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MINIREVIEW

Influence of Growth Rate on Susceptibility to Antimicrobial Agents: Modification of the Cell Envelope and Batch and Continuous Culture Studies

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INTRODUCTION

Those interested in antimicrobial agents and chemotherapy culture microorganisms for a variety of tests. These include susceptibility assessments, bioassays, studies of the interactions between antibiotics and host defenses, and the in vitro simulation of in vivo conditions for purposes of extrapolation (19). The specific physiological state of the organisms used, especially surface properties, greatly influences the outcome of all such tests (7, 12, 13, 37). An underrecognized but major determinant of such physiology is the rate of cell replication. Recent excellent reviews have been concerned with growth rates in nature (54, 79) and the metabolic consequences of slow growth (15).

A characteristic response of a population of replicating microorganisms when an adverse environmental change occurs is to reduce the growth rate, perhaps to zero. Slowly growing organisms generally survive adversity better than do those replicating quickly (12, 13, 93). The precise nature of the physiological response is influenced by the particular nature of the adversity, be it an inhibitor or the lack of an essential nutrient(s). Many environmental changes exert their main effect on the proton motive force, which influences the phenotypic response of the bacterium, including the growth rate (54, 57). The growth rate and nutrient limitation also influence plasmid stability, including that in vivo (13).

Spore formation is the supreme example of biological survival. It appears significant that the probability of spore formation is inversely related to the growth rate of the vegetative culture (20; P. Gilbert, P. J. Collier, and M. R. W. Brown, Antimicrob. Agents Chemother., in press). It also seems probable that several resistance mechanisms are linked to a reduced growth rate for nonsporeformers (13). Thus, the growth rate per se influences physiology, as do the specific cultural circumstances. It is experimentally difficult, but nevertheless possible, to separate the two.

The closed environment of a batch culture is gradually modified by the cells until it no longer supports rapid growth. Controlled changes in doubling times (t_d) are typically brought about by alterations in the medium or temperature; these changes also occur in vivo (84). Such factors may independently influence cell physiology. It is inherently difficult, therefore, to study the influences on cell physiology and associated properties of the growth rate per se in a batch culture. It has long been known that sub-MICs of some antibiotics, when added to logarithmic-phase batch cultures, rapidly change t_d in a reproducible way. Quantitative relationships between antibiotic concentrations and t_d have long been used to assess the possible synergistic action of combinations (9, 32, 33).

The use of population kinetics to define the logarithmic phase of a batch culture can be physiologically misleading. For example, nutrient-depleted batch culture cells are typically in the stationary phase. Changes in cell properties, however, may take place several generations before the onset of the stationary phase because of reductions in the availability of specific nutrients. Magnesium-depleted *Pseudomonas aeruginosa* in batch cultures loses susceptibility to EDTA and polymyxin B, depending on other metal cations in the medium. This susceptibility is fully restored only after about three generations in magnesium-plentiful medium (10). Similarly, in batch cultures iron-depleted media derepress high-affinity iron uptake systems in *Klebsiella pneumoniae* about three generations before the onset of the stationary phase (94). The use of logarithmic-phase cells therefore requires that they are harvested several generations both before the onset of the stationary phase and after inoculation. If not, they often demonstrate some of the properties of stationary-phase cells or of the inoculum or of both. The presence of logarithmic replication is therefore no guarantee of a constant and reproducible cell envelope and hence of associated properties (7, 37).

The presence of iron-regulated membrane proteins in the outer membrane of gram-negative bacteria and the production of siderophores are often taken as evidence of iron deprivation. These and other envelope changes, although related to nutrient deprivation, can nevertheless be produced in vitro by rapidly growing bacteria that have become adapted to acquiring iron or other nutrients under such conditions while maintaining the maximum specific growth rate (μ_{max}) (13, 97). Consequently, long in vivo t_d (see below) may be the result either of deprivation of a nutrient(s) other than iron (7, 84a) or of the presence of growth-inhibitory substances or antibodies directed at surface structures involved in nutrient uptake, e.g., iron-regulated membrane proteins and porins (13).

An open continuous culture can maintain cells growing under steady-state conditions (45, 86). In a chemostat the medium has an excess of all nutrients except for one at a growth-limiting concentration. The existence in a chemostat of an equilibrium cell mass is a consequence of the exponential loss of cells resulting from the dilution rate (D) (volume changes per hour) being equal to the specific growth rate (μ)

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and related to t_d as $D = \mu = \ln 2/t_d$. Thus, the chemostat enables the study of growth rate effects per se. Quantitatively defined salts media have been derived to yield cultures in which a specific essential nutrient is depleted while others are present in controlled excesses (17, 55).

In vivo growth rates appear to vary from slow, perhaps zero, to fast. They may generally correspond to infections that are chronic or in the early stages of the acute phase (13, 16, 84a, 93). The t_d of an invading organism contributes to the outcome of an infection. The ability to take up low levels of a nutrient (93), in addition to generating a characteristic cell envelope, influences the t_d and thus the attainment of a microbial population sufficient to harm the host. Such considerations are significant in selection. A low μ_{max} and a high affinity (low K_s) for the substrate favor selection at low nutrient levels (44, 49).

Many in vivo studies do not distinguish the contribution of host clearance to observed t_d. True t_d can be measured by using nonreplicating genetic markers or temperature-sensitive mutants (not multiplying in vivo) (5, 13, 80, 84a). Recently, the frequency of dividing cells, calibrated with chemostat cultures at different t_d, was used to study t_d in various experimental infections (R. M. Cozens, P. Sulc, B. Hengstler, S. Kunz, E. A. Konopka, and O. Zak, Program Abstr. 26th Intersci. Conf. Antimicrob. Agents Chemother. abstr. no. 572, 1986). In all cases replication was slow relative to that in vitro, especially in chronic infections.

Sub-inhibitory concentrations of antibiotics may significantly influence the host-pathogen interaction (60). Many studies use concentrations which lengthen t_d. The influence of t_d on the observed effects is reduced or eliminated if drug concentrations which have little or no effect on t_d are used (25, 50–53, 66).

The t_d of a culture not only influences cell physiology and hence the outcome of susceptibility tests but also, especially with gram-negative bacteria, may influence sensitivity to handling procedures before and after testing (29). Thus, logarithmic-phase cells are relatively sensitive to rapid changes in temperature or osmolarity because of conventional harvesting and posttest recovery procedures (29, 41).

MODULATION OF SUSCEPTIBILITY THROUGH MODIFICATION OF THE CELL ENVELOPE

Batch culture studies. Given optimal growth conditions, microbes grow rapidly and efficiently with generation times as short as 20 min. In natural environments, rapid division is unlikely to persist for long. More likely the rate and extent of growth are governed by the availability of critical nutrients. The imposition of nutrient deprivation causes the physiology of the cells to adapt in a number of ways. (i) Usage of the nutrient is rationed within the cell, through the use of alternative substrates, modification of the cell composition, and/or reduction in the amounts of cellular macromolecules containing such nutrients. (ii) Alteration of the cell surface occurs and increases the affinity of surface components for the growth-limiting substrate to make uptake into the cytosol more competitive. (iii) The cellular growth rate is reduced to the maximum permissible (given i and ii).

Growth limitation by different nutrients therefore gives rise to cells with reduced growth rates and coincidentally radically altered envelopes (6, 10, 28, 46, 59). This result has been widely reported to influence greatly susceptibility to antimicrobial agents (6, 39) and to antibiotics (12, 24, 90) for a wide range of organisms (8, 21, 22, 36, 46, 63, 65, 87). In gram-negative bacteria susceptibility changes are often associated with modifications of both the outer and cytoplasmic membranes (28, 61, 71). Thus, the growth of gram-negative species under phosphate limitation (P-lim) decreases the cellular phospholipid content yet increases the fatty and neutral lipid content (34), whereas under magnesium limitation (Mg-lim) diphosphatidylglycerol content is slightly increased (8, 34, 35). These changes have been associated with the susceptibility of cells to agents, such as biguanides (14, 47, 56), gentamicin (74), and polymyxin (30, 95), which interact directly with specific envelope lipids. Gilbert and Brown (34) showed that in batch cultures carbon-limited (C-lim) *Escherichia coli* was particularly susceptible to the actions of substituted phenols and 2-phenoxyethanol and related these changes to increased amounts of lipopolysaccharide (LPS).

Al-Hiti and Gilbert (1) demonstrated with USP antimicrobial agent effectiveness test microorganisms that the resistance to a number of commonly used preservatives varied markedly after growth in liquid media producing C-lim, P-lim, Mg-lim, or nitrogen limitation (N-lim). Generally, changes in susceptibility have been correlated with changes in the phospholipid (47, 48, 74, 88), porin protein (43, 94, 97), LPS (85), and cation (10, 40, 64, 69) composition of the cell envelope. These changes are thought to modify the action of chemical antimicrobial agents in a number of possible ways. (i) When the envelope itself contains the primary target for drug action, a reduction in the relative abundance of the target material may reduce the overall susceptibility of the cells (70, 77). (ii) Alterations to the bacterial surface, particularly those affecting the acidic phospholipid content, LPS, or surface charge, can affect the initial binding of antimicrobial agents (62). (iii) Hydrophilic agents must traverse the outer membrane via its porin proteins (23, 73); a variation in the porin protein content may therefore be reflected in the susceptibility to such agents (70). (iv) The cell envelope can be regarded as a series of lipophilic and hydrophilic compartments. Hydrophobic agents that are active at the cytoplasmic membrane or cytosol but unable to utilize porins must pass through these compartments to gain access to their sites of action (70). Passage is influenced not only by the relative lipophilicity of the agent but also by the lipophilicity of each compartment. Thus, changes in envelope composition affect the deposition of the antimicrobial agent throughout the cell (42, 96).

The nutritional status of cells growing within their natural habitats is almost impossible to assess (7, 37, 78). For contaminants found in pharmaceutical agents, cosmetics, and toiletries, the original growth conditions of the organisms may include the manufacturing water, raw materials, and product residues within the manufacturing plant and other parts of the environment. One can only speculate about the nature of particular nutrient deprivations associated with particular localized sites.

Continuous culture studies. The chemostat allows μ to be controlled with minimal changes in the physicochemical environment of the cells. Many workers have used chemostats to evaluate the effects of the growth rate on the susceptibility of cells to antibiotics, disinfectants, and preservatives. A general conclusion to be drawn from such studies is that slowly growing cells are particularly recalcitrant to chemical inactivation (6, 30, 35, 36, 89).

P. aeruginosa becomes particularly susceptible to polymyxin and EDTA as the growth rate is increased (30). The susceptibility of this organism to various substituted phenols altered with changing growth rate when the change was associated with a marked alteration in the cellular LPS

content (35). Increases in LPS resulted in decreased drug uptake by the cells and decreased susceptibility.

Various studies have related changes in gross cell envelope composition, brought about by alterations in the cellular growth rate and the nutritional environment, to changes in polymyxin susceptibility (30, 64). Particular studies have implicated acidic phospholipids (48, 88), LPS (85), and the presence of particular outer membrane proteins such as H1 in *P. aeruginosa* (68, 82) as regulators of polymyxin binding and/or permeation through the envelope. Although these studies have clearly demonstrated the dependence of polymyxin action upon nutrient limitation and the growth rate, the use of only one or two physiological variables may have led to chance covariance. The susceptibility of *E. coli* to the lytic action of polymyxin B was assessed at a variety of μ values and under conditions of C-lim, N-lim, P-lim, and Mg-lim (95). Mg-lim and P-lim cells demonstrated a trend of increased resistance with increasing growth rate, whereas C-lim and N-lim cells demonstrated increased susceptibility as the growth rate increased. Divergent patterns such as these allowed a number of models for resistance to polymyxin to be assessed. It was not possible to attribute polymyxin susceptibility to any single envelope component (phospholipid composition, LPS, 2-keto-3-deoxyoctulosonic acid, cation content, outer membrane proteins, etc.); instead, the patterns of susceptibility reflected, in a complex manner, the presence of envelope proteins and acidic phospholipids. Similar conclusions have been reached with *P. aeruginosa* (11, 81).

The interrelationship of the chlorhexidine susceptibility of *E. coli* and the growth rate for four nutrient limitations was investigated by using chemostats (95). N-lim and C-lim cultures showed an overall increase in susceptibility as the growth rate increased, whereas Mg-lim and P-lim cultures showed an opposite trend of increased resistance. At the extremes of growth rate tested different orders of susceptibility were observed between nutrient limitations. When μ was $\leq$0.08/h, susceptibility was seen to decrease with different nutrient limitations in the sequence C-lim > P-lim > Mg-lim > N-lim, whereas at faster growth rates (μ, $\geq$0.4/h), the sequence was altered to C-lim > N-lim > P-lim > Mg-lim. Overall, C-lim cultures were most susceptible to chlorhexidine, with this limitation showing the least dependency on the growth rate. If chlorhexidine binding and activity were dependent upon acidic phospholipid content or some other cell envelope component, as has been suggested by some earlier studies, then they ought to have demonstrated opposite dependencies on the growth rate for Mg-lim and P-lim versus C-lim and N-lim. No such correlation was observed for specific phospholipids, LPS, outer membrane protein composition, or the phospholipid/fatty and neutral lipid ratio. All of these properties, however, changed significantly with the growth rate and nutrient limitation. The results were not consistent with any simple model for chlorhexidine binding and activity and probably reflected a subtle involvement of phospholipid-LPS complexes and cations in chlorhexidine permeation through the envelope and binding to the cell membrane.

In a related study, the effects of the growth rate and specific nutrient limitations on the activity of a homologous series of *n*-alkyltrimethylammonium bromides against *E. coli* were studied (96). The growth-inhibitory and bactericidal activities of these compounds are parabolically related to the *n*-alkyl chain length of these compounds and thereby to compound lipophilicity (log P) (2). The chain length at which optimal activity is demonstrated varies between different cell types and reflects the lipophilicity and barrier properties of the cell envelopes (42). Wright and Gilbert (96) argued that alterations in envelope lipophilicity through changes in the growth rate and nutrient limitation might be expected to produce changes in optimal lipophilicity (log P_o) and also in the degree of activity demonstrated by the optimally active compound. In all cases resistance was maximal at growth rates of 0.1 to 0.23/h and decreased markedly at faster growth rates. The compounds chosen represented one side of a parabolic relationship between log P and biological activity in which, for nutrient broth-grown cells, activity was maximal for the compound with an *n*-alkyl chain length of 16 (cetrimide; USP). Activity was reduced for all the compounds at slow growth rates (0.05 to 0.2/h) and therefore suggested an overall increase in envelope lipophilicity followed, as the growth rate increased, by a steady decrease. The effects upon activity would be expected and were observed to be greatest for those compounds with a log P closest to the log P_o (cetyltrimethylammonium bromide). Although similar trends were observed for all four nutrient limitations, C-lim cultures were the most resistant to the agents and showed a 10-fold variation in susceptibility, whereas P-lim cultures were the most susceptible and showed an approximate 1,000-fold change in susceptibility. The results of this study therefore supported the hypothesis that the growth rate and nutrient limitation alter the overall lipophilicity of the cell envelope and thereby influence the optimal value of log P required by compounds to traverse it.

Continuous culture techniques have also been extensively used to model natural open-growth systems, such as infections, to control the growth rate and to apply particular nutrient deprivations. With such techniques, the growth rate and nutrient deprivation have been identified as fundamental modulators of antibiotic activity. From such studies it has become apparent that the antibiotics ceftizoxime and ceftriaxone have no activity against slowly growing cultures of *E. coli*, irrespective of the growth-limiting nutrients studied (18, 90). In contrast, the β-lactam CGP 17520 is particularly effective against slowly growing cultures, with activity directed against penicillin-binding protein 7 (18, 91). Since the expression of penicillin-binding proteins is highly growth rate dependent, β-lactam antibiotic susceptibility is affected (13, 18, 90, 92). The activity of polymyxin is governed by nutrient limitation and μ and can be increased by up to 10-fold (27, 95). The aminoglycoside antibiotics tobramycin and streptomycin are also growth rate dependent in their action (67, 76), as are the newer quinolone agents (98, 99; R. C. Cody, G. C. Cuchural, and M. Barza, 28th ICAAC, abstr. no. 86, 1988). Such effects are not restricted to antimicrobial susceptibility and have also been reported to influence profoundly the immunogenicity of microbes (3, 13) as well as susceptibility to host defenses (4, 31, 36) and extracellular virulence factor production (72).

Cell size is altered widely as a function of μ (26, 38, 58, 75, 83). This alteration in turn causes changes in the cell surface area/volume ratio. Exclusion resistance to antimicrobial agents also varies with cell size (83), as does susceptibility to drugs which bind strongly to or act at the cell envelope (e.g., polymyxin and tetracycline).

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