Development of a Five-Hour Radiometric Serum Antibacterial Assay for Gram-Positive Cocci

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A preliminary report on a 5-hr radiometric serum antibacterial assay (ABA) for Gram-positive cocci is presented. The method agreed within ± one twofold dilution with static ABA endpoints in 24/26 (92%) of the assays and with cidal ABA endpoints in 23/26 (88%) of the assays performed.

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The serum antibacterial activity test (ABA) as first described by Schlichter and MacLean (*1*) was designed to determine the adequacy of penicillin therapy in subacute bacterial endocarditis. Recently an antibacterial activity test, expanded to include body fluids such as urine or synovial fluid, has been suggested as a guide for therapy of bacterial infections (*2*).

A problem in the therapeutic utilization of the ABA test is the time lapse of 24 to 48 hr between the receipt of the specimen by the laboratory and the receipt of the results by the clinician. To be of greatest use to the clinician, the assay should permit changes in dose or administration of antibiotics in an expedient, clinically meaningful time frame. We report a preliminary study using Gram-positive cocci that assesses antibacterial activity by employing radiometric detection of C-14-labeled CO_2 from the metabolism of labeled substrates by these bacteria.

MATERIALS AND METHODS

Bacteria. Stock cultures of *Staphylococcus aureus* ATCC 25923, *Streptococcus bovis* CDC 1877-76, and patient isolates of *S. faecalis, S. mitis, S. salivarius, S. Mg-intermedius, S. avium, S. morbillorium, S. sanguis II,* and *S. pyogenes* were used. The viridans streptococci were identified using the physiological tests described by Facklam (*3*). The identification of *S. aureus* was based on the coagulase test. Nonviridans streptococci were identified by standard methods (*4*).

Antibiotics. These were obtained from the United States Pharmacopeia as base powders of known biological activity. In order to simulate serum specimens with antibiotic concentrations that would provide antibacterial end points, the antibiotics used in Phase 1 were selected by determining the minimal inhibitory concentration (MIC) of a panel of antibiotics for each organism using a commercial microdilution MIC panel† according to the manufacturer's instructions. The intent of the experimental design was to use concentrations of antibiotics that would give ABA titers of 1:4 or 1:8. An antibiotic with an MIC value between 0.5 and 8.0 μg/ml against the study organism was selected. The organism antibiotic combinations chosen were *S. aureus:* methicillin; *S. bovis:* penicillin G; *S. faecalis:* ampicillin; *S. mitis:* chloramphenicol; and *S. pyogenes:* gentamicin.

In Phase 2 of the experiment, *S. faecalis* and six species of viridans streptococci were tested against penicillin G. The commercial microdilution panel did not support the growth of all of these isolates and, when the microdilution panels were not workable, minimal inhibitory concentrations of penicillin G were determined by macrodilution methods (*5*).

Diluent. In a prestudy evaluation, the initial diluent used for the antibiotics was tryptic soy broth (TSB). Subsequently, however, in the assays reported in Phase 1 and Phase 2 of our investigation, a diluent that closely

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resembles human serum was used in order to simulate the physiologic characteristics of serum. This diluent (MHB-S/HS) was prepared according to Stratton and Reller (*6*), using normal human serum[‡] that was heat-inactivated as suggested by the authors.

Assay. A stock concentration of the antibiotic was diluted in serial twofold dilutions to two to four dilutions more than the MIC of the antibiotic in a total volume of 1 ml. In the radiometric assay, the bacterial inoculum added to each dilution consisted of 50 μl of a suspension containing approximately 10^8 organisms per milliliter. In Phase 1 this inoculum density was estimated by comparing the bacterial suspension with a 1.0 McFarland density standard. In Phase 2 the inoculum size was confirmed by pour-plate counts. A 1:1000 dilution of the radiometric inoculum was prepared in TSB and 50 μl were added to the tubes for performance of the standard ABA (*7*) in a final volume of 1 ml.

In Phase 1 the radiometric assay was performed by adding 0.3 ml of each dilution to a sterile 25-ml Bactec vial. To each vial, 0.1 ml C-14-labeled glucose (0.1 μCi) and 0.1 ml of C-14-labeled glycine (0.1 μCi) was added. In addition, a growth-control vial containing the bacterial inoculum and substrate, but no antibiotic, was run as well as a substrate control consisting of MHB-S/HS and the C-14-labeled substrates with no organism added. The vials were mixed on a vortex and incubated at 35°C with agitation for 5 hr, after which a digital growth index was determined on the Bactec 301.[‖] The radiometric end point was considered to be the highest dilution that gave a reading less than 25% of the growth-control reading. In our present evaluation, readings taken once at 5 hr after inoculation gave sufficiently high growth-index readings to permit our arbitrary 25% end point to be used.

Phase 2 assays were identical to Phase 1 with two exceptions. First, the empty 25-ml Bactec vials were flushed with a gas mixture consisting of 5% carbon dioxide in air; second, the inoculum density was verified by plate counts.

The bactericidal end point in the standard ABA was considered to be the dilution at which 99.9% of the inoculum was killed. This was calculated for each isolate in Phase 2 according to the pour-plate counts.

Patient specimen. A specimen received for a serum antibacterial activity test was assayed in parallel with standard techniques to preview the possible application of the method with clinical specimens.

Case. A serum specimen was received with a request for a serum bactericidal assay from a 33-year-old woman diagnosed as having subacute bacterial endocarditis. The patient had been admitted with chief complaints of headache and intermittent fever of 1 mo duration following routine dental prophylaxis. On examination a heart murmur was heard and four blood cultures collected at hourly intervals grew *S. sanguis II*. The patient was treated with penicillin G, and the specimen received was collected before one of the doses.

RESULTS

Using TSB as the antibiotic diluent, nine of ten radiometric trials correlated with the ABA bactericidal end point within ± one $\log_2$ dilution. In Phase 1, using MHB-S/HS as the diluent, 13 out of 14 assays gave a bactericidal end point that was ± one $\log_2$ dilution from

TABLE 1. RESULTS OF A 5-HR RADIOMETRIC ANTIBACTERIAL ACTIVITY ASSAY AND STANDARD ABA STATIC AND CIDAL END POINTS (PHASE 1)

Organism	Antibiotic	Radiometric end point	Bacteriostatic end point	Error ($\Delta 1/\log_2$)	Bactericidal end point	Error ($\Delta 1/\log_2$)
S. aureus	methicillin	1:4	1:8	1	1:4	0
		1:8	1:8	0	1:4	1
		1:8	1:8	0	1:4	1
		1:8	1:8	0	1:8	0
S. bovis	penicillin G	1:8	1:16	1	1:8	0
		1:32	1:64	1	1:64	1
		1:16	1:16	0	1:4	2
S. pyogenes	gentamicin	1:4	1:8	1	1:4	0
		1:2	1:8	2	1:4	1
		1:4	1:8	1	1:2	1
S. faecalis	ampicillin	1:4	1:8	1	1:8	1
		1:4	1:8	1	1:4	0
S. mitis	chloramphenicol	1:16	1:16	0	1:16	0
		1:64	1:64	0	1:32	1
				avg. error ± 0.64		avg. error ± 0.64

the cidal end point as determined by plating the clear tubes to blood agar (*7*). In six instances, the radiometric end point was equal to the ABA cidal end point. In five trials the radiometric end point was one dilution more than the ABA cidal end point, and in two instances was one dilution less (Table 1). Likewise, 13 of 14 assays agreed within one tube dilution of the standard bacteriostatic end point, and no trend toward higher or lower end points was noted. The ABA end points higher than anticipated by experimental design with *S. mitis* and *S. bovis* (Table 1) reflect the difficulty experienced in reading the MIC end point in the unsupplemented MMS plate used for selection of antibiotic concentrations in Phase 1. The problem of adding more antibiotic than four times the MIC value, because of indistinct end points, was eliminated in Phase 2 by use of macrodilution MIC techniques in cases where end points were not clear-cut in the microdilution system.

In Phase 2 the streptococci tested against penicillin agreed in ten out of 11 assays within ± one twofold dilution of the cidal end point. In one instance a *S. morbillorium* demonstrated growth on subculture from the undiluted tube but radiometrically had an end point of 1:4 and a bacteriostatic end point of 1:2 (Table 2).

The patient specimen agreed with the standard ABA within ± one twofold dilution. The radiometric end point was 1:4, with a standard static end point of 1:2 or 1:4 (difficult to assess) and a cidal end point of 1:2.

DISCUSSION

An ABA test is the only in vitro test of the effectiveness of antibiotic therapy. It uses the specific organism isolated and accounts for patient-specific factors, such as concurrent medications or body-fluid variables that might affect antibiotic activity. The test has been underutilized because of lack of standardization and the 24–48 hour turnaround time for completion (*8,9*). In order to best predict in vivo action, the diluent should physiologically resemble the body fluid infected (*10*). Stratton and Reller (*6*) proposed the use of a cation-supplemented Mueller-Hinton broth diluted 1:1 with pooled normal human serum as a standard physiologic diluent for serum antibiotic testing. In the standard ABA, the static end point is based on visual determination of turbidity, which may be difficult to assess in a medium containing serum because of inherent cloudiness. This is a particular problem during testing of slow-growing gram-positive cocci. Therefore, a method that uses a standard physiologic diluent but circumvents the need to assess turbidity is desirable.

Since Schlichter's original proposal to use the ABA as a therapeutic guide in the penicillin treatment of subacute bacterial endocarditis, expanded use of serum or body-fluid bactericidal titers has been reported for urinary tract infections (*11*), septic arthritis (*12*), and hematogenous osteomyelitis (*13*). Correlation of bacteriostatic levels and clinical cure have also been reported for serum and urine (*2*). When combination therapy is used, an ABA may be of particular value in determining the synergistic efficacy of the combination chosen. Heineman and Lofton (*14*) and Kunst and Mattie (*15*) have underscored the *a priori* unpredictability of the synergistic action of antibiotics. Optimal use of an ABA would occur if inadequate therapy could be determined before the next scheduled dose of antimicrobic, so that the dose could be adjusted or the therapy altered.

Development of modified serum bactericidal tests (SBT) have been reported. Prober et al. (*16*) developed

TABLE 2. RESULTS OF A 5-HR RADIOMETRIC ABA AND STANDARD ABA STATIC AND CIDAL LEVELS FOR VIRIDANS STREPTOCOCCI AND PENICILLIN G (PHASE 2)

Organism	Radiometric end point	Bacteriostatic end point	Error ($\Delta 1/\log_2$)	Bactericidal end point	Error ($\Delta 1/\log_2$)
S. salivarius	1:4	1:2	1	1:2	1
S. faecalis	1:4	1:8	1	1:4	0
S. faecalis	1:4	1:8	1	1:8	1
S. Mg-intermedius	1:4	1:4	0	1:4	0
S. avium	1:4	1:4	0	1:2	1
S. mitis	1:8	1:2	2	1:1	3
S. mitis	1:2	1:2	0	1:1	1
S. mitis	1:2	1:4	1	1:2	0
S. moribillorium	1:4	1:2	1	N.E.*	N.E.*
S. sanguis II	1:4	1:2	1	1:2	1
S. sanguis II	1:8	1:8	0	1:16	1
S. sanguis II	1:2	1:2	0	1:1	1
			avg. error = ± 0.67 (static)		avg. error = ± 0.91 (cidal)

* N.E. = no end point, i.e., growth on subculture from undiluted specimen.

a microdilution method for performing the SBT that uses less serum and provides cidal end points in 48 hr. Provonchee and Zinner (*17*) suggested a replicator method where the organisms were exposed to serum diluted in brain-heart infusion broth, sampled hourly for 3 hr to agar plates, and read for cidal activity after 18 hr.

In a recent abstract, D'Antonio et al. (*18*) report a method similar to ours. In their study they used a triple substrate of arginine, glucose, and glycine and an unsupplemented Mueller-Hinton broth. They tested *S. aureus, E. coli,* and *Pseudomonas aeruginosa* and found reproducibility and correlation between the SBT and their radiometric procedure.

The assay described herein was developed using nine species of gram-positive cocci that are associated with endocarditis or other serious infections. Alpha streptococci can be problematic to test using assays for antibacterial activity because their slow growth makes a turbidometric determination difficult and an additional 18-24 hr are required to find the cidal end point after plating to solid media.

Our radiometric procedure gives 5-hr results that are not dependent on assessment of turbidity, and correlate within ± one $\log_2$ dilution of standard antibacterial levels. This assay measures inhibition of bacterial respiration as evidenced by the decrease in CO_2 evolution. How this correlates with the irreversible loss of the ability to replicate—i.e., form colonies—is as yet ill-defined. The organisms tested had static and cidal end points that were within a dilution or two of each other. We next intend to test "tolerant" gram-positive organisms (*19*) to determine whether the antibacterial activity detected radiometrically correlates better with static or cidal end points. A limitation of the method is that a growth-control index exceeding 100 in 5 hr is preferable in applying the 25% end point criterion. Insufficient evolution of CO_2, with consequent low readings, would invalidate the procedure. Providing an adequate inoculum size was verified by pour plate, and the bottle contained an atmosphere of 5% CO_2 in air, we encountered only a single isolate of *S. sanguis I* that would not give an adequate growth-index reading. Addition of a third substrate, e.g., arginine, might help to alleviate this problem.

The rapidity of the radiometric system in providing results in 5 hr makes an assay of this type a potentially useful adjunct in the treatment of bacterial infections. Further expansion of the protocol to include gram-negative rods, as suggested by D'Antonio et al. (*15*), and fluids other than serum, is warranted. The utility of the assay must then be determined in clinical trials.

FOOTNOTES

† Micro-Media Systems, Inc., San Jose, CA.
‡ Gibco, Grand Island, NY.
‖ Johnston Laboratories, Cockeysville, MD.

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REFERENCES

1. SCHLICHTER JG, MACLEAN H: A method of determining the effective therapeutic level in the treatment of subacute bacterial endocarditis with penicillin. A preliminary report. *Am Heart J* 34: 209-211, 1947
2. KLASTERSKY J, DANEAU D, SWINGS G, et al: Antibacterial activity in serum and urine as a therapeutic guide in bacterial infections. *J Infect Dis* 129: 187-193, 1974
3. FACKLAM RR: Physiological differentiation of viridans streptococci. *J Clin Microbiol* 5: 184-201, 1977
4. FACKLAM RR: Streptococci and Aerococci. In *Manual of Clinical Microbiology,* EH Lennette, A. Balows, WJ Hausler, Jr., JP Truant, Eds. 3rd ed, Washington, DC, American Society for Microbiology, 1980, pp 88-110
5. WASHINGTON JA, BARRY AL: Dilution test procedures, In *Manual of Clinical Microbiology.* EH Lennette, EH Spaulding, JP Truant, Eds. 2nd ed, Washington, DC, American Society for Microbiology, 1974, pp 410-417
6. STRATTON CW, RELLER LB: Serum dilution test for bactericidal activity. I. Selection of a physiologic diluent. *J Infect Dis* 136: 187-195, 1977
7. JAWETZ E: Assay of antibacterial activity in serum. A useful guide for complex antimicrobial therapy. *Am J Dis Child* 103: 81-84, 1962
8. PIEN FD, VOSTI KL: Variation in performance of the serum bactericidal test. *Antimicro Agents Chemother* 6: 330-333, 1974
9. BRYAN CS, MARNEY SR, ALFORD RH, et al: Gram-negative Bacillary endocarditis. Interpretation of the serum bactericidal test. *Am J Med* 58: 209-215, 1975
10. PIEN FD, WILLIAMS RD, VOSTI KL: Comparison of broth and human serum as the diluent in the serum bactericidal test. *Antimicrob Agents Chemother* 7: 113-114, 1975
11. KLASTERSKY J, HENSGENS C, GERALD M, et al: Comparison of sisomicin and gentamicin in bacteriuric patients with underlying diseases of the urinary tract. *Antimicrob Agents Chemother* 7: 742-747, 1975
12. PARKER RH, SCHMID FR: Antibacterial activity of synovial fluid during therapy of septic arthritis. *Arth Rheum* 14: 96-104, 1971
13. PROBER CG, YEAGER AS: Use of the serum bactericidal titer to assess the adequacy of oral antibiotic therapy in the treatment of acute hematogenous osteomyelitis. *J Pediatr* 95: 131-135, 1979
14. HEINEMAN HS, LOFTON WM: Unpredictable response of *Pseudomonas aeruginosa* to synergistic antibiotic combinations in vitro. *Antimicrob Agents Chemother* 13: 827-831, 1978
15. KUNST MW, MATTIE H: Cefazolin and cephradine: Relationship between antibacterial activity in vitro and in mice experimentally infected with *Escherichia coli. J Infect Dis* 137: 391-402, 1978
16. PROBER CG, DOUGHERTY SS, VOSTI KL, et al: Comparison of a micromethod for performance of the serum bactericidal test with the standard tube dilution method. *Antimicrob Agents Chemother* 16: 46-48, 1979
17. PROVONCHEE RB, ZINNER, SH: Rapid method for determining serum bactericidal activity. *Appl Microbiol* 27: 185-186, 1974

18. D'ANTONIO R, CHARACHE P, WAGNER HN, et al: Rapid radiometric serum test for antibiotic activity. *J Nucl Med* 20: 616, 1979 (abst)

19. SABATH LD, LAVERDIERE M, WILKINSON BJ, et al: A new type of penicillin resistance of *Staphylococcus aureus*. *Lancet* I: 443-447, 1977

ANNUAL SPRING MEETING
Pacific Northwest Chapter
Society of Nuclear Medicine

March 27-29, 1981 **Alderbrook Resort** **Union, Washington**

ANNOUNCEMENT

Drs. Raymond Marty, Program Chairman and Michael Graham, Program Co-Chairman announce the following plans for the Pacific Northwest Chapter Spring Meeting.

Clinical Aspects of single photon emission tomography.	John Keyes, M.D.
Practical aspects and applications of the 400T system.	Dave Williams, M.D., James Ritchie, M.D., James Caldwell, M.D., and Glen Hamilton, M.D.
Combined Imaging Modalities in the evaluation of the Abdomen.	Thomas Davis, M.D.
Nuclear Medicine, Ultrasound, CAT scans and conventional radiography in the evaluation of renal function.	Tom Rudd, M.D.
Evaluation of the gallbladder and biliary tree by various imaging modalities.	John Denney, M.D.
General overview of the various imaging modalities and their appropriateness and cost effectiveness.	Wil Nelp, M.D.

There will also be a Technologist sponsored program.
Application for AMA category I credit for physicians will be on file.
There will be a Chapter General Business Meeting on Saturday, March 28, 1981 at the scheduled lunch.
For further information and hotel and registration cards, please contact: Jean Parker, Administrator, Pacific Northwest Chapter, SNM, P.O. Box 40279, San Francisco, CA 94140.

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May 23-24, 1981 **Hyatt Kuilima Resort Hotel** **Oahu, Hawaii**

The Hawaii Chapter of the Society of Nuclear Medicine will hold its Fourth Annual Memorial Day Weekend Meeting Saturday, May 23 and Sunday, May 24, 1981. The location for the meeting will be the Hyatt Kuilima Resort Hotel on Oahu's beautiful north shore. The program will be divided into two segments with noted speakers from the Mainland presenting talks on Gastrointestinal Imaging and Nuclear Cardiology.

Due to the success of its previous annual meetings the Hawaii Chapter SNM is extending an invitation to all other chapters to join in an enjoyable and interesting weekend. For further information and registration please write:

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