-free medium, re-addition of an aminoglycoside had no bactericidal action. This was a period in which the post-antibiotic effect on growth inhibition had disappeared and bacterial replication was occurring. This refractory pattern to the second exposure to the drug remained the same at increasing concentrations of drug used for the second exposure, although higher concentrations were able to produce a higher rate of killing. After removal of the drug, ≥8 h were necessary before full susceptibility of the bacteria to aminoglycosides was re-established [2,3]. If after the drug was removed 1× MIC of netilmicin was placed in the incubation mixture, the drug-induced refractory state following the first exposure was enhanced and prolonged as shown in Fig. 1. In that experiment, after 5 h bacterial growth rather than bactericidal action occurred in the presence of 4× MIC in the re-exposure medium. Adaptive resistance is induced by all aminoglycosides and, more importantly, resistance induced by one aminoglycoside produces cross-resistance within the class of these agents [2].

The experiments mentioned previously have provided information on the time course of adaptive resistance using static concentrations of aminoglycosides. This type of

resistance, however, could have different characteristics when drug concentrations decrease with first-order kinetics as occurs in humans. To examine this hypothesis, a dynamic in vitro model mimicking human pharmacokinetics was used by Barclay et al. [8]. A strain of *P. aeruginosa* with a gentamicin MIC of 2.5 mg/L was inoculated into a central chamber and was exposed to single gentamicin doses achieving peaks of 2.5, 8 or 25 mg/L. Following the first dose producing a peak concentration of 25 mg/L (10× MIC), bacteria were extremely resistant to the second drug exposure (at concentrations of up to 20× MIC) for 16 h. Re-exposure to higher doses of gentamicin resulted in bacterial killing, but it still was significantly lower than that achieved in cultures that had not been previously exposed to the aminoglycoside [8]. Bacteria reverted to the sensitive phenotype 36–43 h after the first drug exposure. Based on these experiments, the authors concluded that the duration of adaptive resistance in their model was 14–18 drug half-lives.

3.2. *In vivo* studies

Induction of adaptive resistance in *P. aeruginosa* has also been studied in vivo and the results were strikingly similar to those obtained in vitro [9,10]. In an experimental infection with *P. aeruginosa* in the thigh of non-neutropenic mice, three groups of animals received a single dose of 15, 30 or 60 mg/kg netilmicin. Two hours later the number of viable organisms was measured in the thigh of each group of animals. The bactericidal effect of each dose was directly related as a linear function to the amount of drug given. Two hours after the first dose, a cohort of animals initially given a dose of 15 mg/kg was given a second dose of either 15, 30 or 60 mg/kg. The bactericidal rate after the second dose was slow, fixed and independent of the amount of drug

given. An experiment with a more elaborate protocol was performed in neutropenic mice in which a first dose of 30 mg/kg netilmicin was followed by a second dose of the same size given 2, 4, 6, 8 and 10 h after the first dose. The only regimen that enhanced the antibacterial effect of the first dose was the one in which the second dose was given within 2–4 h of the first dose, before the development of adaptive resistance. All doses given between 4 h and 8 h after the first dose were accompanied by an increase rather than a decrease in the number of viable bacteria. Only an interval of 8 h before the second dose showed an antibacterial effect from the dose. Thus, the results recapitulated the kinetics of the bactericidal rate following the first and second exposure to an aminoglycoside *in vitro*.

Similar results were also observed with amikacin in a rabbit model of endocarditis [10]. Using a strain of *P. aeruginosa*, adaptive resistance occurred between 8 h and 16 h after the amikacin dose, with complete refractoriness to *ex vivo* killing by amikacin seen at 12 h post dose. By 24 h post dose, bacteria within excised vegetations had partially recovered their initial amikacin susceptibility. The longer duration of adaptive resistance observed in these experiments compared with *in vitro* studies was attributed by the authors to possible persistence of aminoglycoside within the cardiac vegetations [10].

Finally, adaptive resistance has been demonstrated in humans in one study in which tobramycin was administered by nebulizer to patients with cystic fibrosis [11]. At 1–4 h following the dose of tobramycin, *P. aeruginosa* cultured from sputum specimens became adaptively resistant to the aminoglycoside, reverting to full susceptibility in 24–48 h. Thus, the time course of adaptive resistance observed in humans was very

similar to that observed in the dynamic in vitro model described previously [8]. As has been pointed out by Barclay et al. [8], the time sequence of adaptive resistance is closely related to the drug half-life in the system in which the phenomenon is examined, suggesting that adaptive resistance will persist for as long as bacteria are exposed to the drug plus the time required for reversal to the sensitive phenotype.

3.3. Mechanisms of adaptive resistance

Several features of adaptive resistance militate against it being the result of selection of resistant mutants. First, in the experiments mentioned previously, adaptive resistance was not present immediately; it required some time and occurred during bacterial replication in drug-free medium. Second, the growth rate and colony morphology during the drug refractory period were the same as in control cultures. Finally, reversal of the phenomenon occurred within a few hours, yielding cells fully susceptible and subject to repetition of the cycle [2,15].

Initially it was believed that adaptive resistance was mediated by reduced aminoglycoside transport across the cytoplasmic membrane during the first few hours after drug exposure [2]. Alterations in the protein profiles of the cytoplasmic membrane, abnormal lipopolysaccharide (LPS), or a collapse of membrane electrical potential were among the potential explanations for decrease drug uptake during the adaptive phase [3–7].

More recently, a multidrug efflux pump (MexXY) was considered to be involved in adaptive resistance to aminoglycosides in *P. aeruginosa* [12]. MexXY belongs to the

resistance–nodulation–division (RND) family that uses the proton-motive force for exporting drugs to the external medium and contributes to the mechanism of impermeability resistance in *P. aeruginosa* [16–18]. These systems classically consist of an RND inner membrane drug–proton antiporter, a gated outer membrane protein factor and a periplasmic membrane fusion protein, called MexY, OprM and MexX, respectively, for the MexXY–OprM efflux pump [19–25] (Fig. 2). These proteins form a functional tripartite efflux machinery that is capable of extruding a variety of antibiotics, including aminoglycosides, macrolides, β -lactams (penicillins except carbenicillin and sulbenicillin, cephalosporins except cefsulodin, ceftazidime and carbapenems), quinolones, lincomycin, chloramphenicol, tetracyclines and tigecycline [26–29].

In a series of elegant experiments, Hocquet et al. [12] demonstrated that adaptive resistance is due to aminoglycoside efflux mediated by the MexXY–OprM pump, rather than by decreased drug uptake as was believed previously. The MexXY efflux system is rapidly overproduced in bacteria surviving the first exposure to aminoglycosides and its expression is downregulated when bacteria are no longer in contact with the drug. In contrast to MexXY overproduction, OprM appears to be produced constitutively in *P. aeruginosa* cells grown under laboratory conditions [30], as its corresponding gene (*oprM*) has expression that is independent of that of *mexXY* and also of *mexAB* (OprM also forms a tripartite efflux complex with the MexA–MexB efflux system) [31]. Of note, the degree of adaptive resistance depends on the amount of MexY induced by aminoglycosides. When mutants defective in MexXY or OprM were used in the experiments, the resistance process was abolished. These observations strongly support the notion that the MexXY–OprM

system is, at least in part, responsible for the process of adaptive resistance. As is evident from other experiments, the functional integrity of this efflux system is also responsible for the observed antagonism between aminoglycosides and the divalent cations Ca^{2+} and Mg^{2+} [32].

Expression of the MexXY system and consequently the process and intensity of adaptive resistance is regulated by the *mexXY* operon. Its expression is under positive regulation of a house-keeping gene named *PA5471*, which appears to be indispensable for the production of MexXY proteins, and under negative regulation of the *mexZ* gene that is located upstream of *mexX* and is transcribed divergently as shown in Fig. 2 [33–35]. It is possible that aberrant or oxidatively damaged polypeptides produced after the action of aminoglycosides on the ribosome activate the *PA5471* gene, which in turn, directly or indirectly via inhibition of the activity of the MexZ repressor protein, upregulates the *mexXY* operon leading to extrusion of the drug from bacterial cells. In addition to aminoglycosides, other antibiotics known to target the ribosome may also enhance expression of the *mexXY* operon [35,36]. Possibly the action of these agents on their ribosomal targets induces the expression of *mexXY* in order to alleviate some stress or adverse effect resultant from ribosome disruption [37]. Other genes such as *rplY*, *galU* and *nuoG* may also influence *mexXY* expression [38,39], but with unknown effects on the process and intensity of adaptive resistance.

A significant step towards understanding the molecular mechanisms responsible for adaptive resistance to cationic compounds was made recently by identifying a novel regulatory system in *P. aeruginosa* designated ParR–ParS [13]. This system

contributes to adaptive resistance by LPS modification as described in detail in the next paragraph [13]. Not all cationic compounds are able to induce adaptive resistance with the same efficiency through this regulatory system. It appears that polymyxins and other cationic peptides are stronger inducers, whereas aminoglycosides require the presence of a cationic peptide to upregulate the system and induce resistance to this class of agents. In addition to LPS modification, ParR–ParS may induce adaptive resistance via interactions with other systems (Fig. 2). It is possible that ParR–ParS upregulates several genes involved in anaerobic respiration, such as *nirC*, *norC*, *norB*, *nosZ* and *nosL*, having as a result decreased intracellular accumulation of drug and induction of adaptive resistance to aminoglycosides in this manner [13,40,41].

4. Adaptive resistance to polymyxins

In addition to aminoglycosides, adaptive resistance has been described to other cationic compounds such as the polymyxins. The main representatives of this group of antimicrobials currently in use are polymyxin B and polymyxin E, the latter being more widely known as colistin [42]. Polymyxins are cationic peptides, resembling in this aspect various human antimicrobial peptides including defensins and cathelicidins. The main target of these polar compounds is the cytoplasmic membrane.

Although resistance to polymyxins in clinical settings is not common, adaptive resistance to this group of agents has been described and studied extensively since the 1970s [1,4,5,43]. Detailed examination of adaptive strains has demonstrated a

number of changes in their cell surface. These include alterations in the architecture of the outer membrane, reduced levels of specific outer membrane proteins, reduction of LPS, alterations in the lipid composition as well as reduction in Mg^{2+} and Ca^{2+} content [4–7]. These alterations appear to decrease the permeability of the outer membrane, affording protection to the sites of action of polymyxins on the cytoplasmic membrane.

Extensive studies in recent years have provided significant insight into the mechanisms responsible for adaptive resistance to polymyxins. The most relevant mechanism of resistance against polymyxins is modification of LPS, which probably evolved as a defence against the naturally encountered antimicrobial peptides [4,44]. Central to this mechanism of resistance is the function of two-component regulatory systems that control the expression of the LPS modification operon *arnBCADTEF-pmrE*. These regulatory systems consist of a membrane-bound sensor protein with histidine kinase activity, which responds to various environmental stimuli (e.g. pH, Mg^{2+} concentration or the presence of cationic peptides), and a cytoplasmic effector protein (response regulator), which regulates transcription of the modification genes in response to the sensor protein [45]. These proteins are activated by phosphorylation and inactivated by dephosphorylation. The phosphorylated regulator protein will then activate and/or repress the transcription of its target genes [46]. Such systems have been described in *P. aeruginosa* and other Gram-negative bacteria such as *Salmonella enterica*, but their specific function, both including sensed stimuli and affected genes, vary between species. Until now, three two-component regulatory systems have been described in *P. aeruginosa* involved in the process of adaptive resistance. These systems act by upregulation of *arnBCADTEF*

and *pmrE* genes, which results in the incorporation of 4-aminoarabinose, a sugar with free amino groups, in lipid A leading to a reduction in the negative charge of LPS and consequently decreased binding of polymyxins [45,47].

The first well characterised two-component system is PhoP–PhoQ, which responds to low pH and low Mg^{2+} and Ca^{2+} concentrations [46]. In *P. aeruginosa*, this system also induces the transcription of OprH, an outer membrane protein of the porin family [48,49]. This porin has been associated with resistance to cationic antibiotics through occupation of Mg^{2+} binding sites and subsequent stabilisation of the membrane [48,49]. Although PhoP–PhoQ has been shown to be activated by sublethal polymyxin concentrations in *Salmonella*, in *P. aeruginosa* cationic peptides by themselves are not sensed by this regulatory system [45,49,50].

The second of these systems is PmrA–PmrB, which also senses low Mg^{2+} conditions and induces modification of lipid A through upregulation of the *arnBCADTEF* operon as described previously [45,50,51]. Mutations in this system have been associated with stable resistance to polymyxins, probably through constitutive activation of the system, resulting in permanent modification of lipid A [51]. Of note, in *P. aeruginosa* cationic antimicrobial peptides induce the expression of *pmrAB* and *arnBCADTEF* operons, independently of both the PhoP–PhoQ and PmrA–PmrB systems, indicating that another regulator system may be involved in this process [45,50].

Recently, a novel two-component regulatory system has been identified in *P. aeruginosa*, named ParR–ParS [13]. This system is activated by subinhibitory concentrations of cationic peptides and appears to be the key component in the

cascade that leads to LPS modification and induction of adaptive resistance [13]. In contrast to the other two regulatory systems that require Mg^{2+} -deficient conditions for their activation, ParR–ParS is activated by cationic peptides regardless of Mg^{2+} concentrations [13]. As shown in Fig. 2, the ParR–ParS system upregulates the LPS modification operon (*arnBCADTEF*), directly or indirectly through activation of the *pmrAB* operon. Interestingly, an interaction between PhoP–PhoQ and ParR–ParS may exist via the *PA1797* gene product [13,52].

The outer membrane efflux pump system MexXY–OprM that has been implicated as a main mechanism explaining adaptive resistance to aminoglycosides has not been associated with polymyxin resistance. However, it was recently observed that the MexAB–OprM efflux system is overexpressed in the metabolically active subpopulations of *P. aeruginosa* biofilms, conferring an adaptive resistant phenotype to polymyxins [53].

5. Clinical implications

The phenomenon of adaptive resistance may have important implications in the treatment of Gram-negative infections. The bacteria–drug interactions related to adaptive resistance, along with the other two pharmacodynamic determinants (concentration-dependent killing and post-antibiotic effect), provide a rationale for administering aminoglycosides in larger initial and longer interval bolus dosing. Clinical and experimental data support this view [54–57]. Although it is widely accepted to administer the total daily dose of aminoglycosides every 24 h, the optimum dosing regimen for these agents remains to be determined [58,59]. The

bactericidal kinetics of adaptive resistance suggests early production and maintenance of high drug levels during the time required for development of resistance (ca. 2 h for *P. aeruginosa* in vitro), then time for complete clearance of the drug and reversal to a sensitive phenotype before repeating the cycle. Such a regimen would accomplish maximum bacterial killing, avoid unnecessary administration of drug during adaptive resistance when bacteria are refractory to killing, and reduce the need for monitoring drug levels. In this respect, the optimum dose interval should be at least as long as the time required for clearance of the drug plus the time needed for reversal from adaptive resistance. Despite the fact that adaptive resistance has been studied extensively in vitro, this phenomenon is poorly understood in the treatment of bacterial infections in humans. Adaptive resistance might explain some paradoxical observations in which laboratory susceptibility results do not reflect the clinical effectiveness of aminoglycosides. The disappointing results from monotherapy with aminoglycosides in granulocytopenic patients and breakthrough of 'susceptible' bacteria during treatment may be examples of clinical expressions of adaptive resistance [60].

Despite the fact that polymyxins have been used extensively in critically ill patients infected with multidrug-resistant organisms, the pharmacokinetics and pharmacodynamics of this group of agents remain poorly understood. Neither experimental nor clinical data exist to date on the implications of adaptive resistance to polymyxins. Nevertheless, there are data indicating restricted clinical efficacy of polymyxins compared with other antibiotics despite their excellent in vitro activity. This discrepancy may be associated among other reasons with the development of adaptive resistance. In addition to the concentration-dependent killing and post-

antibiotic effect displayed by polymyxins [61], adaptive resistance may be another reason for administering these compounds in larger dosages at longer intervals, analogously to aminoglycosides.

A better understanding of the clinical implications of adaptive resistance and the molecular mechanisms behind this phenomenon will be essential not only for optimum use of cationic antibiotics but also for developing new compounds with an ability to counteract the detrimental effects of adaptive resistance. Inhibition of the MexXY–OprM efflux pump and the ParR–ParS regulatory system represents a promising strategy to enhance the antibacterial activity of cationic antibiotics and improve their clinical efficacy in the treatment of Gram-negative infections [12,62–65].

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Fig. 1. Schematic presentation of the time course of adaptive resistance to aminoglycosides in *Pseudomonas aeruginosa* (modified from [2]). Upper graphs show the number of viable bacteria [colony-forming units (CFU)] at different times without prior drug exposure (left panel), with 1-h prior drug exposure to 8× the minimum inhibitory concentration (MIC) of netilmicin (middle panel), and with continuous drug exposure to 1× MIC after the conditioning exposure to 8× MIC of netilmicin for 1 h (right panel). Lower graphs show the bactericidal rate measured 1 h after addition of 4× MIC of netilmicin at successive hourly intervals. Left panel, control culture given first drug exposure. Middle panel, development and recession of adaptive resistance. At 1 h after removal from the initial drug, 4× MIC of netilmicin killed as it did in the control culture (see left lower panel); from 2–5 h after removal of the initial drug and growth in drug-free medium, addition of 4× MIC of netilmicin had decreasing bactericidal activity (almost none after 3–4 h); and at 6 h after the culture was removed from the first drug exposure bacteria had reverted to the sensitive phenotype. Right panel, adaptive resistance is enhanced and prolonged when bacteria grow in the continuous presence of drug.

Fig. 2. Schematic illustration of the mechanisms involved in adaptive resistance to cationic compounds in *Pseudomonas aeruginosa* (modified from [24] and [45]). (A) The MexXY–OprM efflux pump consists of three components: the MexY inner membrane antiporter; the gated outer membrane protein factor OprM; and the periplasmic membrane fusion protein MexX. Activation of the MexXY efflux system and consequently the process and intensity of adaptive resistance to aminoglycosides is regulated by the *mexXY* operon. Its expression is under positive

regulation of the *PA5471* gene and under negative regulation of the *mexZ* gene. It is possible that aberrant or oxidatively damaged polypeptides produced after the action of aminoglycosides (AG) on the ribosome activate the *PA5471* gene, which in turn, directly or indirectly via inhibition of the activity of the MexZ repressor protein, upregulates the *mexXY* operon leading to the extrusion of drug from bacterial cells.

(B) The *P. aeruginosa* ParR–ParS, PhoP–PhoQ and PmrA–PmrB two-component regulatory systems involved in adaptive resistance to cationic antimicrobial peptides, including polymyxins. Cationic peptides activate the ParR–ParS regulatory system, which in turn upregulates the lipopolysaccharide (LPS) modification operon *arnBCADTEF*, directly or indirectly through activation of the *pmrAB* operon. This has as a result a reduction of the net negative charge of LPS by incorporating 4-aminoarabinose in lipid A. As a consequence, interaction of cationic peptides with the outer membrane is limited and the self-promoted uptake of these compounds is decreased, leading to increased resistance to cationic peptides. In addition, cationic peptides under Mg^{2+} -deficient growth conditions activate PhoP–PhoQ and PmrA–PmrB regulatory systems that lead to upregulation of the *arnBCADTEF* operon. It is possible that there is ‘cross-talk’ between ParR–ParS and PhoP–PhoQ via the *PA1797* gene product. ParR may also upregulate several genes involved in anaerobic respiration such as *nirC*, *norC*, *norB*, *nosZ* and *nosL*, having as a result decreased intracellular accumulation of aminoglycosides. Continuous black lines indicate gene upregulation or positive influence; broken black lines indicate gene downregulation or negative influence; red x symbol indicates inhibition.



