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MINIREVIEW

Testing the Susceptibility of Bacteria in Biofilms to Antibacterial Agents

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The temporary or permanent implantation of various bioprosthetic devices, such as cardiac pacemakers, peritoneal Tenckhoff catheters, etc., has become a common and indispensable part of modern medical care. However, the use of bioprosthetic devices has created problems related to clinical infections which have caused significant distress to those whose lives are dependent on these devices. The devices are frequently colonized by bacteria which form biofilms that have been demonstrated by direct electron microscopic studies (14–16, 26, 33, 34, 40, 41). Instantaneous nonspecific adherence of microorganisms to the surfaces of these devices has been observed in the laboratory (4, 6, 12, 42). The potential sources of bacteria that are likely to be associated with device-related infections have been discussed (18–20). Microbiological analysis has shown that skin floras such as *Staphylococcus aureus* and *Staphylococcus epidermidis* from patients or hospital staff are commonly implicated in these infections (18–20, 26). The physiology of bacteria in biofilms has been reviewed (12). In this minireview, we summarize recent studies of the interaction of bacteria in biofilms with antibacterial agents and we discuss the parameters that should be considered in the development of experimental protocols for study of the efficacy of antibacterial agents against pathogenic bacteria in biofilms.

FACTORS AFFECTING INTERACTION OF BACTERIA WITH ANTIBIOTICS

An antibacterial molecule must interact with the surface components of the bacterial cell to gain access to its targets located inside the cell. It is logical to suggest that any changes of the surface composition of the bacterial pathogen may cause dramatic alterations in the ability of antibacterial molecules to cross the cell envelope. Studies from a number of laboratories have concluded that the surface composition of microorganisms is remarkably flexible and is regulated by the nature of the growth environment (9, 11, 21). The protein (1, 7, 38), phospholipid (35), and divalent metal cation (29) components of the outer membrane have been shown to be influenced by the conditions used in the cultivation of microorganisms. It is of pivotal importance for the cell to maintain a certain degree of plasticity in the composition of its envelope to respond to the frequent changes in its growth environment (9, 11). Pathogenic bacteria encounter an extremely hostile environment when they enter the host. They are constantly under attack by humoral and cellular defenses, and they have adopted several survival mechanisms which involve changes in cell surface composition (9, 11, 21).

Research from several laboratories has concluded that the susceptibility of pathogenic bacteria to antibacterial agents (8, 38, 43) and also to killing by serum and phagocytes (2, 22, 46) is profoundly influenced by the conditions used in the cultivation of the organisms. Growth of *Pseudomonas aeruginosa* under conditions of magnesium depletion often results in resistance of the organism to a number of antibacterial agents, such as polymyxin B, EDTA, and aminoglycosides (8, 38, 43). Interaction of bacteria with subinhibitory concentrations of antibacterial agents has been shown to alter the exposure and expression of surface antigens (28), the production of microbial iron-chelating compounds (28), and the yield of extracellular virulence factors (25). Thus, the growth conditions used in the cultivation of the test organism can greatly influence the experimental results. The physiological status of bacterial pathogens in vivo is very different from that observed when the same strains are cultivated in the laboratory (9, 11). It can be misleading to draw conclusions regarding the performance of antibacterial agents in vivo based on data obtained with cells cultivated in a complex laboratory medium. Therefore, it is of paramount importance to identify the growth parameters that are likely to affect the physiology of cells in vivo so that they can be incorporated into protocols designed to test the efficacy of the antimicrobial agents more realistically in vitro.

PARAMETERS AFFECTING PHYSIOLOGY OF BACTERIAL PATHOGENS IN VIVO

(i) Iron-regulated OMPs. As soon as an invading pathogen enters the host, it must multiply to form a population sufficient to mount an attack on the host. The environmental signals in vivo immediately instruct the invading cell to respond accordingly, and this may involve the expression of certain virulence determinants which are important for the survival of the pathogen. Information obtained from bacteria isolated directly, without subculture, from human infections has revealed that several high-molecular-weight outer membrane proteins (OMPs) that are expressed in cells of the same species grown in batch cultures in nutrient-rich media in vitro are expressed by a number of gram-negative bacteria growing in vivo (1, 5, 7, 23, 24, 30, 44, 48). Specific antibodies that strongly recognize these antigens can be detected in convalescent-phase sera (1, 5, 44, 48). These studies demonstrate the importance of these OMPs for the survival of infecting pathogens in vivo and the determination of the immune system to eradicate them by mounting a vigorous antibody response against these surface antigens.

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The expression of these OMPs was found to be regulated by the amount of free iron present in the growth environment, and in vitro cultivation of the organisms under conditions of iron restriction resulted in the expression of these OMPs (1, 5, 7, 23, 24, 30, 44, 48). These OMPs are commonly referred to as iron-regulated OMPs (1, 5, 7, 23, 24, 30, 44, 48). Iron-binding proteins such as transferrin and lactoferrin are synthesized by the host to inhibit bacterial growth by sequestering free iron (23). Under these circumstances, pathogenic bacteria respond by secreting a number of iron chelators (siderophores) which compete with transferrin and lactoferrin for the acquisition of iron (23, 37). Iron-regulated OMPs function as receptors for iron siderophore complexes in the uptake of iron (23).

(ii) Growth rate. Growth rate is an important parameter that has been suggested to affect the physiology of pathogenic bacteria in vivo (9). The purpose of restricting the availability of iron to the infecting pathogen is to reduce the growth rate. Under iron restriction, the metabolic activity of the cell has to be readjusted to synthesize siderophores and iron-regulated OMPs to optimize the supply of iron required for growth (36). It has been shown that the growth rate of pathogens is significantly reduced when transferrin or lactoferrin is added to their growth medium (23). Reducing the growth rate of the invading pathogen is one of the important strategies used by the host to combat infection. This parameter has commonly been ignored in experimental designs to study the susceptibility of microbial pathogens to antibacterial agents. In the laboratory, the basic principle used by microbiologists in the development of media for the cultivation of pathogenic bacteria is that the medium must contain all the essential nutrients required to support the growth of the organism at a maximum rate. Growth rate has been shown to affect the physiology of microbial pathogens which, in turn, can cause dramatic changes in the susceptibility of the cells to antimicrobial agents (9). The convention of using rapidly growing cells as target organisms in determination of the efficacy of antimicrobial agents should be abandoned if we are to implement an in vitro system which closely mimics the growth conditions of pathogenic bacteria in vivo.

(iii) Biofilm formation. We now return to the main point of our discussion, i.e., growth of bacteria in biofilms. Electron microscopic studies of infected medical devices have revealed heavy colonization of the devices by multiple layers of bacterial cells (11, 13, 32–34, 40, 41). The cells bind firmly to the surface of the device by producing exopolysaccharide glycocalyx polymers, forming a matrix inside which microcolonies develop (12, 47). As the size and number of the adherent microcolonies increase, they coalesce to form biofilms (12, 47). Even though adherent bacterial cells produce a very wide variety of exopolymers, most of which are carbohydrates but some of which are polypeptides (12, 45), biofilms are typically composed of cells embedded at various levels in a highly hydrated polyanionic matrix (12). Observations of living biofilms by the scanning confocal laser microscopic technique have established that biofilms produced by all bacterial species examined to date have similar structures and that cells typically occupy 5 to 35% of the space within these adherent structures, with the largest space being occupied by the matrix (10, 32). This type of growth confers on the cells the ability to avoid attack by host defenses and an increased resistance to antimicrobial agents (11, 12). Antibacterial agents are used for the control of biofilm-associated infections; however, the dosages of antibacterial agents used are, in many instances, below the concentrations required for the elimination of bacteria growing in glycocalyx-protected biofilms (4, 6, 12, 16).

NEED TO TEST INTERACTION OF BACTERIA IN BIOFILMS WITH ANTIBACTERIAL AGENTS

In vitro susceptibility testing is employed to determine the spectrum and potency of antibacterial agents, and the data obtained, along with pharmacokinetic information, are used to formulate guidelines for the use of these agents in the treatment of infections. These in vitro tests do not take into account the growth parameters that are likely to affect the physiological functions of bacterial cells in vivo, e.g., low growth rates, iron restriction, and growth in the presence of biofilms (4, 6, 9, 23). In our opinion, in vitro tests incorporating these parameters should be developed to provide more realistic assays on which to base the dose and frequency of antibacterial agents for treating infections involving bioprosthetic devices. There is no monograph in the U.S. Pharmacopoeia or any other pharmacopoeia describing such tests. Nor are descriptions of such tests provided in the performance standards for susceptibility testing by the National Committee for Clinical Laboratory Standards. Consequently, pharmaceutical companies do not perform testing to determine the efficacy of new agents against bacteria in biofilms.

Ideally, one would prefer to test the activity of new antibacterial agents by using colonized devices removed from infected patients as the source of biofilms because bacteria grown in this manner would give us the most relevant information regarding the relative potency of antibacterial agents for eradication of these glycocalyx-protected pathogens. However, this is obviously unethical and impractical, and alternatives must be developed.

The target organisms should be grown on an inert surface similar to that used to manufacture common medical devices, in a circulating system that allows replenishment at the menstruum and continuous exposure to actively growing planktonic cells (42). The composition of the menstruum should mimic, as much as possible, the composition of the body fluid concerned and should be iron depleted (4, 6, 9, 23, 48). If in vivo conditions are carefully mimicked, in these improved in vitro test systems we will be able to study the effects of antibacterial agents on slowly growing, iron-depleted cells in biofilms that closely resemble those that have been studied (4, 6, 9, 23, 48) after removal from patients in whom they have been the foci of device-related infections.

IN VITRO SYSTEM FOR TESTING ANTIBACTERIAL AGENTS AGAINST BACTERIA IN BIOFILMS

An in vitro chemostat system in which test organisms are cultivated under conditions of iron restriction, low growth rate, and biofilm formation was recently developed for the study of the interaction of bacteria in biofilms with tobramycin (4, 6). Any materials that are used in the construction of medical devices can be used as the physical support for formation of the bacterial biofilm. Mucoid and nonmucoid strains of *P. aeruginosa* were used as test organisms (4, 6). The results indicated that planktonic cells (free floating) of both mucoid and nonmucoid *P. aeruginosa* were susceptible to tobramycin. Cells in young biofilms (sessile bacteria) of both mucoid and nonmucoid *P. aeruginosa* harvested on day 2 were equally susceptible to tobramycin. However, cells in old biofilms (harvested on day 7) were extremely resistant to this antibiotic (4, 6). This study indicated that the eradication

of cells in biofilms is best done as early as possible (4, 6). Any delay in implementing chemotherapy may result in failure of the antibacterial treatment. We recently investigated the susceptibilities of mucoid and nonmucoid *P. aeruginosa* cells in biofilms to combinations of tobramycin and piperacillin (3). Combinations of high concentrations of tobramycin (100 μg/ml) and piperacillin (200 μg/ml) resulted in an enhancement of the killing of these sessile bacteria. This in vitro chemostat system has already given us some clues about the approaches that we should use in obtaining useful data relevant to biofilm bacteria growing in vivo. We believe that the physiology of the invading pathogen in vivo is of paramount importance and should be incorporated in the experimental design for evaluation of the efficacy of antibacterial agents.

The chemostat is a useful system in which the growth rate of the test organism can be controlled, and then there are a number of approaches one can use to obtain iron-deficient media. Some microorganisms can be cultivated in an iron-deficient chemically defined medium, but a large number of bacterial pathogens in which we are interested do not grow in iron-deficient chemically defined medium. Therefore, other means of restricting the iron available to these pathogens must be developed. Iron-binding proteins such as transferrin and lactoferrin (23, 24) or synthetic chelators such as Desferal (CIBA-GEIGY Corp., Basel, Switzerland) (27) and 2,2′-dipyridyl (17) have been incorporated in growth media to restrict the availability of iron to the test microorganisms. In our studies (4, 6, 27), Chelex 100 was used to remove 85 to 95% of the iron from commercially available complex laboratory media. It is important to note that other divalent cations such as magnesium are also removed by Chelex 100 and that these metals must be added to the Chelex-treated media to avoid the imposition of other metal depletions on the test pathogens (27).

CONCLUSION

It has been reported in a number of studies that bacteria growing in biofilms are more resistant to antibacterial agents than are those grown in culture (4, 6, 9, 12, 31, 39, 42). These observations appear to be correct because, clinically, it is extremely difficult to resolve biofilm-associated infections with antibacterial agents alone (12, 14–16, 18–20, 26, 40–42). We believe that the lack of effective antibacterial therapy, once bioprosthetic devices have become colonized, is due to inadequate dosing of the antibacterial agents required for the eradication of the sessile bacteria. It is clear that the current regulatory tests for determining the activity of antibacterial agents, as described in the U.S. Pharmacopoeia or by the National Committee for Clinical Laboratory Standards, are not sufficient to evaluate the efficacy of antibacterial agents against bacteria in well-formed biofilms. We feel that pharmaceutical companies should provide more relevant information regarding the dosages or concentrations of antibacterial agents that will control or cure device-related infections. It is important to incorporate the key growth parameters that are likely to influence the physiology of bacterial cells growing in vivo into the experimental system used to determine antibacterial activity. These are low growth rate, iron restriction, and the biofilm mode of growth. An in vitro chemostat system which incorporates these parameters has been successfully used in the study of interactions of bacteria in biofilms with antibacterial agents (4, 6). The concentrations of antibacterial agents required to eradicate bacteria in biofilms could be determined in the chemostat and provided by the manufacturers as part of their new drug applications to allow clinicians to calculate more accurately the dosages of antibacterial agents needed actually to cure these infections.

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