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Serum Bactericidal Test

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INTRODUCTION

The susceptibility of microorganisms to antimicrobial agents is most often estimated in the laboratory by measuring the inhibitory activity of the agent (92). Tests which measure only inhibition of growth may not always provide sufficient information to guide the therapy of certain infections such as infective endocarditis. Moreover, certain antimicrobial agents such as beta-lactam agents, which are considered to be "bactericidal," can no longer be assumed to kill all isolates (33, 54, 82, 103). There is the need, therefore, for additional laboratory methods that can assess the bactericidal activity of an antimicrobial agent.

The serum bactericidal test (SBT) is a modification of the broth dilution method that measures the bactericidal activity of the patient's serum during antimicrobial therapy against the bacterial pathogen isolated from that patient. It is one of the few in vitro tests performed in the clinical microbiology laboratory that incorporates the interaction of the pathogen, the antimicrobial agent, and the patient. Although the use of such a test for assessing the bactericidal activity in a patient's serum has been widely used for 40 years, its performance, results, and interpretation have been subject to question and controversy (12, 38, 113).

HISTORICAL REVIEW OF THE SBT

Of the laboratory methods for assessing bactericidal activity, it is not surprising that the SBT has received the most attention. Tests to estimate the bactericidal activity of blood antedate the antimicrobial era (53). A logical extension of this concept is the estimation of the bactericidal activity of blood during the antimicrobial treatment of serious infections. Although Schlichter and MacLean (84) are generally given credit for the SBT, they tested only the inhibitory activity of serum and did not define criteria for the determination of bactericidal endpoints. In fact, it was Fisher (20) who subsequently modified Schlichter's method by subculturing the tubes that failed to exhibit macroscopic evidence of growth after overnight incubation. Although Fisher addressed the need for determining the serum bactericidal concentration, there have been no universally accepted criteria for microdilution or macrodilution methods for performing bactericidal testing despite the use of the SBT for 40 years. It is no wonder that critical reviews of the SBT (12, 38, 113) have not found this test clinically useful and have stressed the need for standardization of the methodology. As the SBT is presently done, there are marked differences from laboratory to laboratory in every major variable in the test (66). Inadequate descriptions and variation in test methods

among clinical laboratories preclude objective assessment of results from clinical studies. Therefore, the National Committee for Clinical Laboratory Standards has recently published proposed guidelines for both bactericidal testing (56) and the SBT (55). These guidelines review aspects of bactericidal testing and the SBT which require standardization. The use of these guidelines through the National Committee for Clinical Laboratory Standards consensus mechanism should allow agreement about standardized methods. This will then enable the appropriate prospective clinical trials to be done to provide a rational data base for clinical interpretive criteria for the SBT in serious infections.

PROBLEMS WITH THE ASSESSMENT OF BACTERICIDAL ACTIVITY

Although the in vitro evaluation of the killing effect of an antimicrobial agent is attractive in a conceptual sense and appears to be necessary in a clinical sense for certain infections, there are a number of factors which have been shown to interfere with the ability of the laboratory to measure essentially complete killing after overnight incubation. These include both biological and technical factors and must be appreciated when using any bactericidal test such as the SBT.

Biological Factors

Persistence phenomenon. A small number (usually <0.1% of the final inoculum) of bacterial cells, termed "persisters," are able to survive the lethal activity of an antimicrobial agent (30). This appears to be especially true for cell wall-active agents. If the persisters are subcultured and retested, they are just as susceptible as the original isolate, and no greater proportion of cells persists (28). The ability of these persisters to survive appears to be because these cells are either dormant or multiplying slowly; i.e., the rate of antimicrobial agent-induced killing is decreasing in strict proportion to the decrease in the rate of bacterial growth (102).

Paradoxical effect. Another factor known as the "paradoxical effect" occurs when the proportion of surviving cells increases significantly as the concentration of the antimicrobial agent increases beyond the minimum bactericidal concentration (MBC) (17, 59). This too is especially common for cell wall-active agents. It has been postulated that a high concentration of the antimicrobial agent can inhibit protein synthesis to a degree which prevents the growth necessary for expression of the lethal effect of the drug (57, 59).

Tolerance. In antimicrobial agent tolerance, bacteria evade only the lethal action of the antimicrobial agent; there is no change in the minimum inhibitory concentration (MIC) (33, 103). Tolerant isolates exhibit unusually high antimicrobial agent MBCs relative to their MICs (MBC/MIC ratio of ≥32 after 24 h). Tolerance has been investigated mainly with beta-lactam agents. Initial reports found that decreased autolytic activity of the organism was a mechanism for tolerance (100). In addition, both the persistence phenomenon and the parodoxical effect appear to be related to tolerance (103). At least four mechanisms have already been found which enable clinical isolates of bacteria to survive during therapy with cell wall-active agents (103). The common feature of these mechanisms is that the behavior of the microorganisms does not result from an increase in the MIC, but from some secondary mechanism related to cidal and lytic effects. The first of these mechanisms is phenotypic tolerance, which is a property of virtually all strains of bacteria and is manifested only under certain growth conditions (36, 101). It is the most common form of tolerance for antimicrobial agents encountered in vivo and is exemplified by the nongrowing dormant bacterium. Another form of tolerance is genotypic tolerance in which a microorganism possesses a unique property such as a defective autolytic system which is restricted to certain mutants (100). The other forms of tolerance have already been mentioned, namely, persistence and the paradoxical response to beta-lactam antibiotics.

Development of resistance. It is possible for microorganisms to develop resistance during the performance of a susceptibility test. This resistance may be a phenotypic phenomenon (27) such as that seen in vitro and in vivo with rifampin (3, 11, 111). Genotypic resistance (47, 58) is related to either a chromosomal mutation or acquisition of a plasmid or a transposon. The likelihood of detecting either phenotypic resistance or genotypic resistance increases with a higher absolute number of microorganisms (>10^6 cells) (21, 25). Tests for bactericidal activity simply may select such strains from the population. Unlike persisters, these survivors will demonstrate an increase in antibiotic MICs when retested.

Technical Factors

Growth phase of the inoculum. Cells in rapidly growing cultures (mid-logarithmic phase) are killed more effectively than cells in slowly growing cultures (stationary phase) because most antimicrobial agents are more active against multiplying cells than against nongrowing cells (102). Therefore, the growth phase of the inoculum can have a marked effect on the results of bactericidal testing (37, 39, 46, 49, 95). The use of overnight cultures for the inoculum will result in MBCs which are much higher than the MIC, although the MICs will not be altered (7).

Inoculum size. Increasing inoculum size can diminish bactericidal activity for some antimicrobial agents against certain microorganisms. This is especially so for microorganisms producing beta-lactamase that are tested against beta-lactam agents (22, 95). Although the clinical significance of the inoculum effect is unclear, the inoculum size has been recognized as the single most important variable in susceptibility testing. The inoculum size should be thought of as both the concentration of microorganisms per milliliter and the absolute number of microorganisms tested. The concentration per se appears to be more important in terms of the effect of inactivating enzymes such as beta-lactamase. On the other hand, the absolute number of organisms appears to be more important in terms of the selection of resistant mutants.

Insufficient contact between organism and drug. Adherence of viable microorganisms to the surface of the container above the meniscus can result in insufficient contact between test microorganisms and the antimicrobial agent (31, 46, 95). Such adherence is more likely with plastic than with acid-treated borosilicate glassware (31, 95). Mixing at 20 h of incubation for tests done in test tubes or continuous shaking for tests done in flasks allows better contact between all cells and the antimicrobial agent.

Volume transferred. The final inoculum and the transfer volume for bactericidal testing should be such that after the defined percentage of killing (usually 99.9%) at least 10 colonies are present. For a 99.9% killing of a final inoculum of 5×10^5 colony-forming units/ml, approximately 100 colonies will be growing on subculture if 100 μl is trans-

ferred. Transferring more than 100 μl is not recommended because of drug carryover. This problem occurs at higher concentrations and can be controlled by spreading the subculture onto agar plates or by adding an inactivating agent (e.g., beta-lactamase) to the agar. Smaller transfer volumes (<10 μl) can result in too few colonies because of pipetting error and intrinsic sampling error due to the Poisson distribution of sample response (not all microorganisms can be assumed to be equally distributed in a broth prior to sampling) (63).

Choice of media. There is no question that the media used in susceptibility testing may have a profound effect on the result (40, 65, 89). By definition, the SBT has some human serum in it. The amount of human serum depends upon whether human serum or broth medium is used as the diluent. Among the variables in either human serum or broth known to affect the bactericidal activity of certain antimicrobial agents and microorganisms are proteins (4, 24, 52, 78, 79), pH (109), phosphates (51), osmolality and salt concentrations (51), and divalent cations (23). The use of human serum as the diluent in the SBT has been shown to be important for certain antimicrobial agents and microorganisms (10, 48, 67, 91). Human serum, however, also has disadvantages including instability of pH (9), risk of transmission of hepatitis B virus or human immunodeficiency virus, inherent antibacterial activity (16, 32), cost and lack of availability, poor support of growth in comparison with broth media (88, 91), and irreversible binding or increased degradation for some antimicrobial agents (61). Nevertheless, when the bactericidal activity of an antibiotic in human serum is being tested, a reasonable and logical diluent to use is human serum. Mueller-Hinton broth (with supplementation if needed) is combined with human serum to achieve a final testing ratio of 1:1. The performance and chemical characteristics of both Mueller-Hinton broth and human serum must be routinely monitored. Pooled human serum can be obtained from commercial sources or from volunteers. It must be quality controlled as rigorously as any other medium used in a clinical microbiology laboratory (55). For the safety of laboratory personnel, it should be screened for hepatitis B virus antigen and for antibodies to human immunodeficiency virus. Human serum may be heated to 56°C for 1 h to inactivate any human immunodeficiency virus.

METHODOLOGY FOR THE SBT

It is not the purpose of this review to discuss in detail the methodology of the SBT. The methodology of the SBT is fully described in the recent National Committee for Clinical Laboratory Standards document (55). There are, however, certain aspects of the methodology that are worth mentioning. Attention to these particular details will enhance the clinical usefulness of the SBT.

Collection of Patient's Serum

The timing of the collection of peak and trough specimens attempts to ensure that the peak level is obtained approximately 30 to 60 min after the drug is absorbed and distributed and the trough level is obtained within 30 min or less of the next dose. The exact timing will depend on the pharmacokinetic properties of the antimicrobial agent(s) used. The most critical aspect of collection is to coordinate the time of collection of the serum with the time of administration of the antibiotic as these agents may not be given at the exact time and rate ordered. Much of the confusion with peak and trough SBTs or antimicrobial assays is related to inaccurate collection.

Broth Medium and Diluent

There is no question that the use of human serum as the diluent will present problems to the laboratory in regards to obtaining the serum and appropriate quality control for the serum. Unfortunately, there seems to be no substitute which has the same performance characteristics (92). Consequently, human serum combined with Mueller-Hinton broth (with supplementation if needed) to achieve a final testing ratio of 1:1 is preferred (55). The amount of human serum needed is small if the microdilution method is used. Serum can be obtained most easily from appropriate volunteers. It is convenient to make the initial dilutions with pooled human serum and then to add the inoculum in an equal volume of Mueller-Hinton broth.

Dilution Methods

Although it is theoretically difficult to assess a bactericidal endpoint of 99.9% killing of the final inoculum with the microdilution method (1, 7, 84), the microdilution method appears to be least influenced by technical variations (64, 98, 114). More importantly, clinical studies suggest that the results obtained are useful (112, 112a). The microdilution method is also better suited for research purposes when testing of serum from a volunteer receiving an antibiotic is done against a large number of different pathogens (74).

Inoculum

The patient's isolate must be saved and used to prepare the inoculum. This inoculum must be standardized and be from an actively growing broth culture (logarithmic phase). The final inoculum size should be approximately 5×10^5 colony-forming units/ml and confirmed by a surface colony count method. The inoculum is added to the wells in the microdilution method in a 0.05-ml sample size, resulting in a 1:2 dilution of the serial dilutions of the specimen.

Incubation

Microdilution trays should be sealed with a plastic cover to prevent evaporation. To maintain the same incubation temperature for all cultures, do not stack microdilution trays more than four high.

Determining Endpoints

The most efficient way to determine the microdilution endpoint is to use a multipoint inoculator or a calibrated pipette to remove 10 μl from each well. This sample is spotted on an agar plate, with the first "no growth" being considered the lethal endpoint. This should be done in duplicate.

Quality Control

The use of the microdilution method decreases the cost of quality control. MIC-MBC performance characteristics can be determined for each lot of pooled human serum by using reference strains with known characteristics (72). For each clinical test, a positive growth control should be done. This can be used to assess purity as well.

CLINICAL RELEVANCE OF THE SBT

Despite the widespread use of SBT titers for a variety of serious infections, the clinical data base for interpretation of these titers has been sparse and the use of the SBT remains controversial. The clinical relevance of the SBT has been suggested in several experimental in vivo studies (2, 83, 97) and disputed in another (19). It must be remembered that the SBT combines in theory only the results of determinations of the antimicrobial agent level in serum and of the MBC. Serum bactericidal activity has been shown to correlate with antimicrobial agent levels and MBCs (48, 91). In addition, the killing rate of serum has been compared with the serum bactericidal activity in several studies (105–107). In these studies, the rank correlations between actual serum bactericidal activity and calculated serum bactericidal activity were excellent and were confirmed by the study of the killing rate in serum. Thus, one cannot embrace MIC-MBC results as being clinically relevant, yet reject the results of the SBT.

In fact, there are few studies that have critically evaluated the outcome of clinical therapy with results obtained by any standard antimicrobial susceptibility testing methods (18, 27, 50). There is much evidence to the contrary; the correlation between any susceptibility test result and clinical outcome is so poor that it has been suggested that such tests actually have little clinical relevance (27, 81, 110). One must remember, however, that antimicrobial agents act in a complex in vivo environment that bears little resemblance to the conditions under which susceptibility tests are performed in the laboratory. Factors that susceptibility tests cannot measure include (i) the cellular and humoral defenses of the host, (ii) the site and severity of the infection, (iii) the virulence and quantity of bacteria present, and (iv) the changing concentrations of the antimicrobial agent(s).

Treatment with an appropriate antimicrobial agent as determined by a susceptibility test method does not ensure a good clinical outcome as other factors may negate this therapy. Nevertheless, the in vitro demonstration of resistance has generally been a good predictor of unsatisfactory clinical response (50). There is increasing evidence that the more precise quantitation of bactericidal activity offered by the SBT is important (74), although the actual titers of activity correlating with good outcome are still not well defined in the literature. Unfortunately, data supporting the clinical utility of the SBT remain sparse. Even in those studies which have been done, the relative number of patients who were successfully treated versus those that had treatment failures is small (112, 112a). Other studies (42, 85) are somewhat difficult to evaluate because the methodology used in different studies by these investigators has varied. Finally, although it is attractive to think in terms of bactericidal levels which are not effective, there have been cures of endocarditis with titers measured at 1:2 and 1:4 (12, 112). Clearly, the serum bactericidal titer cannot be used to define what levels are not effective.

CURRENT ROLE OF THE SBT

Infective Endocarditis

The SBT is most often used to guide antimicrobial therapy in infective endocarditis. There is agreement that antimicrobial therapy for bacterial endocarditis must be bactericidal, achieve adequate levels in serum, and be prolonged to be successful. Serum bactericidal titers of 1:8 or greater are often recommended for optimal treatment of infective endocarditis (113). Nonetheless, there have been cures of endocarditis with titers measured at 1:2 and 1:4 (12). A multicenter study of infective endocarditis (112) found that peak serum bactericidal titers of 1:64 or greater and trough titers of 1:32 or greater by the microdilution method were associated with 100% bacteriologic cure. The often-recommended peak bactericidal titer of 1:8 was associated with less predictive accuracy and had no statistically significant association with outcome. An arbitrary peak or trough titer should not be sought at the cost of unwarranted toxicity. Also, surgical intervention may be needed for the complications of infective endocarditis despite high serum bactericidal titers. Finally, the SBT cannot accurately predict either bacteriologic failure or clinical outcome (112).

In summary, the use of the SBT alone in infective endocarditis will not accurately predict therapeutic success or failure. Serum bactericidal titers cannot be expected to predict anything that serum antibiotic levels or MBCs cannot predict. However, the SBT is clinically useful in infective endocarditis to document therapeutic concentrations of drugs in serum and to demonstrate that the regimen is bactericidal. Although the available data do not fully support the need to attain a specific titer with the SBT, there are many case studies of patients with difficult-to-treat microorganisms that provide useful data to support the value of achieving and monitoring bactericidal activity in serum at a titer of 1:8 or greater (34, 68, 73, 75).

Osteomyelitis and Suppurative Arthritis

Skeletal infections remain difficult infections to treat successfully despite the availability of effective antimicrobial agents; inadequate therapy carries a great risk for chronic infection. Successful therapy requires prolonged use of antibiotics and, in addition, often involves drainage of pus, debridement of necrotic tissue, and removal of foreign bodies. The SBT has been recommended as a tool to assess and monitor the adequacy of antimicrobial therapy of osteomyelitis and suppurative arthritis (44, 60, 68, 88). Tetzlaff and McCracken (96) treated successfully 29 of 30 children with acute hematogenous skeletal infections (19 osteomyelitis, 3 osteoarthritis, and 8 suppurative arthritis) with a brief (1- to 13-day) period of parenteral antibiotic therapy followed by oral antibiotics to complete a usual 4-week course. Adjustments in dosage were made when necessary to ensure a peak serum bactericidal titer of at least 1:8. Weinstein et al. (112a) have recently completed an analysis of patients (mostly adults) with osteomyelitis (acute and chronic) who were monitored at multiple medical centers with the same SBT methodology (76) used in their earlier study of infective endocarditis (112). In this series, trough serum bactericidal titers of 1:2 or greater predicted medical cure in patients successfully treated for acute osteomyelitis. In patients with chronic osteomyelitis, peak serum bactericidal titers of 1:16 and trough titers of $<1:2$ in patients in whom therapy failed accurately predicted this failure. The relative importance of the trough serum bactericidal activity in acute osteomyelitis reflects the delay in achieving effective drug concentrations in bone, the lower concentrations achievable in relation to the serum levels, and the greater need for prolonged therapy. The importance of both peak and trough serum bactericidal activities in chronic osteomyelitis may be related to the need for higher concentrations to penetrate the glycocalyx (29). The relationship between the glycocalyx and foreign bodies such as prosthetic devices is less well studied, and current thought

(14) suggests that removal of the device is needed regardless of what concentration of antimicrobial agent is achieved.

Further work with specific serum bactericidal activity is needed to evaluate the efficacy and economy of initial parenteral therapy for specific microorganisms and sites of bone infection. Delineation of which adult patients could safely be treated by such a regimen (which is widely used at present by pediatricians) (70, 96) is needed.

Combination Therapy in Cancer Patients

Selection of a bactericidal regimen becomes more difficult when several antimicrobial agents are combined. The usual methods for demonstrating synergy between agents are the checkerboard titration method and the quantitative time-kill curve (41, 45, 71). The checkerboard method and the time-kill curve technique measure different phenomena and show a poor correlation in terms of the frequency of bacterial strains showing synergy (60). The time-kill curve method appears to correlate best in animal models with cure (15). Unfortunately, the time-kill curve method is not widely available, nor is it practical for monitoring therapy with combinations of antimicrobial agents.

The assessment of combinations of antimicrobial agents is important for immunocompromised patients with bacterial infections (71). In these patients, high serum bactericidal titers are associated with effectiveness of the combination of agents against the patient's pathogen, and such effectiveness seems to correlate with a favorable clinical outcome (43). The SBT is a convenient method for evaluating the efficacy of combination antibiotic therapy (74). The SBT has been used clinically to assess therapy with combinations of antimicrobial agents in bacteremic patients with cancer. Klastersky and co-workers have shown a correlation of 1:8 or greater peak serum bactericidal activity with successful clinical outcome (79 versus 47%) in nongranulocytopenic cancer patients with gram-negative bacillary septicemia (42). In addition, Sculier and Klastersky have shown that bacteremic patients with granulocytopenia have a more favorable clinical outcome if peak serum bactericidal titers of 1:16 or greater are achieved (85). Additional experience with the SBT in immunocompromised patients is needed to better delineate the target guidelines for successful combination antimicrobial therapy.

FUTURE USES FOR THE SBT

The SBT occasionally has been used to detect antimicrobial activity in infected body fluids other than blood. Activity in cerebrospinal fluid (35, 104) and joint fluid (62) has been assessed, although such data are limited. Another future application of the SBT is to test the bactericidal activity of a patient's serum after the drug doses have been changed from parenteral to oral administration. This use of the test will probably be more commonly encountered with increased efforts to shorten hospital stay by utilizing home therapy. As an experimental tool, the SBT has been used for evaluating new drugs and drug combinations (6, 77, 87, 108). When the SBT is used for such evaluations, the antimicrobial agent is administered to a healthy person, serum is collected, and serum bactericidal titers are determined for the pathogen under study. Analysis of the results can be done by using the titer to directly measure the effectiveness of the agent(s) tested or by a subtraction method for antimicrobial combinations (99). Additional information may be obtained by measuring the area under the curve (5).

Finally, a number of investigators have been evaluating rapid methods for performing the SBT. Both radiometric methods (8, 13) and the Autobac system (78) (General Diagnostics, Raleigh, N.C.) have been shown to correlate with dilution methods. The use of such rapid methods may be useful in permitting earlier adjustment of antibiotic therapy in serious infections.

SUMMARY

The assessment of bactericidal activity of an antimicrobial agent is not easily accomplished because of a number of biological and technical factors (56). An appreciation of these factors is needed to evaluate the results of such testing. To minimize the influence of these factors, the methodologic aspects of the SBT have been addressed (55) and should place this test on firmer ground. New information on the clinical utility of this test is becoming available; additional data are needed to establish more clearly the usefulness of the SBT in specific infections. Such clinical trials from multiple centers will enable firmer recommendations for future use of the SBT.

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