

Rapid Microbiological Methods in the Pharmaceutical Industry

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11

***Limulus* Endotoxin Test**

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Associates of Cape Cod, Inc.

H. Donald Hochstein

United States Food and Drug Administration (retired)

DISCOVERY

Howell (1885) observed that the blood of *Limulus polyphemus*, the horseshoe crab, formed a solid clot when withdrawn from the crab. Loeb (1902) noted that when the blood cells of *Limulus* were collected and exposed to foreign substances, they coagulated. These were the first papers detailing various aspects of coagulation with reference to amebocytes, which are the only circulating cells found in the blood of *Limulus*.

In 1964 Levin and Bang (1964a, 1964b) reported that *Escherichia coli* endotoxins and a marine *Vibrio* induced the extracellular coagulation of *Limulus* blood. Although endotoxin did not cause coagulation of the cell free serum, clotting activity could be restored by the addition of amebocytes to the serum. The following conclusions were drawn from this study: amebocytes were required for coagulation, disruption of the amebocytes enhanced the reaction, and quantities of endotoxin were possibly inactivated by the coagulation process. Amebocytes coagulating in the presence of bacterial endotoxin is not limited solely to *Limulus* but has been demonstrated in the oyster, crab, and lobster also. In a subsequent study, Levin et al. (1970) developed a sensitive assay for endotoxin using the lysed amebocyte from *Limulus polyphemus*. As little as 0.0005 µg of endotoxin per milliliter could be detected, and the rate of the reaction depended on the concentration of endotoxin. Yin et al. (1972) refined Levin's original endotoxin assay to detect picogram amounts of endotoxin and demonstrated that the lipid A part of the lipopolysaccharide was responsible for lysate gelation.

Comparison of Blood Coagulation in *Limulus* and Mammals

A most exciting aspect of the increasing knowledge of the basis for the reaction between amebocyte lysate and endotoxin has been recognition of the remarkable similarities between the biochemistry of coagulation in *Limulus*, a primitive and ancient invertebrate, and mammalian blood coagulation. Most of the knowledge in reference to the biochemical aspects of coagulation in horseshoe crabs has been provided by a series of outstanding Japanese researchers, who have studied all four of the species of the horseshoe crab (Morita et al. 1984). All of the enzymes that have been identified are serine proteases, and at least three serine proteases are required for the activation of *Limulus* blood coagulation by endotoxin (Nakamura and Levin 1982). It has been reported that the same enzymatic inhibitors block mammalian and horseshoe crab coagulation (Young et al. 1972). In fact, some of the synthetic substances designated for the assay of human coagulation factors also are excellent substrates for the components of the coagulation system in *Limulus* (Torano et al. 1984).

Preparation of *Limulus* Amebocyte Lysate

The horseshoe crabs are either captured by commercial fisherman (in nets) or by persons on the beach or in shallow water during the crab mating season. The crabs are then either placed in a tank of ocean water for a few hours, to release excess dirt and sand, or are scrubbed with a brush in tap water. When the crab has air dried, it is flexed and placed in bleeding racks. The muscular hinge between the cephalothorax and abdominal region is then scrubbed with iodine or 70% alcohol, and a sterile, endotoxin-free 18-gauge needle is inserted into the heart chamber. Hemolymph can then flow freely into an appropriate container (usually a 250 mL glass centrifuge bottle), which is either empty or contains about 70 mL of an anticlotting agent, such as 0.125% N-ethylmaleimide (NEM) in 3% sodium chloride solution (Levin and Bang 1968). The NEM is a sulfhydryl

inhibitor that stabilizes the amebocyte cell membrane sufficiently to permit washing. The hemolymph that is mixed with NEM can be centrifuged when time allows; however, the hemolymph that is not mixed with NEM must be centrifuged within 15 or 30 minutes or the hemolymph will clot in the bottle. The large white cells are gently sedimented in the 250 mL tubes by centrifugation at 300 G for six minutes. After discarding the blue (hemocyanin-containing) supernatant, the amebocytes are washed with warm 3% sodium chloride solution. The cells are then transferred to 50 mL graduated centrifuge tubes and washed with warm sodium chloride solution. The amebocytes are then lysed by adding sterile water for injection, to cells in a 2:1 ratio and vortexing vigorously at least three times daily for one or two days. Finally, the amebocyte lysate is separated from the cellular debris by centrifugation at 1200 G for ten minutes, the supernatant is removed, and the lysate is stored at 4°C. Most lysate preparations remain stable for at least nine months at 4°C. The lysate is then tested for potency by using EC-6 endotoxin and freeze-dried. The expiration date of freeze-dried lysate is about five years.

EARLY REGULATORY INTEREST IN AN ALTERNATIVE TO THE RABBIT PYROGEN TEST

In early 1970 the personnel in the Division of Biologic Standards (DBS), National Institutes of Health (NIH), were made aware that a new test was being developed for detecting endotoxin. This test used the lysed amebocytes from the *Limulus polyphemus* (horseshoe crab) and could detect nanogram quantities of endotoxin (lipopolysaccharide, LPS) located in the cell wall of gram-negative bacteria. Laboratory personnel were performing the rabbit pyrogen test on at least four licensed biological products per day and decided to incorporate the Limulus Amebocyte Lysate (LAL) test when testing certain biological blood products. These products were 5 and 25% Normal Serum Albumin (Human, NSA), Purified Protein Factor (PPF), Immune Serum Globulin (ISG), and Antihemophilic Factor (AHF) (Hochstein et al. 1979).

The first several batches of lysate were prepared at the Johns Hopkins University. After about five months of trial and error, it was determined that the *in vitro* test really was of value in detecting endotoxin. Because the DBS laboratory was about 150 miles from the ocean, horseshoe crabs were difficult to obtain, so it was decided to have a crew spend one week each year, for the next several years, at the NASA base in Chincoteague, Va., bleeding horseshoe crabs. About 800 to 1,000 crabs were bled per week, and the crew would return to the laboratory with several liters of lysate and 200 to 300 live crabs. These crabs were put into three-tier fiberglass lobster tanks (Bergson Products, Warren, R.I.). Each tank was 10 feet long and 20 inches deep. The tanks from bottom to top were 6, 5, and 4 feet wide. This arrangement provided total visibility of the interior of the bottom two tanks and also provided steps for reaching the top tank. Each tank contained 7 inches (depth) of synthetic seawater (Instant Ocean). A pump connected to the bottom tank pumped the salt water to the top tank. From there it ran out the overflow into the middle tank and then back to the bottom tank. Each tank held between 70 and 100 crabs, depending on their size. If the tanks were full and there were extra crabs, they were put into plastic boxes and placed in the refrigerator at 4°C to 8°C. If the crabs were kept moist, they would live in the refrigerator for at least six months without food or water. The crabs did not move in this environment as long as the light was off, but when the light in the refrigerator went on, the crabs responded to this stimulus by moving their tails. The crab tanks were housed in the rabbit holding room. Following a pyrogen test, the rabbits were sacrificed and their livers removed. These livers were rinsed in tap water, sliced, and fed to the crabs. The crabs thrived on this diet.

Each crab in the tanks was numbered, so that a bleeding record could be kept. The crabs were bled semiannually. If they were bled more frequently, the crabs produced hemolymph but very few amebocytes, indicating that it took at least six months for the horseshoe crabs (in captivity) to regenerate their normal amebocyte count.

In 1972 the LAL test began to be widely used in the medical community. At the same time, the U.S. Food and Drug Administration (FDA) received complaints that some people were using lysate to diagnose septicemia, meningitis, and urinary tract infections. The FDA decided that regulations had to be written to control the sale and use of this product. Because LAL was a blood product and the expertise for using it was in the Division of Control Activities, it was decided to assign this product to the newly created Bureau of Biologics (BoB); that replaced the DBS.

FDA's Experience with Bacterial Endotoxin

It soon became apparent that in order to determine the lysate sensitivity, a standard endotoxin was needed. In 1974 the FDA contracted with the University of Montana to prepare 30 g of purified endotoxin from *E. coli* O1 13:H10:K negative (Rudbach et al. 1976). This bulk endotoxin was labeled EC and was stored in a desiccating jar at room temperature.

In 1976 a portion of this bulk EC endotoxin was used to prepare lot EC-1. Before freeze-drying, NSA (human) at 0.1% concentration was added as a stabilizer. This small lot, consisting of several hundred vials, lasted about one year. It was then decided to prepare a large lot of endotoxin in the same way EC-1 was prepared. This lot, EC-2, consisted of 1,500 vials, each containing 1.0 µg of EC endotoxin. After extensive data were collected on this very stable standard preparation, it was decided to assign a unit value to the preparation. Most control authorities agree that it is better to use units of activity rather than a concentration based on dry weight in expressing the strength of standard preparations. On the basis of a collaborative

study involving the FDA and several licensed LAL manufacturers, a value of 5.0 endotoxin units (an endotoxin unit is a measure of potency) per nanogram (EU/ng) was assigned to lot EC-2 (Rastogi et al. 1979). The only criticism of lot EC-2 was that it contained NSA (human) that might bind endotoxin. To eliminate this hypothetical problem, in 1980 lot EC-3 was prepared as a pilot lot with no fillers or stabilizers. After several months of use, this lot appeared satisfactory, and lot EC-4 was prepared in the same way. After EC-4 was freeze-dried (with no filler or stabilizer), not only did the vials appear empty, but it was quite difficult to dissolve all the endotoxin into the water used for reconstitution. This lot was depleted in 1981.

Each new lot of endotoxin created problems in that the in-house standards had to be revalidated to reflect the new endotoxin; therefore, it was decided that to avoid the necessity of recalibration each year or two, a large lot of endotoxin would be prepared that would last 10–15 years.

This large lot of EC-5 endotoxin was prepared from the EC bulk powder. Because lot EC-2 was the best that had been prepared, it was decided to prepare lot EC-5 in the same way. A licensed lysate manufacturer was contracted to freeze-dry 30,000 vials from a single bulk. The protocol used for lot EC-5 was prepared jointly by the U.S. Pharmacopoeia (USP) and the FDA; lactose and polyethylene glycol were specified as stabilizers (Hochstein et al. 1983).

This lot was stored at -20°C at the USP facility in Rockville, Md., and was distributed by the agency as Endotoxin Standard Lot F. Several hundred vials of this material were stored at the Center for Biologics Evaluation and Research (CBER) facility (Kensington, Md.) and distributed upon request to licensed manufacturers as the U.S. Standard Endotoxin lot EC-5. There has been a great interest in endotoxin testing by using both the amebocyte lysate test and the rabbit pyrogen test. An endotoxin rabbit study (Hochstein et al. 1983) indicated that it required about 10 EU/kg of EC-5 to elicit a 1°C rise in a rabbit. A similar study in humans to determine the threshold to pyrogenic stimulation by EC-5 was the next step. This study was done at the Clinical Research Center in New Orleans, La. (Hochstein et al. 1994). Human male volunteers were divided randomly into 5 groups of 12. Each group was given one intravenous injection of lot EC-5 at a level of 0, 2, 4, 8, or 16 EU/kg of body weight. Oral temperatures were taken and recorded every 15 minutes for 8 hours. The pyrogenic variations of the U.S. Standard Endotoxin in humans over the test period were determined. The results indicated that there was a direct correlation between endotoxin units per kilogram administered and temperature rise. The threshold pyrogenic dose (1.0°F rise in 50% of volunteers) in this study was approximately 4.1 EU/kg. This was the first report that describes the human dose response to the intravenous administration of a pyrogen-free water control and four dose levels of U.S. Standard Endotoxin, Lot EC-5. Backed by this information, the FDA guideline on use of the lysate test states that the maximum human dose of endotoxin should be 5 EU/kg. This dose may cause a slight fever in about half of the patients, but it should not cause shock or death. EC-5 was the best endotoxin lot; however, by 1995 it was rapidly being depleted, so a replacement was needed quickly. The USP stated that if CBER furnished the bulk EC powder and some expertise on the preparation of EC-6, the USP would have it prepared at the National Institutes of Biological Standards and Controls (NIBSC). In late 1996 about 60,000 vials, each containing 10,000 EUs, were prepared. This lot was labeled EC-6 (Poole et al. 1997). After an extensive collaborative study involving more than 20 testing facilities worldwide, this lot was accepted as the World Health Organization (WHO) standard endotoxin and is being distributed by the USP as lot G endotoxin. This lot should last until about 2007.

Licensing the LAL Test

The FDA is responsible for licensing all *Limulus* or *Tachypleus* amebocyte lysate manufacturers. To be licensed, a manufacturer must complete the FDA forms for an establishment license for the manufacture of biological products. The licensing procedure comprises the following. After all the forms and samples are received, an ad hoc committee is established to review the submission in detail. While the paperwork is being reviewed, the product samples are distributed to specific laboratories for moisture, sensitivity, and sterility testing. When the paper review and sample testing are complete, an on-site inspection of the manufacturing facility is scheduled at a time when the appropriate personnel will be present for answering questions. One of the inspectors is usually a committee member with expertise in the testing of the product. The inspection involves not only checking the physical plant, but also reviewing daily records, including standard operating procedures for each production step. The validation records of equipment such as freeze-dryers, flow hoods, and autoclaves are also reviewed. If any exceptions to the federal regulations are noted during the inspection, the inspector discusses them with management before leaving the facility. The manufacturer then has the responsibility to reply in writing to each observation. The licensing committee reviews all data and submits a recommendation to the CBER director regarding licensure.

A manufacturer that has been licensed must submit to CBER each lot of lysate prepared for sale. If the lot passes, the manufacturer is notified that the lot is released and may be marketed. The FDA has recently specified that the lot to lot release approval can be waived if some parameters (e.g., track record) are consistent. However, if a later problem develops (e.g., sterility, potency) the lot to lot release approval can be reinstated. In 1976 three lysate manufacturers applied for a license to produce and market LAL. They completed all necessary requests for an establishment license for the manufacture of biological products and were licensed in 1977. Since then twelve additional U.S. companies and one Japanese company have been licensed.

LAL Licensed Manufacturers

Today there are only five licensed amebocyte lysate manufacturers remaining, all in the United States. These are Associates of Cape Cod, Inc., Baxter Healthcare, Charles River Endosafe, Haemachem, and BioWhittaker. Due to an ever increasing regulatory burden, low market growth (increased competition), and difficulty securing horseshoe crabs, one or two of these may soon relinquish their licenses.

After a manufacturer is licensed, a sample of each lysate lot must be submitted to CBER for release and must be accompanied by two forms. One is a protocol that lists all the manufacturer's test results, including sterility and chemical and physical data. The second form is the raw data potency sheet that shows the test results on the contents of four final containers in parallel with four replicates of the FDA reference lysate. Both lysates are tested with U.S. Standard Endotoxin Lot EC-6. Because the sensitivity of the reference lysate and the potency of Lot EC-6 are known, one can determine if the testing laboratory is in control. Both the reference lysate and endotoxin are supplied to LAL manufacturers for their in-house release testing. FDA laboratories test the sensitivity of each lot of lysate before it is released. If the test value is within twofold of the manufacturer's sensitivity test, the lot is released. If the testing is outside the twofold range, four more samples are tested; if the test value is still outside the range, the manufacturer is notified of the problem and requested to retest more samples. If the manufacturer is unable to match FDA results, the lot is rejected, and the manufacturer is notified that the lot cannot be released. The testing of samples and the review of the product protocol usually takes two to three weeks.

Each manufacturing facility (both manufacturing area and horseshoe crab bleeding areas) is inspected at least every two years. During these inspections, the inspectors review many standard operating procedures (SOPs) and current good manufacturing practices (cGMPs); interview persons directly involved with day-to-day activities; review books containing data of laboratory testing of the product; review all temperature charts for deviation from set ranges; make sure that all released products are segregated from unreleased products; review records indicating that the crabs were returned to their natural habitat within 48 hours after bleeding; check to see if all processing steps have been dated and initiated as completed; and determining if any new major personnel, equipment, or renovations have taken place since the last inspection and if these were reported to the FDA as major changes. If any exceptions are noted during the inspection, an annual inspection is performed until these exceptions are corrected.

COMMERCIALIZATION

In addition to the interest by the DBS in the LAL test, numerous research laboratories, both academic and commercial, in the United States and Japan initiated research projects involving LAL (and its Asian counterpart, *Tachypleus* amebocyte lysate, or TAL). Significant among these were Travenol Laboratories; Mallinckrodt, Inc.; and the Woods Hole Oceanographic Institution in the United States, and Seikagaku Corporation (and their university collaborators) in Japan. Out of this group came four licensed LAL manufacturers (Travenol Laboratories, licensed manufacturer for internal use only; Mallinckrodt, Inc., no longer in the LAL business; Associates of Cape Cod, Inc., spun off from the Woods Hole Oceanographic Institution; and Seikagaku Corporation, who merged with Associates of Cape Cod, Inc., in 1997). Along with Fredrik Bang, Jack Levin, several U.S. university laboratories, and those involved with the FDA, researchers associated with these groups are a Who's Who of LAL.

Of the private organizations interested early in LAL, four groups figured prominently with the FDA in the commercialization of this product. These were Associates of Cape Cod, Inc.; Mallinckrodt, Inc.; Microbiological Associates; and Travenol Laboratories. Associates of Cape Cod, Inc., founded by Stanley Watson specifically to manufacture and sell LAL, was the first company licensed by the FDA. Mallinckrodt and Microbiological Associates soon followed. Stanley Watson, Marlys Weary, and Phil Griffiths of Mallinckrodt, along with one of us (Hochstein) and Ed Seligmann at the FDA figured prominently in formulating a consensus set of rules for manufacturing and quality control for LAL. James Cooper, who developed methods to apply LAL to the testing of pharmaceutical products while a researcher with Hochstein at the FDA, later went on to found Endosafe, Inc. Although the LAL reagent became commercially available in 1977, research on the components, reactivity, and application of LAL continued in earnest. Jack Levin at the Marine Biological Laboratories continued his investigations into clinical applications of LAL (Levin et al. 1970). Stanley Watson and his group including one of us (Novitsky) at the Woods Hole Oceanographic Institution worked on environmental applications of the LAL test (Watson et al. 1977), while Watson's group at Associates of Cape Cod, Inc., worked on improving sensitivity, stability, and specificity (Sullivan and Watson 1974). Weary at Baxter Travenol was quite active in pharmaceutical applications and regulations (Weary and Baker 1977). Over the years other companies decided to commercialize LAL; 13 were granted establishment licenses for LAL by the FDA. These included, in addition to those already mentioned, then Microbiological Associates, a division of Dynasciences Corporation; CooperBiomedical, Inc.; Difco Laboratories; Millipore Corporation; Diagnostic Isotopes, Inc.; Worthington Diagnostic Systems, Inc.; Marine Biologicals, Inc.; Neumann Biotechnologies, Inc., and Endosafe, currently a division of Charles River Laboratories. In Japan, Seikagaku Corporation commercialized a TAL reagent

in 1978 but did not decide to pursue a U.S. license until later, receiving the first foreign license to manufacture LAL (actually TAL) in 1995. From the beginning LAL has been a fascinating product, as witnessed by the number of companies getting into the business. The reality, however, is that the world market is relatively small, which is one reason more than half of the companies receiving FDA licenses for LAL are no longer in the business.

Evolution of the Test Methodology

The first LAL test methodology approved by the FDA was the gel-clot assay. This assay is based on the observations of the clotting phenomenon of horseshoe crab blood by Levin and Bang (1964a). In their description of the assay, Levin and Bang refer to the LAL reagent as *pre-gel*, an extract of horseshoe crab amebocytes. This assay was later modified for commercialization through the addition of salts, for activation, and stabilizers, so the reagent could be freeze-dried. The assay was also standardized with respect to time and temperature and later to reactivity with a standard endotoxin preparation. Otherwise, the gel-clot assay is little changed from its original description. Today this version of the LAL test is still the most widely used, because of its simplicity. The gel-clot assay, however, is subjective owing to the manual reading of the test, and its semiquantitative nature. Because of this, much research has been conducted on alternative test methods (Novitsky 1993). Of the dozens that have been described since 1970, only two beside the gel-clot assay are in widespread use today: the kinetic turbidimetric method, first described by Levin and Bang (1968) and commercialized by Associates of Cape Cod, Inc., and the chromogenic method, first described by Nakamura et al. (1977) and commercialized by Seikagaku Corporation. In the United States, however, BioWhittaker, Inc. (then M.A. Bioproducts), was the first to introduce a chromogenic LAL approved by the FDA. Other methods worth noting include the colorimetric test and the end-point turbidimetric assay. For a short time, Travenol Laboratories used the colorimetric assay (Nandan and Brown 1977). This assay employed a protein determination of the insoluble gel-clot formed during the LAL reaction with endotoxin. The colorimetric assay eliminated the subjectivity of the gel-clot test and made the assay quantitative. It was, however, somewhat cumbersome and was replaced (at Travenol) by a chromogenic assay. The first commercially available quantitative assay was the end-point turbidimetric assay introduced by Worthington Biochemical (Bondar et al. 1979). This test employed an ordinary spectrophotometer with a 360 nm filter and read the turbidity that developed after a fixed period of incubation at 37°C. The need for precise timing and the lack of inexpensive multi-sample incubating spectrophotometers made this assay unpopular. Although the first chromogenic assays also required precise timing, unlike the turbidimetric assay, the end-point chromogenic test could be stopped with the addition of acid and the result read later. Addition of acid to a gel-clot or turbidimetric assay lead to the dissolution of the clot or turbidity. The first kinetic assay was a turbidimetric LAL test described by Levin and Bang (1968). This assay was most likely not readily commercialized because of lack of adequate equipment for performing kinetic assays on multiple samples. With the advent of machines like the MS-2 (Abbott Laboratories) adapted for use with the LAL assay as described by Jorgenson and Alexander (1981), incubating microplate readers by Albaugh and Chandler (1982), and the purpose-designed LAL-4000 by Novitsky et al. (1987), kinetic LAL assays became feasible. They were commercialized with the introduction of an LAL specifically designed for kinetic turbidimetric testing by Associates of Cape Cod, Inc. The final assay to be developed was the kinetic chromogenic test. This assay development was difficult for several reasons. Early chromogenic substrates were invariably contaminated with endotoxin. To circumvent this problem, the end-point assay consisted of two steps. The first involved incubation of a sample (containing endotoxin) with LAL under optimal conditions for activation. The second step involved adding the chromogenic substrate and allowing color to develop. In this two-step assay, the first step, activation, was slow (about 10 to 15 minutes) compared to the second, color development (1 to 3 minutes). Because the activation part of the assay was separated from the development part, it did not matter if the substrate was contaminated with small amounts of endotoxin. To make a single-step kinetic assay, however, the substrate needed to be as endotoxin-free as possible. Two companies involved in early research with the chromogenic assay, Kabi AB and Seikagaku Corporation, succeeded in producing clean chromogenic substrates. In addition, other factors that affected the performance of the reagent were worked out (Lindsay et al. 1989), and a kinetic chromogenic assay was finally commercialized by BioWhittaker.

Current Technology and Automation

Today the majority of LAL tests are still conducted by using the gel-clot method. However, use of one of the automated, kinetic methods is growing rapidly. The kinetic test employing a turbidimetric LAL is still in widespread use. The LAL-4000 has since been replaced with the LAL-5000, and other machines have appeared on the market, including the Toxinometer® (Wako), the ATI-6000®, the PUR-32 (Pyroquant), the Pyros® KinetiX, and various incubating microplate readers. At least two LAL manufacturers now market LAL reagents especially formulated for kinetic turbidimetric assays.

The chromogenic assay is also in widespread use, both as an end point and a kinetic test. The LAL successor to Kabi AB (Chromogenix) markets Chromo-LAL®, a kinetic LAL test manufactured by Associates of Cape Cod, Inc., but incorporating a Chromogenix (IL Laboratories) substrate. Newcomer Charles River Endosafe also has a kinetic chromogenic reagent that

employs a Chromogenix substrate. Seikagaku markets a dual-use (kinetic and endpoint) reagent, Toxicolor, with an additional diazo option for reading the end-point version. Associates of Cape Cod, Inc., also markets a similar reagent under license from Seikagaku but employing lysate from *Limulus* rather than *Tachypleus*. BioWhittaker continues as the U.S. market leader, with its chromogenic LAL coupled with a microplate reader and computer software system that greatly facilitate data collection and analysis. All manufacturers are working on new software that will be compliant with current federal regulations (Part 11).

Although users seem satisfied with the current LAL assay choices, improvements continue to be made in reagent performance (sensitivity, specificity, stability), instrumentation, and automation. BioWhittaker, for example, has teamed with Beckman to offer a complete liquid handling/assay system for those users with large testing requirements.

Seikagaku was first to offer an endotoxin-specific LAL. Because all LAL, unless specifically altered to remove or inhibit the glucan-reactive factor G, reacts to various degrees with beta 1, 3 glucans, there is a potential problem with false positive tests. Fortunately, false positive tests related to glucan contamination are relatively rare in pharmaceutical products. Problems do occur in clinical testing, dialysis, certain medical devices, and processed blood products. Currently two manufacturers offer products purported to “block” glucan activity when added to their lysates. It is likely that endotoxin-specific as well as glucan-specific lysates will be developed.

INDUSTRY ACCEPTANCE

Replacement of the Pyrogen (Rabbit) Test

Because a standardized test for pyrogens using rabbits was not implemented until 1942 with publication of USP XII, the pyrogen test was relatively new when Levin and Bang were making their initial discoveries regarding LAL in the 1960s. It wasn't until 1955 that the actual pyrogenic dose of endotoxin was determined in the laboratory of Otto Westphal (Eichenberger et al. 1955). Westphal was also able to confirm that the minimal pyrogenic dose of a purified *Salmonella* endotoxin was comparable in rabbit and humans. A dilemma for regulators, however, is that not all endotoxin species are equal as pyrogens, and, perhaps more important, not all pyrogens are endotoxins. For example, the threshold pyrogenic dose in human and rabbit was tenfold less for *Salmonella typhosa* endotoxin than for *E. coli* endotoxin, whereas *Pseudomonas* was 50 times greater (Greisman and Hornick 1969). Arguments ensued over selection and care of rabbits, assay protocol, and pyrogen standards. These have continued to a lesser degree to the present time. In 1978 the Health Industry Manufacturers Association (HIMA) established, in a collaborative study, the pyrogenicity of a well-characterized, readily available endotoxin standard from *E. coli* 055:B5 (Dabbah et al. 1980). This study established that, at the 95% confidence interval, a laboratory will attain a 50% pass or fail result at concentrations of *E. coli* 055:B5 (Difco) above 0.098 ng/mL (0.98 ng/kg dose at 10 mL/kg). This translated roughly to an LAL test pass or fail limit of 1.0 ng/mL using the same standard. HIMA concluded that if a laboratory could demonstrate an LAL test failure rate significantly greater than 50% using LAL sensitive to 0.1 ng/mL of the 055:B5 endotoxin, the test could be considered equivalent to the pyrogen (rabbit) test. A Parenteral Drug Association (PDA) *Limulus* Amebocyte Lysate Task Force also concluded that the USP pyrogen test approaches a 1 ng sensitivity and that this level could serve as a reference point for an endotoxin limit in small-volume parenterals. Large-volume parenterals were not addressed. The PDA also extended their review to the then current FDA reference endotoxin standard, EC-2, and found that the threshold pyrogenic dose (TPD) for EC-2 was about 1.0 ng/kg. This was significant because the FDA guideline recommended 0.5 ng/kg as the limit for endotoxin concentration in parenteral drugs.

The acceptance of a reliable standard as well as a reasonable endotoxin limit was considered essential for industry acceptance of the LAL test. In 1983 the FDA published the results of its collaborative study on a reference endotoxin standard. This study also established a potency for the new EC-5 standard (from *E. coli* O113:H10:K-) of 10 EU/ng. Earlier the FDA had established the EU to facilitate comparison of different endotoxin preparations. In 1985 the HIMA published the results of another collaborative study (Pearson et al. 1985). This one compared several control standard endotoxins, those used routinely by quality control and assurance laboratories, with the then current FDA reference standard EC-5. The *E. coli* O55:B5 standard was also included in this study, and all preparations were assigned EUs according to the reference standard. This study confirmed the similarity in potencies between the reference standard and O55:B5 and also added validity to the selection of 5.0 EU/kg as the limit of endotoxin (activity) in devices, parenteral drugs, and biological products.

After publication of the FDA's draft guideline for validation of LAL in 1983 and the USP's Bacterial Endotoxins Test chapter in 1985, industry acceptance was virtually complete. Implementation, however, except for in-process control, followed slowly.

Sensitivity, Reproducibility, Sample Interference

Sensitivity of the LAL test has always been greater than that of any other endotoxin detection test. From an industry standpoint, this was not an endearing feature of the assay. For those accustomed to the pyrogen test, which was not designed

to be quantitative, a positive test was a failure. With the LAL test, validation of sensitivity, use of graded standards, and dilution of sample became absolutely necessary to properly compare LAL with rabbit results. The first commercially available LAL had an average sensitivity of 0.025 ng/mL (equivalent to 0.25 EU/mL). Thus, to use this LAL as a pass-fail test, one had to dilute the drug 1:2 to meet the endotoxin limit of 5.0 EU/kg based on a dose of 10 mL/kg. As manufacturing of LAL improved, and with the introduction of the alternative methods, LAL sensitivity improved. Typical gel-clot sensitivities today are 0.03 EU/mL, and chromogenic and turbidimetric results reach 0.005 and 0.001 EU/mL, respectively.

The reproducibility of the test has always been within those expected with other enzyme assays employing biological standards and dilutions. Thus the error associated with the gel-clot test employing twofold dilutions of sample or standard is accepted at plus or minus one dilution. The error limits with the quantitative methods are usually tighter, approximating those associated with ELISA-type assays. Thus coefficients of variation (CVs) around 10% or less are readily achievable, with recovery of endotoxin added to samples approaching 25%.

Sample interference remains the single largest problem associated with the use of LAL. With the rabbit pyrogen test, sample interference was never controlled; i.e., the test did not require validation using known amounts of endotoxin added to samples. Therefore, little information on sample interference in the pyrogen test is available. With LAL, sample interference is easy to measure and is quite common (Dawson 1996, Dawson 1997a). It could be said that all samples tested with LAL when compared to pure water probably interfere with the test to some degree. Basically, any component in the sample that affects the LAL enzyme cascade or the availability of endotoxin can and will interfere with the test. Common examples are pH, ions, proteins that bind endotoxin, and detergents. Interference can manifest itself both as an inhibition of the reaction—i.e., lower than expected endotoxin concentration measured—or as enhancement—i.e., higher than expected endotoxin concentration measured. Apart from the use of buffer or other pH adjustment, dilution is the method of choice for elimination of sample interference. If dilution is used, however, sensitivity of the LAL becomes more important, especially if the endotoxin limit of the sample under test is close to the sensitivity of the LAL reagent being used. Thus the value of the very sensitive LAL available today can be seen when samples with extreme interference are encountered.

Non-Endotoxin Pyrogens

From the beginning, industry was concerned that the LAL test was not an adequate replacement for the pyrogen test, because not all pyrogens are endotoxins. Thus pyrogens such as viruses, proteins (bacterial toxins), and various chemicals would be missed by the LAL test. As it turned out, however, endotoxins are the most ubiquitous pyrogens and those most likely to be encountered in pharmaceuticals. This was shown convincingly by the FDA (Twohy et al. 1984) and is now given only slight attention during the validation phase of new drugs. Thus the rabbit pyrogen test has not gone away completely but is required at least once during the development of a new drug to prevent the occurrence of nonendotoxin pyrogens. Recently, an *in vitro* pyrogen test has been introduced that uses human monocytes or cell culture (Novitsky 2002). This test, once adequately validated, may prove to be a viable replacement for the rabbit.

False Positives

Less important than missing a nonendotoxin pyrogen or, for that matter, obtaining a false negative result, is finding a false positive. False positives affect the user by requiring repeat tests, explanations to the regulatory authority, and possibly even discard of product. With the exception of certain types of glucans, however, the LAL test is quite specific for endotoxin (Roslansky and Novitsky 1991). LAL reactive glucans, although often associated with cellulose, are (1,3)-D-glucans, which are mostly of fungal origin. They are found in cellulose because this material is readily colonized by fungi during processing. Since all glucans, whether biologically reactive or not, are basically similar chemically, LAL reactive glucans co-process with the cellulose and end up in various concentrations in the final product as a contaminant. Fortunately, the glucan reaction with LAL has been thoroughly documented and therefore can be readily addressed if it occurs. Chemical procedures and even LAL reagents that have been modified so as not to react with glucans can be used if glucan reactivity is suspected. Of course, the presence of (1,3)-D-glucan in a product that does not include this biologically active glucan as a component is indicative of a contamination event. Therefore, a positive LAL test can be viewed as a tool for controlling contamination in addition to testing for endotoxin. Several facts act in the favor of pharmaceutical manufacturers: (1) LAL positive tests are rare on products produced in GMP facilities, especially if the water used has already passed an LAL test; (2) very few LAL positive tests on final products result from glucan, with the exception of products filtered using cellulosic filter media and certain cellulose-containing medical devices—e.g., certain artificial kidneys; and (3) it is very easy to differentiate between glucan and endotoxin if a positive LAL test occurs. Because of these facts, it would be wise for a pharmaceutical manufacturer to use an LAL that is reactive for glucans when in-process testing and when evaluating raw materials for suitability. In certain validated cases, i.e., for the release of artificial kidneys that have been shown to shed LAL reactive glucans that may not affect patient safety—a nonglucan reactive LAL is warranted. Users should be worried, however, that certain LAL formulations containing

Zwittergent®, although showing reduced reactivity to glucans, may also be poorly reactive with lipid A and environmental endotoxins low in polysaccharide content (Roslansky and Novitsky 1991). It is also too soon to know whether the “glucan blockers” sold by some lysate manufacturers to remove glucan reactivity affect the LAL’s ability to detect naturally occurring endotoxin.

FDA GUIDELINES

In 1973 (FDA 1973), the FDA announced that *Limulus* amebocyte lysate (LAL), derived from circulating blood cells (amebocytes) of the horseshoe crab (*Limulus polyphemus*), is a biological product (U.S. Food and Drug Administration 1973). As such, it is subject to licensing requirements as provided in section 351 of the Public Health Service Act (42 U.S.C. 262). Since 1973, LAL has proved to be a sensitive indicator of the presence of bacterial endotoxins (pyrogens). Because of this demonstrated sensitivity, LAL can be of value in preventing the administration or use of products that may produce fever, shock, and death if administered to humans or animals.

When the 1973 notice was published, available data and experience with LAL were not adequate to support its adoption as the final pyrogen test in place of the rabbit pyrogen test, which had been accepted and recognized for many years. To establish a database and gain experience with the use of LAL, that notice permitted the introduction of LAL into the marketplace without a license on the condition that its use be limited to the in-process testing of drugs and other products. The decision to use it was reached voluntarily by affected firms, and the labeling on LAL stated that the test was not suitable as a replacement for the rabbit pyrogen test.

Since that time, production techniques have been greatly improved and standardized so that they consistently yield LAL with an endotoxin sensitivity over 100 times greater than originally obtained. Moreover, it is widely recognized that the LAL test is faster, more economical, and requires a smaller volume of product than does the rabbit pyrogen test. In addition, the procedure is less labor intensive than the rabbit test, so it is possible to perform many tests in a single day.

In 1977 the FDA described conditions for the use of LAL as an end-product test for endotoxins in human biological products and medical devices (FDA 1977). The notice stated further that the application of LAL testing to human drug products would be the subject of a future *Federal Register* publication.

In 1980 FDA announced the availability of a draft guideline that set forth procedures for use of the LAL test as an end-product testing method for endotoxins in human and animal injectable drug products (FDA 1980). This draft guideline was made available to interested parties to permit manufacturers, especially those who had used the LAL test in parallel with the rabbit pyrogen test, to submit data that could be considered in the preparation of any final guideline.

The Guideline

The guideline (U.S. Department of Health and Human Services 1987) sets forth acceptable conditions for use of the *Limulus* amebocyte lysate test. It also describes procedures for using this methodology as an end-product endotoxin test for human injectable drugs (including biological products), animal injectable drugs, and medical devices. The procedures may be used in lieu of the rabbit pyrogen test.

Legal Effect

This 1987 guideline was issued under section 10.90(b) (21 CFR 10.90[b]) of the FDA’s administrative regulations, which provides for use of guidelines to outline procedures or standards of general applicability that are acceptable to the FDA for a subject matter within its statutory authority. Although guidelines are not legal requirements, a person who follows an agency guideline may be assured that the procedures or standards will be acceptable to the FDA. The following guideline has been developed to inform manufacturers of human drugs (including biologicals), animal drugs, and medical devices of procedures that the FDA considers necessary to validate the use of LAL as an end-product endotoxin test. A manufacturer who adheres to the guideline would be considered in compliance with relevant provisions of the applicable FDA Current Good Manufacturing Practice (cGMP) regulations for drugs and devices and other applicable requirements. As provided in 21 CFR 10.90(b), persons who use methods and techniques not provided in the guideline should be able to assure, through validation, that the method or technique they use is adequate to detect the endotoxin limit for the product.

USP BACTERIAL ENDOTOXIN TEST AND RELATED MONOGRAPHS

The bacterial endotoxin test is a means of estimating the concentration of bacterial endotoxins present in or on the sample of the article(s) to which the test is applied. This test uses the LAL reagent obtained from the aqueous extracts of the circulating amebocytes of the horseshoe crab, *Limulus polyphemus* (U.S. Pharmacopeia 1995).

The determination of the test end point is made with dilutions from the material under test in direct comparison with parallel dilutions of a reference endotoxin. Quantities of endotoxin are expressed in defined EUs.

Because amebocyte lysate has been formulated to be used for turbidimetric or colormetric readings, such tests may be used if shown to comply with the requirements for alternative methods. The tests require the establishment of a standard regression curve, and the endotoxin content of the test material is determined by interpolation from the curve. The procedures include incubation, for a preselected time, of reacting endotoxin and control solutions with LAL reagent and reading of the spectrophotometric light absorbance at suitable wavelengths. In the case of the end-point turbidimetric procedure, the reading is made immediately at the end of the incubation period. In the kinetic assay (turbidimetric and colorimetric), the absorbance is measured throughout the reaction period, and rate values are determined from those readings. In the endpoint colorimetric procedure, the reaction is arrested at the end of the preselected time by the addition of enzyme-reaction-terminating agent prior to the readings.

Reference Standard and Control Standard Endotoxin

The USP Endotoxin Reference Standard has a defined potency of 10,000 USP EU per vial. It is prepared as follows: constitute the entire contents of 1 vial of the RSE with 5 ml of LAL reagent water (LRW), mix intermittently for 30 minutes, using a vortex mixer, and use this concentrate for making appropriate serial dilutions. Preserve the concentrate in a refrigerator, for making subsequent dilutions, for not more than 14 days. Mix vigorously, using a vortex mixer, for not less than 3 minutes before use. Mix each dilution for not less than 30 seconds before proceeding to make the next dilution. Do not store dilutions, because of loss of activity by adsorption, in the absence of supporting data to the contrary. A control standard endotoxin (CSE) is a preparation other than the RSE that has been standardized against the RSE. Each new lot of CSE is to be standardized prior to use in the test. Calibration of a CSE in terms of the RSE must be with the specific lot of LAL reagent and the test procedure with which it is to be used. Standardization of a CSE against the RSE using an LAL reagent for the gel-clot procedure may be effected by assaying a minimum of one vial of the CSE and one vial of the RSE, as directed under Test Procedure, but using 4 replicate reaction tubes at each level of the dilution series for the RSE and 4 replicate reaction tubes similarly for each vial or aliquot of the CSE. The antilog of the difference between the mean $\log_{10}$ end point of the CSE is the standardized potency of the CSE, which then is to be converted to and expressed in EUs per nanogram under stated drying conditions for the CSE, or in EUs per container, whichever is appropriate.

A suitable CSE has a potency of not less than 2 EU/ng and not more than 50 EU/ng.

Preparatory Testing

The following procedure is used for preparatory testing. Use an LAL reagent of confirmed label sensitivity. Treat any containers or utensils employed so as to destroy extraneous surface endotoxins that may be present. Heating in an oven at 250° C or above for sufficient time is one way to do this.

The validity of test results for bacterial endotoxins requires an adequate demonstration that specimens of the article, or of solutions, washings, or extracts thereof to which the test is to be applied do not of themselves inhibit or enhance the reaction or otherwise interfere with the test. Validation is accomplished by performing the Inhibition or Enhancement Test as described below. Appropriate negative controls are included. Validation must be repeated if the LAL reagent source or the method of manufacture or formulation of the article is changed.

Test for Confirmation of Labeled LAL Reagent Sensitivity

Confirm the labeled sensitivity using at least one vial of the LAL reagent lot. Prepare a series of twofold dilutions of the RSE (or CSE) to give concentrations of $2 \times$, $1 \times$, $0.5 \times$, and $0.25 \times$, where $\times$ is the labeled sensitivity of the LAL reagent in EU/mL. Perform the test on the four standard concentrations in quadruplicate and include negative controls. The geometric mean end-point concentration (see Calculations and Interpretation) must be $0.5 \times$, and $2.0 \times$. Confirm the labeled sensitivity of each new lot of LAL reagent prior to use in the test.

Inhibition or Enhancement Test

Perform the test on aliquots of the specimen, or a dilution not to exceed the maximum valid dilution (MVD), in which there is no detectable endotoxin. Perform the test on the specimen without added endotoxin and with endotoxin added to give final concentrations of $2.0 \times$, $1 \times$, $0.5 \times$, and $0.25 \times$. Perform the test as directed under Test Procedure, but using not fewer than 4 replicate tubes for the untreated specimen and for each specimen to which endotoxin has been added. In parallel with the above, test in duplicate the same endotoxin concentrations in water and untreated negative controls. Calculate the geometric

mean end-point endotoxin concentration for the specimen as described under Calculations and Interpretation. The test is valid for the article if the geometric mean end-point concentration in the specimen is 0.5 , and 2.0 .

If the result obtained for the specimens to which endotoxin has been added is outside the specified limit, the article is unsuitable for the Bacterial Endotoxins Test.

Repeat the test for inhibition or enhancement after neutralization, inactivation, or removal of the interfering substances or after the specimen has been diluted by a factor not exceeding the MVD. Use a dilution, not to exceed the MVD, sufficient to overcome the inhibition or enhancement of the known added endotoxin, for subsequent assays of endotoxin in test specimens.

If endogenous endotoxin is detectable in the untreated specimens under the conditions of the test, the article is unsuitable for the Inhibition or Enhancement Test; it may be rendered suitable by removing the endotoxin present by ultrafiltration, or by appropriate dilution. Dilute the untreated specimen (as constituted, where applicable, for administration or use) to a level not exceeding the MVD, at which no endotoxin is detectable. Repeat the test for inhibition or enhancement, using the specimens at those dilutions.

Maximum Valid Dilution

The MVD is appropriate to injections or to solutions for parenteral administration in the form constituted or diluted for administration, or where applicable, to the amount of drug by weight if the volume of the dosage form for administration could be varied. Where the endotoxin limit concentration is specified in the individual monograph in terms of volume (in EU per milliliter), divide the limit by , which is the labeled sensitivity (in EU per milliliter) of the lysate employed in the assay, to obtain the MVD factor. Where the endotoxin limit concentration is specified in the individual monograph in terms of weight or of units of active drug (in EU per milligram or in EU per unit), multiply the limit by the concentration (in milligram per milliliter or in units per milliliter) of the drug in the solution tested or of the drug constituted according to the label instructions, whichever is applicable, and divide the product of the multiplication by , to obtain the MVD factor. The MVD factor so obtained is the limit dilution factor for the preparation for the test to be valid.

Test Procedure

In preparing for and applying the test, observe precautions in handling the specimens to avoid gross microbial contamination. To quantify the amount of endotoxin in a specimen, an assay is performed on decreasing concentrations of specimens prepared by serial dilution. Select dilutions so that they correspond to a geometric series in which each step is greater than the next by a constant ratio. Include negative and positive controls and a positive product control.

Use not fewer than 2 replicate reaction tubes at each level of the dilution series for each specimen under test. A standard endotoxin dilution series involving not fewer than 2 replicate reaction tubes is conducted in parallel. A set of standard endotoxin dilution series is included for each block of tubes, which may consist of several racks for incubation together, provided the environmental conditions within blocks are uniform.

Preparations

Because the form and amount per container of standard endotoxin and of LAL reagent may vary, constitution and/or dilution of contents should be directed in the labeling. The pH of the test mixture of the specimen and the LAL reagent is in the range 6.0 to 8.0 unless specifically directed otherwise in the individual monograph. The pH may be adjusted by the addition of sterile, endotoxin-free sodium hydroxide or hydrochloric acid or suitable buffers to the specimen prior to testing.

Procedure

Into 10×75 mm test tubes or single test vials, dispense the specified volumes of negative controls, standard endotoxin concentrations, specimens, and positive product controls. Positive product controls consist of the article, or of solution washing or extract thereof to which RSE, or a standardized CSE, has been added to give a concentration of 2 . Add appropriately constituted LAL reagent, unless single test vials are used. Mix the specimen-LAL reagent mixture, and place in an incubating device such as a water bath or heating block, accurately recording the time at which the tubes are so placed. Incubate each tube, undisturbed, for 60±2 minutes at 37±1 C°, and carefully remove it for observation. A positive reaction is characterized by the formation of a firm gel that remains when inverted through 180°. Record such a result as positive (+). A negative result is characterized by the absence of such a gel or by the formation of a viscous gel that does not maintain its integrity. Record such a result as negative (–). Handle the tubes with care, and avoid subjecting them to unwanted vibrations, or false negative observations may result. The test is invalid if the positive product control is negative or the endotoxin

standard does not show the end-point concentration to be within ± 1 twofold dilutions from the label claim of sensitivity of the LAL reagent or if any negative control is positive. Proceed to Endotoxin Content Calculation to determine the amount of endotoxin present in the test specimen.

Calculation and Interpretation

Geometric Mean Calculation

The end point is the last positive test in a series of decreasing concentrations of endotoxin, specimen, or specimen to which endotoxin has been added. Record the end-point concentration, E , for each replicate series of dilutions. Determine the lot end-point concentration, e , and calculate the geometric mean end-point concentration by using the following formula:

$$\text{geometric mean end-point concentration} = \text{antilog} (e/f),$$

where e is the sum of the log end-point concentrations of the dilution series used and f is the number of replicates.

Endotoxin Content Calculation

Calculate the concentration of endotoxin (in units per milliliter, gram, or milligram) in or on the article under test. First, calculate the end-point concentration, E , for each of a series of dilutions by multiplying the reciprocal of each end-point dilution factor by S , where S is the labeled sensitivity expressed in EUs per milliliter of the lysate used in the test. The geometric end-point concentration of the article under test is thus the antilog of ef , where e is the log of the end-point concentration, and f is the number of replicate reaction tubes read at the level for the specimen under test.

Interpretation

The article meets the requirements of the test if the concentration of endotoxin is not more than that specified in the individual monograph.

EUROPEAN AND JAPANESE PHARMACOPOEIA ACCEPTANCE OF THE LAL TEST

The European Pharmacopoeia (EP) closely followed the USP in describing an endotoxin test using LAL. Basically, the EP test is similar to the USP Bacterial Endotoxins Test, with the exception that the EP may place more emphasis on a pass or fail decision than on quantitation. The EP Guidelines also state that the substitution of the LAL test where a rabbit pyrogen test is specified in the product monograph requires validation and the agreement of the competent authority. Pyrogenic batches, if available, should be tested with the LAL test as part of the validation. In any case, the gel-clot test is emphasized (as with the USP and Japanese Pharmacopoeia [JP] tests). The latest version of the EP Endotoxin Test (ET) and accompanying guidelines can be found in the 1998 EP Supplement (European Pharmacopoeia 1998). Apart from an incubation requirement of 37°C, the EP-ET is the most flexible with respect to gel-clot methodology. For example, the EP-ET allows choice of sample and reagent volumes (thus allowing micromethods, i.e., methods that employ lesser amounts of lysate than the "standard" assay) as well as Incubation time (20–60 minutes). Otherwise, the sample under test cannot be diluted past the MVD; a negative control must be included, as well as a 2 positive product and positive control. The EP Guidelines provide details on determination of MVD and endotoxin limits. In addition to the controls, samples must be replicated. A retest is allowed only if one replicate of the sample passes and the other fails. A detailed review in a comparative format (USP vs. EP vs. JP) is provided by Dawson (1997b).

The Japanese Pharmacopoeia also closely follows the USP and EP with respect to LAL, although the JP pays more attention to the "photometric methods," perhaps because they are more widely used in Japan than in the United States and Europe. Like the EP, the JP allows use of lysates from either *Limulus* (LAL) or *Tachypleus* (TAL). For the gel-clot method, the JP is quite specific, but very similar to the USP; i.e., 37±1°C, 60±2 minutes, negative, positive, and positive product controls, and so forth. Retest is similar to that specified by the EP; i.e., one is allowed if one replicate is negative and the other positive. Although there is a Japanese reference endotoxin standard, the endotoxin units used in the JP are in terms of the USP RSE, to which the JP endotoxin was compared.

International Standard

As should already be apparent, many endotoxin standards have been used over the period of time the LAL test has been gaining acceptance. It is very likely that too many standards prevented adequate comparison of LAL in some of collaborative studies. In many cases, the LAL reagent(s) were blamed instead of the standards. Pharmacopeial jealousy and national pride also played a role in slowing the acceptance of the LAL test, by insistence on “official” standards. At one point, the U.S. FDA and USP, the EP, the WHO, and the JP each had its own “reference standard.” Unlike a platinum meter stick or an atomic clock, endotoxin is a biochemical subject to influences of drying, ions, pH, detergents, and so forth. Thus collaborative studies comparing one official reference to another became statistical nightmares in which the users, often given only the “average” unitage of the standard, could not understand why their LAL did not perform according to label claim. The World Health Organization, under the guidance of the NIBSC, teamed with the USP and the FDA to manufacture and test a new international standard (IS) (Poole et al. 1997). This standard, designated EC-6 by the FDA, Lot G by the USP, the second IS (preparation 94/580) by the WHO, and BRP-3 by the EP, is now accepted by these organizations. The JP has indicated it will maintain its own reference standard.

The common standard and acceptance of the EU goes a long way toward harmonization. Although the pharmacopoeias are not entirely in agreement, the gel-clot methodology is close enough for comfort, and true pharmacopeial harmonization for the LAL test may be within sight.

METHODOLOGY

Gel-Clot Assay

The gel-clot assay is the simplest LAL method. The basic test protocol requires mixing 0.1 mL of sample with 0.1 mL of reconstituted LAL in a 10×75 mm test tube, mixing gently, and incubating undisturbed in a 37°C water bath or dry block for 60 minutes. To read the test, the tube is gently inverted. If a gel is formed that can withstand an inversion of 180°, the test is positive. Anything less is negative. Particular details of the test can be found in the manufacturer’s instructions and in the various pharmacopoeias. It should be noted that the manufacturer’s instructions take precedence and should be followed exactly for expected results. Reagent storage, reconstitution, and glassware or plastic ware used with the assay can all influence the test.

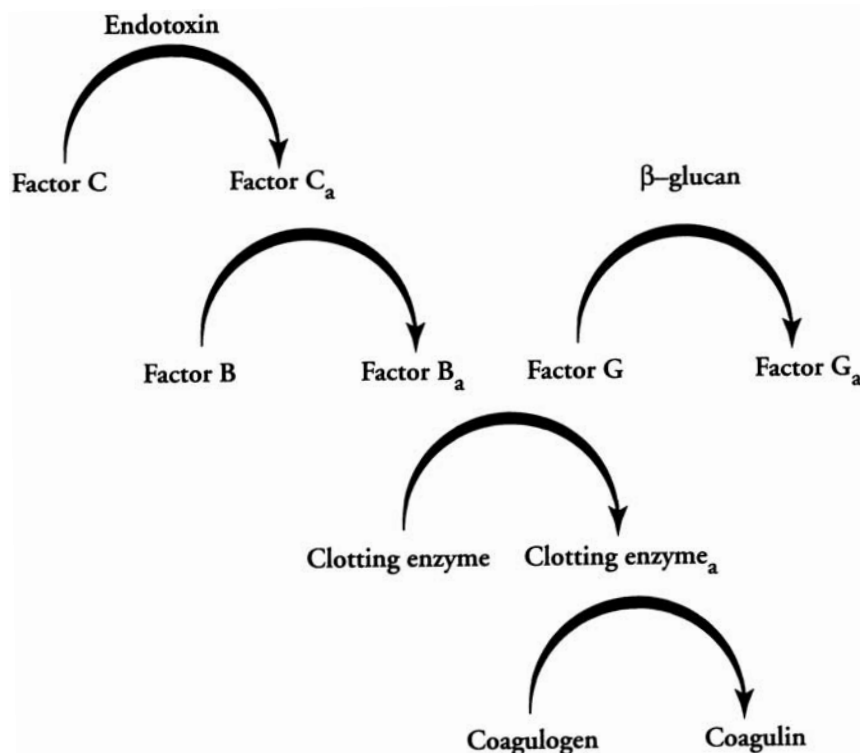
Turbidimetric Assay

The turbidimetric assay relies on the fact that the protein formed in the development of a gel clot also forms a turbid solution. The turbidimetric assay can therefore be viewed as an objective way of following clot formation. However, degree of turbidity and the formation of a solid gel clot are not tightly correlated, although attempts have been made to relate the two so that certain machines can be accepted as “gel-clot readers.” The ability of the turbidimetric method to accurately measure endotoxin however has been adequately demonstrated. Likewise, correlations among the turbidimetric, chromogenic, and gel-clot methods have been shown to be excellent within the error of the methodologies.

For the turbidimetric assay, 0.1 mL of LAL is mixed with 0.4 or 0.1 mL of sample in a tube (e.g., for assays employing the LAL-5000 or Toxinometer) or microplate (e.g., for assays employing the KQCL) and placed in the machine (or incubator for the end-point method). For the kinetic method, incubation varies but since reading is continuous or semicontinuous, only a maximum time must be determined. For the end-point method, a maximum time must be determined from experiment, and subsequent readings must be made precisely on time. Most readers now can be coupled with computer programs to collect and analyze data. Most programs perform a linear regression, using the time it takes to reach a certain optical density or percentage transmission and the endotoxin concentration. At least one program uses a curve-fitting algorithm because some lots of LAL do not show a precisely linear response when a spectrophotometer is used to measure turbidity. Non-curve-fitting programs avoid this problem by selecting a 1 or 2 log range of standards rather than trying to force a 4 or 5 log range on the assay. Although the original kinetic assay was developed to allow for a range of sensitivities (gel clot is set at one concentration, while end-point tests are limited to a one log range), for pharmaceutical applications where a one concentration limit or spike control is used, greater precision can be achieved and time saved by using a smaller standard range. Incubation of the turbidimetric test is also 37°C, only the time of incubation varying.

Chromogenic Assay

The chromogenic assay is basically a turbidimetric assay with an added chromogenic substrate. These substrates mimic the coagulogen component of the assay and release a chromophore, paranitroaniline (pNA), when cleaved by activated

Figure 11.1 *Limulus* amoebocyte lysate cascade.

From LAL Update, September 1995, Vol. 13, No. 3. (Reprinted with permission of Associates of Cape Cod, Inc.)

proclotting enzyme (see [Figure 11.1](#)) The chromogenic assay can be performed as an end point, end point with diazo coupling, or kinetic, depending on the reagent or test kit. The end-point test is performed two ways, depending on the kit. If the reagent is supplied as a combined LAL-substrate, the reconstituted LAL, 0.05 or 0.1 mL is added to 0.05 or 0.1 mL of sample in a tube or microplate and incubated at 37°C for a period of time. At the end of incubation, the sample is either read directly or the reaction is stopped with the addition of acid and read later. In the case of the diazo-coupled end point, the reaction is converted to the diazo derivative with the chemistry provided with the kit and then read. For pNA, a spectrophotometer or plate reader equipped with a 405 nm filter is required. For the diazo-coupled reaction, 540–550 nm is required. Some older kits supply the chromogenic substrate separate from the LAL. For these end-point tests, a primary incubation is performed with the LAL-sample followed by addition of the substrate and a short secondary incubation, all at 37°C. After the second incubation, the reaction is read immediately or stopped with acid and read later. For the kinetic test, the sample and LAL are mixed and the reaction placed immediately in an incubating plate or tube reader. Data collection for the kinetic chromogenic test is similar to that for the kinetic turbidimetric test.

AUTOMATION

The kinetic turbidimetric and chromogenic tests described above have been considered “automated” in that the operator need only load the test and walk away. The computer, in concert with the plate or tube reader, times and incubates the test and collects and analyzes the data. With these methods, a user could load a test prior to leaving for the day and retrieve the results the following morning or a week later.

The turbidimetric and chromogenic tests both lend themselves to robotics—i.e., eliminate the operator in the preparation of samples and standards, and in loading the machine. The LAL-5000 has been successfully coupled to a Zymate robot and the KQCL has been hooked up to a Beckman system. These methods, however, lend themselves only to very large operations and require extensive validation and attention to detail due to short reconstituted shelf life of lysate, propensity of standards to adsorb to the walls of containers and tubing, and the ubiquitous nature of bacteria and endotoxin in the environment (facilitating contamination).

RESEARCH AND CLINICAL APPLICATIONS

It was always hoped that the LAL test would prove useful as a clinical diagnostic tool. Studies in the literature indicated that the LAL test could detect gram-negative spinal meningitis and urinary tract infections. A test for gonorrhea was also proposed. LAL manufacturers were actually approved to market tests for spinal meningitis and gonorrhea but never did. Although the LAL test is used as a diagnostic in Japan, a clinical trial for an LAL as an aid in the diagnosis of gram-negative sepsis was done in the United States, but the results did not indicate clinical utility (Ketchum et al. 1997). In light of additional findings that antiendotoxin therapies are not efficacious in reducing mortality or morbidity in septic patients, it is surprising that the LAL test is still considered by some to hold diagnostic promise for this condition. One reason given for the lack of clinical utility of the LAL test is its exceptional sensitivity to endotoxin. The many interfering factors present in clinical samples are thought to mask the ability of LAL to adequately measure endotoxin. In addition, especially in blood, endotoxin can occur in the absence of bacteria, and too few bacteria may be present to cause a positive LAL test. It appears unlikely that a clinical application for LAL will appear since first proposed almost 30 years ago. Excellent reviews of clinical applications have been published (Novitsky 1996; Prior 1990).

LAL as a Research Tool

The LAL test has been used extensively as a research tool to follow the fate of endotoxin in animal models, environmental systems, water, and food processing. As a tool for the detection of endotoxin, the LAL test is unsurpassed; no other test comes close to its sensitivity and specificity. For reviews see Watson et al. (1982, 1987) and Pearson (1985).

FUTURE OF THE ASSAY

We are amazed that the LAL assay is still the dominant, indeed almost the only practical, test for endotoxin. However, there are indications that the horseshoe crab's very existence may be threatened due to overharvesting the animal for use as eel and whelk bait (Kerlinger 1998). Ever since the LAL test was first described, scientists have endeavored to elucidate the reaction components. These have now been thoroughly described. Not surprisingly, recombinant analogues of at least factor C have been developed and have been cloned in yeast (Ding 1997) and in insect cells (Ding and Ho 2002). A chromogenic substrate for the coagulin component has been in use for more than 15 years. In one of our laboratories (TN), an endotoxin binding protein (rENP) from *Limulus* has been cloned and has been developed into an assay (Novitsky et al. 2001). This new assay utilizes the principle of fluorescence polarization to detect and quantify endotoxin bound to fluorescently labeled rENP. It is very fast (10 seconds) and easy to perform. However, the sensitivity of the current reagent and reader is lower than for LAL. Should research succeed in producing an adequate sensitivity with either a true recombinant LAL replacement or a completely new endotoxin assay, given the history of acceptance of the LAL test, it may be a while before an "official" replacement assay appears, however exciting the science may be.

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