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MINIREVIEW

Establishment of Aging Biofilms: Possible Mechanism of Bacterial Resistance to Antimicrobial Therapy

HOSMIN ANWAR,[1]* JANICE L. STRAP,[1] AND J. WILLIAM COSTERTON[2]

Department of Microbiology, University of Alberta, Edmonton, Alberta, Canada T6G 2E9,[1] and Department of Biological Sciences, University of Calgary, Calgary, Alberta, Canada T2N 1N4[2]

INTRODUCTION

Few discoveries in the history of medicine have had such a profound impact on human life and society as the discovery of antibiotics to combat bacterial infections. In the last few decades, there has been an observed exponential increase in the number of antibacterial compounds with diverse chemical structures introduced clinically to control infectious diseases. The most significant development in antibiotic research has been the ability to extend and improve the action of naturally occurring compounds by chemical modification. An example of this is the chemical modification of the penicillin molecule such that the new derivatives are immune to attack by bacterial enzymes (e.g., β-lactamases).

Despite efforts in the search for new antibiotics as well as the improvement of existing antibiotic performance, bacterial resistance to antimicrobial agents remains a problem in the treatment of infections. Before a new antibiotic is introduced into clinical practice, extensive laboratory studies are performed to prove that the new compound is superior to existing compounds in killing the many species of pathogens for which the antibiotic is intended. These species of pathogens, which are grown planktonically in test tubes, are often used to test the effectiveness of the compound in their eradication. If the results are promising, then carefully designed in vivo animal models and clinical trials are used to obtain data regarding the efficacy of the compound. Undoubtedly, this approach has been extremely successful, and many new antibiotics are introduced into clinical practice annually. However, resistance to the antibiotic may be detected soon after its release for clinical use. We believe that this is partly due to the inadvertent use of sublethal concentrations of antibiotics in the treatment of chronic infections such as medical device-related infections. It is therefore of great importance to consider the strategies that are being used to deal with medical device-related infections. It is hoped that this minireview will help to stimulate the formulation of effective strategies to combat these chronic infections.

THE PHYSIOLOGY OF BACTERIA IN BIOFILMS

The formation of biofilms is an important strategy used by a large number of microbes for survival in natural environments (12, 13, 32, 54). For example, microbes have a marked tendency to adhere to the surfaces of rocks in rivers. The microbe lands on the surface and the physicochemical properties of the surfaces of the cell and the rock determine the affinity of interaction and the degree of adherence of the cell to the inert surface. The exopolysaccharides, also known as glycocalyx (13), produced by the bacterial cell assist the cell in firmly gluing itself to the inert surface. Cell division inside the glycocalyx matrix results in the formation of microcolonies. Microcolonies coalesce to form biofilms as the size and number of adherent microcolonies increase (12, 13, 32, 54). A similar mechanism is believed to be used by pathogenic bacteria in the colonization of mucosal and catheter surfaces (3, 13).

The physiology of biofilm cells is extremely complex and is profoundly different from those grown planktonically (3, 13, 54). It is generally believed that the attachment of microbes to a solid surface can profoundly influence their metabolic activities in a way that is not easily predicted on the basis of current knowledge (6, 13, 54). The influence of a solid surface on microbial behavior has been summarized in a recent review (54). The physiological status of biofilm cells is heterogeneous and is determined by the location of each individual cell within the multiple layers of cells that form the biofilm. Cells located in the upper regions of the biofilm (surface biofilm cells) may have easy access to nutrients, including oxygen, and have fewer problems with the discharge of metabolic waste products. These cells are metabolically active and are normally large in size. The cell envelope is likely to be permeable to nutrients. The surface biofilm cells are likely to have properties very similar to those of cells grown planktonically. In contrast, cells enmeshed within the thick glycocalyx matrix (embedded biofilm cells) are likely to be less metabolically active because of poor access to essential nutrients, including oxygen. These cells also have problems associated with the accumulation of waste metabolites in their surroundings. Embedded biofilm cells are at the stage of dormancy, and these cells are likely to be smaller than the surface biofilm cells since they are not actively engaged in cell division. Planktonic cells (suspended bacterial cells) do not have to deal with the accumulation of metabolic products, because the products are constantly being removed from the milieu. However, planktonic cells are subjected to a variety of stresses of physical, chemical, and biological nature (3, 13, 54). They are vulnerable to antimicrobial agents and to lethal attacks by phagocytes. The formation of biofilms, however, generates a sheltered encapsulated community of cells in which these environmental stresses are greatly reduced.

The restriction of the availability of certain essential nutrients is known to influence the physiology of bacterial pathogens which, in turn, can profoundly affect their susceptibilities to antibiotics (3, 10, 13). For example, the

* Corresponding author.

cultivation of *Pseudomonas aeruginosa* under conditions of magnesium depletion has been shown to result in an increase in the resistance of the organism to polymyxin B, EDTA, and aminoglycosides (9, 39, 47). It has been suggested that changes in envelope composition cause alterations to the susceptibility of the organism to antibiotics (3, 10, 39, 47). It is therefore extremely important to identify the environmental stresses that are likely to influence the physiology of bacteria in vivo so that they may be incorporated into experimental designs that can be used to study the interaction of bacteria in biofilms with antibiotics (3, 10).

At present, it remains a mystery why *P. aeruginosa* colonizes and persists in the lungs of patients with cystic fibrosis (CF). The organism does not normally colonize the lungs of individuals without CF. Electron microscopic analysis of postmortem lung tissues has revealed that *P. aeruginosa* forms microcolonies in the lungs of patients with CF (14, 31), and it is believed that this mode of growth may play a profound role in the pathogenesis of the disease (14, 29, 35). The virulence determinants produced by *P. aeruginosa* have been reviewed (28, 36, 40, 44, 57). Alginate is one of the virulence determinants produced by the organism and has been shown to play important roles in the adherence of the organism to trachael epithelium (33, 46), the avoidance of phagocytosis by polymorphonuclear leukocytes and macrophages (48, 50), and resistance to antibiotic therapy (49). Mucoid and nonmucoid forms of this organism are commonly isolated from patients with CF. The production of alginate is known to be affected by environmental signals such as medium osmolarity and nutrient limitation (16, 18, 23, 24, 51). The genetic regulation of alginate biosynthesis has recently been reviewed (35). The *algR* gene product, which controls transcription of a key alginate biosynthetic gene, *algD*, has been shown to be a class of transcriptional regulators that controls global cellular functions in response to environmental stresses (16–18). An increase in the production of alginate has been observed when *P. aeruginosa* PAO 568 (the *muc-2* cells) is cultivated in a medium with an increase in the NaCl concentration (18). Antibiotics continue to play a profound role in the care of patients with CF, and antibiotics with excellent activities against *P. aeruginosa* are being found (5). It is also interesting that biofilm cells of *P. aeruginosa* have been shown to produce 32-fold more β-lactamase than cells of the same strain grown planktonically (22). Although eradication of *P. aeruginosa* is not achievable with antibiotics at present, aggressive antibiotic treatment reduces the planktonic and surface biofilm cell populations of *P. aeruginosa* in the lungs of patients with CF so that the clinical symptoms of pulmonary infections are curtailed and the damage to the lungs of patients with CF is delayed temporarily. It remains to be studied whether the biofilm mode of growth and antibiotics have any effect on the activation of the transcriptional regulation of alginate biosynthesis that causes any changes in the production of alginate in the lungs of patients with CF.

The influence of growth rate on the physiology and sensitivity of bacterial pathogens to antimicrobial agents has been discussed recently (3, 8). The susceptibilities of chemostat-grown planktonic cells of *P. aeruginosa* to the bactericidal actions of phenolic compounds have been shown to be influenced by the growth rate used in the cultivation of the organism (21). Further studies have revealed that this is due to a marked alteration in cellular lipopolysaccharide content (21). An increase in the lipopolysaccharide content as a result of changes in growth rate results in a decrease in the uptake of compounds correlating with a decrease in susceptibility to the drug (21). However, it is important that the methods used in the quantitation of lipopolysaccharide in intact cells may influence the results. It is extremely difficult to draw general conclusions on the influence of a surface on the growth rate of biofilm cells because the results are often dependent on the nature of the organism, the kind and concentration of substrate, and the nature of the solid surfaces (3, 13, 54). Although fast-growing planktonic cells have traditionally been used in the evaluation of the susceptibilities of bacterial pathogens to antimicrobial agents, we have observed an increase in the use of slow-growing cells in the study of bacterial susceptibility to antibiotics. Several β-lactam antibiotics have been found to have good activity against slow-growing bacteria, and further studies have revealed that this can be attributed to the binding of the antibiotics to penicillin-binding protein 7 (15, 52, 53).

CHEMOSTAT STUDIES OF BACTERIA IN BIOFILMS

Chemostats are open systems which can maintain cells growing at a specific growth rate under steady-state conditions. In shake flasks, it is difficult to control the growth rate of bacteria in an ever-changing environment. It has been suggested that physiological adjustments to the lack of availability of nutrients such as the expression of iron-regulated outer membrane proteins under conditions of iron depletion are made by bacterial cells several generations before the cells enter the stationary phase in shake flasks (10).

The purpose of using chemostat systems in antibiotic research is to control the growth rate and the availability of essential nutrients to target organisms so that the bacterial cells cultivated under these defined conditions resemble as closely as possible those growing within an infected host (3, 8, 10). It is known that iron is not readily available to infecting pathogens for growth in vivo (7, 11, 25, 26, 58), because this metal ion is strongly bound to transferrin and lactoferrin (11, 25, 58). To obtain iron, many bacterial pathogens respond by secreting iron-chelating compounds (siderophores) which compete with transferrin and lactoferrin for iron (25, 37, 38). Iron-regulated outer membrane proteins are expressed to facilitate the uptake of iron-siderophore complexes (9, 27, 28, 39, 40). The biosynthesis of siderophore, the expression of iron-regulated outer membrane proteins, and the production of enzymes to destroy iron-siderophore complexes within the cell interior have been suggested as ways of reducing the growth rates of pathogens in vivo, especially at the initial stage of infection (10, 11, 25). It is therefore useful to apply chemostat models to control the growth rate of bacteria in the study of the interaction of biofilm bacteria with antibiotics under these defined conditions (3, 10).

An in vitro chemostat system in which bacterial pathogens are allowed to adhere to solid surfaces and grow as biofilms under iron-restricted conditions at slow growth rates has been developed to study the effectiveness of a number of clinically available antibiotics in the eradication of biofilm bacteria (1, 2, 4). This system provides kinetics of biofilm formation that allow experimenters to evaluate the degree of biofilm establishment and the effectiveness of antibiotics in the eradication of bacteria in biofilms. This is extremely important, because several days or weeks may have elapsed before clinical symptoms of medical device-associated infections are diagnosed and antibiotic therapy is implemented in clinical situations.

In the study of the kinetics of biofilm formation, it was

found that the population of biofilm cells of *P. aeruginosa* that adhered to a solid surface increased exponentially from days 1 to 5 and remained constant after day 5. When the biofilm bacteria were removed on day 2 (young biofilms) and exposed to tobramycin and piperacillin, the results indicated that the young biofilm cells were susceptible to these agents (1, 2, 4). If the cells were allowed to continue the colonization of the surface until day 7 (old biofilms) and were then exposed to tobramycin and piperacillin, the results indicated that old biofilm cells were very resistant to killing by these agents. From our studies, it is clear that the chemostat system offers experimenters the opportunity to investigate the degree of biofilm establishment on the susceptibilities of sessile bacteria to antimicrobial agents. The results obtained with this system enable us to conclude that the eradication of bacteria in biofilms is best accomplished as early as possible and any delay in implementing antibiotic therapy may result in the failure of the antibiotic treatment (1, 2, 4).

The use of the in vitro chemostat system has allowed us to study the interaction of bacteria in biofilms with antibiotics under more realistic conditions, because the system allows us to study the behaviors of aging biofilms for an extended period of time (3). Results of our studies indicate that the establishment of aging biofilms is a possible mechanism of bacterial persistence despite antimicrobial therapy in medical device-associated as well as chronic *Pseudomonas* bronchopulmonary infections, as observed in patients with CF. The way in which this mechanism works is likely to be very complex and may involve changes in the permeability barrier, the production of bacterial enzymes, and the molecular targets of antibiotics, depending on the locations of the individual cells in the biofilms. In view of the complex nature of the physiology of bacteria in biofilms, it is logical to propose that biofilm cells embedded in the glycocalyx matrix may have different degrees of susceptibility to antibiotics, depending on the sites where each individual cell is located within the multiple layers of cells forming the biofilm (Fig. 1). Planktonic and biofilm cells are known to coexist at the site of infection. When these cells are exposed to antibiotics, the planktonic and surface biofilm cells are quickly inactivated, because these actively growing cells are very susceptible to antibiotics. The number of antibiotic molecules entering these cells is likely to be greater than that actually needed to inactivate the cells. The excess antibiotic molecules that have entered the cells and which are not engaged in cell inactivation are probably destroyed by antibiotic-degrading enzymes or are involved in a nonspecific interaction with other cellular components. This results in a significant reduction in the number of antibiotic molecules that are available to kill the biofilm cells that are embedded in the thick glycocalyx matrix (embedded biofilm cells). The antibiotic molecules that do not interact with the planktonic and surface biofilm cells continue their journey to reach embedded biofilm cells. The glycocalyx produced by biofilm cells (exopolysaccharides) is negatively charged and is known to function as an ion-exchange resin which is capable of binding a very large number of the antibiotic molecules which are attempting to reach the embedded biofilm cells. Antibiotic-degrading enzymes such as β-lactamases may also be immobilized on the glycocalyx matrix so that the incoming antibiotic molecules can be inactivated effectively. The purpose is to reduce the rate and also the number of antibiotic molecules that reach the embedded biofilm cells. Furthermore, the embedded biofilm cells are generally not actively engaged in cell division and are smaller in size. Slow-growing cells are generally less susceptible to antibiotics, presumably because the membranes of these cells are less permeable. Under these circumstances, embedded biofilm cells may have sufficient time to switch on the expression of antibiotic-resistant factors such as antibiotic-degrading enzymes (22) to counteract the presence of high concentrations of these agents. The antibiotic targets of these slow-growing embedded biofilm cells may become altered so that they are less vulnerable to attack by antibiotics. The reduction in the populations of planktonic and surface biofilm cells curtails the clinical symptoms of infections and patients may temporarily feel better. At that time, clinicians may decide to terminate antibiotic treatment. The embedded biofilm cells sense that the growth environment has changed when the antibiotic treatment is removed, and the cells again become engaged in cell division. A few weeks later, recurrent infection occurs. From this discussion, it is clear that the mechanism of resistance of biofilm cells to antibiotics is extremely complex. We believe that it is of paramount importance to study the behaviors of bacteria in biofilms so that antibiotics that are effective against biofilm cells can be found. The discovery of antibiotics with activity against

FIG. 1. A proposed model for describing the complex nature of the resistance of bacteria in biofilm to antibiotics. Step 1, Planktonic cells around the infected catheter. These cells are large in size, and the cell membrane is permeable to nutrients and antibiotics. The number of antibiotic molecules entering these cells is likely to be greater than that actually required to kill the cells. Step 2, Surface biofilm cells. These cells resemble the planktonic cells. They are large in size, and the cell membrane is permeable to nutrients and antibiotics. The number of antibiotic molecules entering these cells is likely to be greater than that actually required to kill these cells. This phenomena significantly reduces the number of antibiotic molecules available to inactivate the biofilm cells deeply embedded in the glycocalyx matrix (embedded biofilm cells). Step 3, Binding of antibiotic molecules to the exopolysaccharide (glycocalyx) produced by biofilm cells. Step 4, Immobilization of antibiotic-degrading enzymes on the glycocalyx matrix. Step 5, Inactivation of antibiotic molecules by antibiotic-degrading enzymes immobilized on glycocalyx matrix. Step 6, Embedded biofilm cells. These cells are at the stage of dormancy because of a lack of accessibility to essential nutrients. They are smaller in size, and the cell membrane has been physiologically adjusted to be less permeable to antibiotic molecules. The events described in steps 1 to 5 significantly reduce the rate and the number of antibiotic molecules that reach the embedded biofilm cells. These cells will therefore have sufficient time to switch on the production of antibiotic-degrading enzymes to facilitate the inactivation of antibiotic molecules. The embedded biofilm cells in old biofilms survive the attack of antibiotic molecules. The stars indicate antibiotic-degrading enzymes.

bacteria in biofilms will open the door for detailed study on the relationship of the structures and biological activities of compounds that show good activities against bacteria in biofilms.

CONCLUSION

Medical device-related infections present a major problem for those who are engaged in the treatment of infectious diseases (19, 20, 30, 34, 42, 55, 56). Biofilms are known to be associated with medical device-related infections, and studies have unequivocally concluded that bacteria in biofilms are more resistant to antibiotics than are those that are grown planktonically (13, 41, 43, 45, 49). From our in vitro studies, it appears that one is still able to eradicate bacteria biofilms if antibiotic therapy is implemented as early as possible and if sufficiently high concentrations of antibiotics are used (1–4). Any delay in implementing antibiotic therapy may result in treatment failure. From our studies, it can be concluded that old biofilms are extremely resistant to antibiotics, and it is therefore appropriate for us to propose that the establishment of aging biofilms is a possible mechanism of bacterial resistance to antibiotic therapy. Some efforts should be made to study the physiology and biological properties of old biofilms so that effective measures may be taken to combat their existence.

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