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Automation of the *Limulus* Amoebocyte Lysate Test by Using the Abbott MS-2 Microbiology System

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A rapid, automated method for the performance of the *Limulus* amoebocyte lysate endotoxin assay has been developed by using the Abbott MS-2 Microbiology System. This instrument automatically determines sequential changes in the optical density of up to 176 samples at 1- or 5-min increments during a 1-h assay period. Graphic representation of optical density changes can be viewed on a cathode-ray tube or reproduced by using a hard-copy printer. *Limulus* amoebocyte lysate preparations that were obtained from different commercial producers and that had similar endotoxin sensitivities by the conventional gelation method varied somewhat in reactivity when determinations were based upon rate changes in optical density. Lysates from Associates of Cape Cod, Difco Laboratories, and M. A. Bioproducts were the most readily adaptable to the MS-2 System. Use of the MS-2 system increased the sensitivity of these preparations from 60- to 250-fold, and as little as 1 pg/ml was detected. Adaptation of the MS-2 instrument for this purpose provides an objective, reproducible, automated method for the performance of *Limulus* amoebocyte lysate tests on a variety of fluids.

Since the introduction of the *Limulus* amoebocyte lysate (LAL) endotoxin assay by Levin and Bang (11), the test has realized numerous and varied applications. It continues to be the most sensitive technique available for the detection of bacterial endotoxins. The LAL test has shown great utility during its use in the pharmaceutical industry for both in-process and final pyrogen testing of injectable pharmaceutical and biological products (4, 23) and for similar testing of medical devices before release (5). The test has also been shown to be useful in the detection of gram-negative bacteria, particularly coliforms, in water and wastewater (2, 9), as well as in the detection of spoilage organisms in certain food products, such as ground beef (6). Numerous studies have demonstrated the usefulness of the LAL test in the diagnosis of gram-negative bacterial infections, including meningitis (8, 16, 18), significant bacteriuria (7), endotoxemia (12), ocular infections (13, 25), and gonorrhea (19, 20). Although the number of uses of the LAL test has increased in the years since its first description, critics of the test have pointed to a lack of standardization of the lysate itself and to the subjective method of manually reading the gelation endpoint (1, 24). The time required to manually perform each determination has also been a limiting factor when large numbers of tests have been performed, such as in the pharmaceutical industry.

Several modifications of the basic LAL test have been made to increase the sensitivity of the test, to reduce the subjectivity of endpoint determinations, or to mechanize the performance of the test. Spectrophotometric methods (11, 21) of determining the endotoxin-LAL reaction seem particularly encouraging due to their sensitivity and potential for automation.

The MS-2 Microbiology System (Abbott Laboratories, Diagnostics Division, Irving, Tex.) was originally developed for rapid automated antibiotic susceptibility testing (22). More recently, this instrument has been applied to the screening of urine samples for the detection of bacteriuria [L. R. McCarthy, C. L. Corlett, and J. A. Robson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, C(H)95, p. 362] and for rapid identification of gram-negative bacilli (14). The instrument offers fully automated, repetitive measurements of changes in the optical density (OD) of up to 176 test fluids simultaneously. This report describes the use of the MS-2 System for automated LAL testing.

MATERIALS AND METHODS

LAL preparations. Amoebocyte lysates were obtained from six different manufacturers to determine their performance characteristics and suitability for use in the MS-2 System. The manufacturers evaluated were Associates of Cape Cod (Cape Cod, Mass.), Difco Laboratories (Detroit, Mich.), Mallinckrodt, Inc., (St. Louis, Mo.), Limulus Laboratories (Horseshoe Beach,

Fla.), M. A. Bioproducts (Walkersville, Md.), and Millipore Corp. (Freehold, N.J.). The storage and reconstitution instructions of each manufacturer were followed explicitly.

Reference endotoxin. So that test results of all LAL preparations could be objectively compared, a reference endotoxin was used for all conventional manual and MS-2 *Limulus* assays. A phenol-extracted *Escherichia coli* O55:B5 preparation (Difco) was used for all experiments.

Performance of conventional LAL tests. Conventional LAL gelation tests were performed by using the procedures established by the manufacturers. Reaction endpoints were graded in accordance with methods described previously (10). The only exception involved the LAL preparation manufactured by Millipore, which is not intended for gelation-type testing. Millipore lysate was therefore evaluated only in the MS-2 system.

Performance of MS-2 *Limulus* assays. The Research System, a specialized version of the MS-2 System, was employed for this study. The Research System includes, in addition to the analysis and control modules, a cathode-ray tube console and hard-copy graphics printer which allows the visualization of plots of OD-time relationships, including plots of the logarithm of changes of OD versus time. There are four different cuvette devices available for use in the MS-2 System. They include a polystyrene research cuvette which contains 11 reaction chambers, a biphasic cuvette used for antibiotic susceptibility testing, a bacterial identification cartridge which is also polystyrene and contains 20 small reaction chambers (this cuvette was developed for identification of *Enterobacteriaceae* as to species), and disposable glass Ampvettes (disposable, single-use containers intended for urine screening in the MS-2 System).

Preliminary experiments demonstrated that the optimum mixture of LAL to total sample volume was a 1:5 ratio. With the research cuvette or Ampvettes, this ratio represented 200 μl of LAL and 800 μl of test sample; 40 μl of LAL and 160 μl of sample were utilized in the smaller bacterial identification cartridge. Later in the course of this study, a modified Ampvette holder was developed by Abbott which allowed the use of one-half the normal volumes of reagents (100 μl of LAL and 400 μl of sample). Test conditions normally included a 60-min incubation in the MS-2 Analysis Module at its normal temperature of 35.5°C. Alternate reaction temperatures from 32 to 39°C were also evaluated for optimal reaction rates.

Since the opaque gel formed when LAL is reacted with bacterial endotoxin is easily disrupted, the shaker device normally employed in the MS-2 Analysis Module was disarmed to provide stationary incubation of all cuvettes. This was accomplished by maintaining one of the cuvette chamber doors of the analysis module in an open position during the period of each experiment, which caused the shaker mechanism to stop. The MS-2 spectrophotometer system examined each sample position at either 1- or 5-min intervals during the test period with light-emitting diodes of 670-nm wavelength. The program allowing readings to be taken every minute was specially developed for LAL testing by Abbott for the purpose of this study. The computerized control module stored information regarding the tests to be performed, controlled the steps required for reading and recording each test, and finally stored the data on magnetic cassette-type tapes. Data could then be retrieved by a hard-copy printout of light transmission values (or changes in transmission values) using the control module printer, or graphically depicted on the cathode-ray tube console and graphics module (hard-copy printer).

RESULTS

Our initial experiments demonstrated that the MS-2 System was very well suited to the spectrophotometric determination of the LAL reaction. Kinetics of OD changes that occurred during the endotoxin-LAL reaction could be visualized with the cathode-ray tube display. Hard copies of results could be readily produced with the graphics module. During the initial experiments, a positive reaction in the MS-2 System was defined as any obvious, continual increase in OD which clearly surpassed any activity observed in a simultaneous negative control (pyrogen-free water plus LAL in an identical reaction vessel).

Kinetic curves of the endotoxin-LAL reaction were quite similar, irrespective of which of the three cuvette devices was employed. The plastic bacterial identification cartridge plastic cuvette had the advantage of requiring smaller sample and lysate volumes and was thus potentially more economical for performing large numbers of tests. However, occasional background endotoxin activity was observed with both of the plastic cuvettes. The glass Ampvettes were most convenient for single-use applications, since one or many could be used at the same time. Moreover, the glass Ampvettes seemed to be completely free of endotoxin contamination due to the high temperatures employed in forming the Ampvettes from glass tubing. Thus, Ampvettes could be used directly for LAL testing without the usual requirement of depyrogenation.

Initial experiments showed that lysate preparations differed markedly in the degree of turbidity formed during reactions with endotoxin. Table 1 lists the results of LAL reactions when standard gelation end points and the MS-2 method were used. LAL preparations of similar potency, as determined by the conventional gelation method, varied somewhat in OD measurements in the MS-2 System. LAL from Associates of Cape Cod, Difco, and M. A. Bioproducts produced the greatest turbidity and thus were most suitable for use in the MS-2 System. Preparations from Mallinckrodt, Inc., and Limulus Laboratories were less suitable for turbidimetric use in the MS-2 System, since their composition favored the formation of a firm gel rather than

TABLE 1. *Comparative sensitivities of LAL preparations by using the MS-2 and manual gelation methodologies*

LAL manufacturer	Sensitivity (ng/ml) by[a]:	
	Gelation method	MS-2 method
Associates of Cape Cod	0.06	0.001
Difco Laboratories	0.25	0.001
Limulus Laboratories	0.125	0.01
M. A. Bioproducts	0.25	0.001
Mallinckrodt, Inc.	0.06	0.01
Millipore Corp. (with 10:1 ratio of LAL to sample)[b] ...		0.1
Millipore Corp. (with 1:5 ratio of LAL to sample)[b] ...		0.005

[a] Based upon Difco *E. coli* O55:B5W endotoxin.
[b] See text for explanation of different ratios.

intense turbidity. The lysate produced by Millipore was the least sensitive of the products tested when the manufacturer's instructions for reconstitution and use of the LAL were followed. Millipore recommends reconstitution of each vial of lysate with 11 ml of diluent and a final test ratio of 10 parts LAL to 1 part sample. The use of these procedures in the MS-2 System resulted in a lysate sensitivity of only 0.1 ng of endotoxin per ml. However, rehydration of the lysate with 1.1 ml per vial and test ratios of 1 part LAL to 4 parts sample provided greatly improved sensitivity (detection of as little as 0.005 ng/ml).

The use of lysates from Associates of Cape Cod, Difco, and M. A. Bioproducts in the MS-2 System provided a 60- to 250-fold amplification of lysate sensitivity as compared with conventional gelation methods (Table 1). Whereas lysates from Difco and M. A. Bioproducts could detect 0.25 ng/ml by gelation, the use of either lysate in the MS-2 System allowed the detection of 0.001 ng/ml. Moreover, concentrations of endotoxin above the minimal detectable amount could be detected very rapidly; for example, 1 ng/ml could be detected by the MS-2 System in approximately 5 to 10 min with LAL from Associates of Cape Cod, Difco, or M. A. Bioproducts.

Recognition of varying quantities of endotoxin could be accomplished in one of two ways. Figure 1 shows the kinetic LAL-endotoxin reaction curves obtained by using the MS-2 graphics program. Both OD versus time and the natural logarithm of OD versus time were determined by the MS-2 System and could be displayed on the cathode-ray tube. The natural logarithm of OD is intended for use in calculating bacterial generation times when the MS-2 System is used for antibiotic susceptibility testing. The rapidity of the OD changes when LAL is reacted with endotoxin in the MS-2 System results in similar-appearing OD and natural logarithm of OD curves. Direct OD-versus-time curves were more convenient for routine, standard dose-response curves for this reason.

Both 1- and 5-min OD determinations provided highly reproducible reaction curves. However, 1-min determinations were slightly more reliable for the precise quantitation of endotoxin. The time required for the initiation of the sharp increases (or a rapid rise) in OD was inversely related to the amount of endotoxin present. A concentration of 1 ng of endotoxin per ml produced an almost immediate increase in OD, with a very steep slope of the reaction curve. Lower concentrations of endotoxin required longer pe-

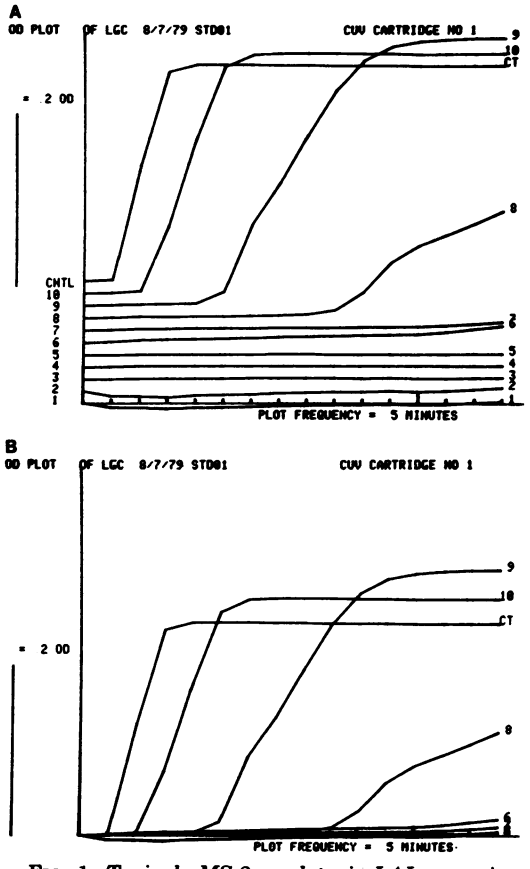

FIG. 1. *Typical MS-2 endotoxin-LAL reaction curves. OD readings are indicated on the ordinate, and time is indicated in 5-min increments on the abscissa. Curves can be staggered (A) for better resolution, or they can be superimposed from the origin (B). Endotoxin concentrations were 1 ng/ml (control [CT]), 0.1 ng/ml (10), 0.01 ng/ml (9), 0.001 ng/ml (8), and negative control (7), and positions 1 to 6 were empty.*

riods of time for the initiation of the reaction curve. The slope of the OD-versus-time curve was similar over a range of 0.1 to 1,000 ng of endotoxin per ml.

A more precise method of endotoxin quantitation was achieved with the 1-min reading program and manual plotting of the time required for the first major change in transmission values (change ≧10 transmission units) versus endotoxin concentration. Transmission values were directly available as a printout on the MS-2 Control Module. Concentration was scaled on the logarithmic axis of semilog paper, and the time periods required to achieve 10 or more transmission units were indicated on the arithmetic scale (Fig. 2). With this method of quantitation, a relatively linear relationship was achieved over a range of 0.01 to 100 ng of endotoxin per ml (Fig. 2). Although concentrations of endotoxin as low as 1 pg/ml (0.001 ng/ml) could be recognized by using the cathode-ray tube OD plots as described above, the marked and abrupt increase in OD which occurred during the LAL-endotoxin reaction with higher endotoxin concentrations was not seen. Thus, the manual method of plotting the time required for the initiation of the reaction burst was reliable only with endotoxin concentrations of ≧5 to 10 pg/ml. For an assessment of the reliability of this method of endotoxin quantitation, the time required to achieve the reaction burst was determined for 22 replicate samples of two different endotoxin concentrations. This determination was accomplished by placing samples of the same endotoxin standard in all 11 positions of two different cuvettes, followed by a simultaneous assay of all 22 positions. This assay was performed with endotoxin standards of 50 and 0.1 ng/ml. The mean time of detection for the 50-ng/ml samples was 9.04 min (standard deviation = 0.25 min; mode = 9 min), and the mean time of detection for the 0.1-ng/ml samples was 26.8 min (standard deviation = 1.10 min; mode = 27 min).

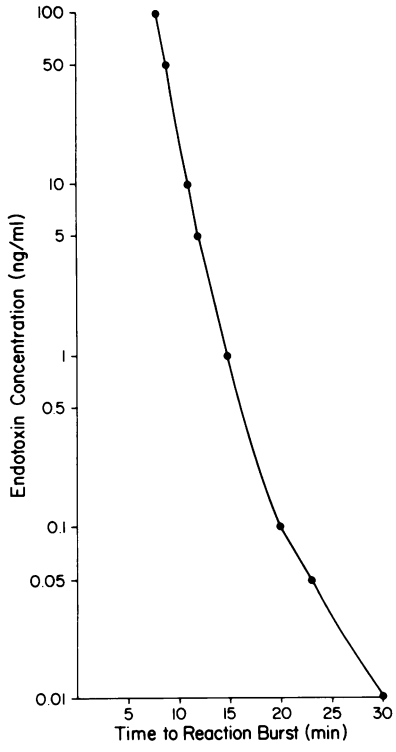

FIG. 2. *Typical endotoxin standard curve plotted off-line by using the time required for the LAL reaction burst to occur versus the concentration of endotoxin in the standard.*

Increasing the incubation temperature in the MS-2 System to as high as 39°C did not provide an increase in the sensitivity of the assay system. However, reaction bursts did occur 1 to 3 min faster at 39 than at 35.5°C. Temperatures below 35.5°C delayed results, with no observable advantage in reproducibility.

A limited number of experiments were conducted to determine the effect of colored or opaque samples on the performance of MS-2 *Limulus* assays. Samples containing red, yellow, green, and purple dyes did not seem to hamper endotoxin determinations. Moreover, initial opacity did not interfere with endotoxin determinations unless initial MS-2 transmission values were below 25% of the available range.

DISCUSSION

This study demonstrated that a currently available automated microbiology instrument, the Abbott MS-2 Research System, can be readily applied to the automation of LAL testing. By using the MS-2 System, up to 176 samples per analysis module can be simultaneously examined for endotoxin content. This creates the feasibility of large-scale pyrogen testing in the pharmaceutical industry and screening large numbers of food or water samples, as well as the possibility of performing one or many simultaneous determinations of patient samples in a clinical laboratory. Moreover, a clinical laboratory using the MS-2 System for antibiotic susceptibility testing, urine screening, or gram-negative bacterial identification could easily perform LAL tests on cerebrospinal or other fluids without any modification of the instrument other than the addition of the research program to the control module. A distinct advantage of the MS-2 System is the flexibility to perform

several different types of clinical microbiology analyses with a single instrument. The use of an automated instrument for interpretating LAL reactions allows a degree of objective standardization which has not existed previously with the LAL test. Furthermore, the use of the MS-2 System provides a 60- to 250-fold amplification of the sensitivity for the LAL test, allowing the detection of levels well below those possible with manual gelation methods.

Previous attempts at instrument-assisted performance of LAL testing have included manual spectrophotometric methods (11, 21) or the measurement of incorporated radioisotopes (15). Other modified procedures for the reading of LAL test endpoints or methods intended to conserve lysate have included the use of microdilution trays (17) or microslides (3). However, the MS-2 System provides the first procedure for automated spectrophotometric performance of the LAL tests. The use of the MS-2 System allows convenient visualization of the kinetics of the reaction between bacterial endotoxin and lysate, a possibility which has not existed previously. A reasonable extension of this study will involve the programming of the MS-2 microprocessor for the interpretation of LAL-endotoxin reaction curves, including automated calculations of endotoxin levels.

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