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LAL UPDATE®

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Dear LAL User,

This LAL UPDATE covers the discovery, development, current status and future of LAL. An earlier article on this subject, "Discovery to Commercialization: The Blood of the Horseshoe Crab" proved to be quite popular and has been reprinted numerous times. The new article expands on the topic and is designed as a replacement.

Melissa Juntunen has joined our staff as Marketing Coordinator. She was previously employed at ZynoCyte, Ltd.

As of June 1, ACC products will be available in Ireland through Associates of Cape Cod International (Ireland) Ltd. Avril MacNamara, formerly with Bioprep Technologies, is the LAL expert and General Manager. ACCI also has a new office in Barcelona, Spain. The contact there is Pelai Fontsare. (Addresses on page 5).

Sincerely,

Thomas J. Novitsky, Ph.D.
Editor

LAL: Discovery and Commercial Development

by Thomas J. Novitsky

Most people familiar with the reagent *Limulus* amebocyte lysate (LAL) have some knowledge or at least some curiosity regarding the source animal, *Limulus polyphemus*, or North American horseshoe crab. The natural history of this unique animal begins with its fossil record. Often referred to as a "living fossil" because it has changed very little in over 150 million years, the horseshoe crab is well documented in the fossil record. An excellent collection of Mesolimulus from the late Jurassic limestone of Bavaria is housed in the Jura-Museums Eichstätt, Germany, near the quarries where these fossils were discovered. Although most of the specimens are juveniles, some full-sized adults have been preserved as well as trace fossils of the horseshoe crab's tracks. Closer to home, the American Museum of Natural History in New York City has some nice specimens. In a recent exhibit on drugs from the ocean, this museum featured LAL from ACC! Although the Smithsonian's Natural History Museum has no horseshoe crab fossils displayed, they do have excellent exhibits on some relatives, the giant water scorpion (eurypterid) and the trilobite. Two features of these, the tail or telson (in eurypterids and some trilobite species) and the head or cephalothorax of both animals are easily recognizable as related to the horseshoe crab. Although the eurypterids and trilobites are long extinct, the horseshoe crab has exhibited amazing survival skills.

Whether this fascinating animal can survive humankind remains to be seen. Horseshoe crabs were used as fertilizer and are even eaten in some societies. See LAL UPDATE, Vol. 3, No. 4 (July 1985). Anyone familiar with blood chemistry of this animal can't help but wonder whether the reaction(s) of the amebocyte to bacterial endotoxin has played a major role in the horseshoe crabs' survival.

Fascination with the horseshoe crab first led W. H. Howell of Johns Hopkins University to examine the clotting of *Limulus* blood in the 1880's. He observed that blood

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removed from the animal formed a clot. Although others, in particular Leo Loeb at the Marine Biological Laboratory in Woods Hole, Massachusetts, later studied the blood of *Limulus*, it was an observation by Dr. Frederik B. Bang that ultimately led to the characterization of the clotting phenomenon. During a summer at Woods Hole, while attempting to observe phagocytosis after injecting various bacteria into *Limulus,* Bang found the presence of *Vibrio* species caused intravascular coagulation in his animals. Years later, joined by another Hopkins researcher, Dr. Jack Levin, Bang was able to demonstrate the cause of the clotting phenomenon (bacterial endotoxin) and the source of the clotting material (*Limulus* amebocyte). In a presentation at an LAL conference held in Woods Hole in 1981, Dr. Bang referred to his discovery as "serendipity," defined as a "combination of chance and sagacity," with sagacity often "a collective effort." Indeed, many investigators were able to reproduce the clotting phenomenon *ex vivo* and Levin prepared an extract of amebocytes (PreGel) which could be used as an *in vitro* test for endotoxin---hence *Limulus* amebocyte lysate (LAL) came into being.

Following publication in scientific literature of the properties of the *Limulus* lysate, the Food and Drug Administration's Bureau of Biologics (BoB, then part of the National Institutes of Health), became interested in LAL as an alternative to the Pyrogen (rabbit) test for endotoxin. Dr. Ed Seligmann, Director of Control Testing at the BoB initiated a number of in-house studies beginning in 1971. Assisted initially by Dr. H. Donald Hochstein and later by Dr. James Cooper, the BoB made and tested LAL. Once a year a crew from Bethesda would drive to a NASA installation on Chincoteague Island, Virginia, where they set up a small laboratory and bled crabs for a week. According to Dr. Hochstein: "we were very pleased with ourselves if we could bleed 1200 crabs the week we were there." The lysate and as many live crabs as could fit in the space that remained in their car were taken back to Bethesda. The lysate was then processed and the live crabs placed in an aquarium which shared space in the rabbit facility. According to Hochstein, the crabs survived quite nicely and could be used to prepare additional lysate if needed before the next trip to Chincoteague.

The FDA soon realized that an endotoxin standard was needed as a control and contracted with Dr. A. Rudbach, then at the University of Montana, to prepare a bulk preparation of endotoxin from *Escherichia coli*. This became the "EC" preparation and has been used to prepare several lots of "Reference Standard Endotoxin" (RSE). The current lot is EC-6 and serves both as a control for the LAL manufacturers to determine label claim or sensitivity and is commercially available as Lot G from the USP.

In 1973, the FDA announced in the Federal Register the first guideline regulating LAL. The early 70's also saw interest in commercializing the LAL test. Dr. Stanley Watson, who had been using the LAL assay in his research at the Woods Hole Oceanographic Institution and consulting for a number of companies, decided to make and market LAL when he could not convince anyone else to do so. He incorporated Associates of Cape Cod in 1974 shortly after the FDA announcement. ACC was the first company to be licensed by the FDA (corresponding to the Federal Register notice setting the conditions for replacing the Pyrogen test with LAL in 1977). Other individuals who played important roles in the early commercial development of LAL were Don Mills at Mallinckrodt, Fred Pearson and Marlys Weary at Travenol, Tai Yin at Haemachem, Aaron Lane at Difco, R. Bondar and J. D. Teller at Worthington, and Gene Lindsay at Microbiological Associates. Some of the early LAL manufacturers are no longer in this market, e.g., Worthington, Difco, Mallinckrodt, and others have changed names, e.g., Microbiological Associates spun-off the part of their business dealing with LAL into M.A. Bioproducts (now BioWhittaker). Other companies have grown and prospered to be acquired by large companies. For example, Endosafe, Inc. was recently acquired by Charles River Laboratories. Despite a relatively small and mature market (a licensed clinical application for LAL has yet to appear), the horseshoe crab seems to attract as much business interest as it does scientific.

In Japan, Seikagaku Kogyo Corporation was marketing a gel-

clot reagent in the late 70's. By the early 80's they introduced the first chromogenic reagent. Although all horseshoe crab products are usually referred to as "LAL", these reagents employed a lysate from the Asian horseshoe crab, *Tachypleus tridentatus.* Recently, Seikagaku Corporation received a license from the FDA to market some of their "TAL" products in the US. It should be noted that Japanese researchers have made major contributions to our understanding of LAL biochemistry.

In addition to the Japanese, many researchers have studied LAL and its applications. All of those who have been most instrumental in the LAL field can be found as contributors to a series of books resulting from conferences held in Woods Hole and later in Amsterdam. I feel quite privileged to have known most of these scientists.

Currently there are three basic variations of the LAL test on the market: the gel-clot, the turbidimetric, and the chromogenic. All three come in various shapes and sizes. Although the FDA regulates the product through a license procedure in the US, some variation in the test methodology does exist. In Europe, where less regulation exists, the methodology is less uniform. For example, in the US most gel-clot tests are performed in glass tubes using 0.1 ml of LAL and 0.1 ml sample (total volume 0.2 ml). A microtest is allowed in Europe which uses a 0.01 ml of LAL and 0.01 ml of sample mixed on a glass microscope slide. Although this microtest seems to work, there is no way for the user to know if the test is "in control" since current manufacturers define label claim according to "standard" conditions, i.e. 0.1 ml LAL, 0.1 ml standard, 60 ±2 minutes incubation at 37 ±1°C.

Perhaps the chromogenic test methods vary the most with several manufacturers recommending different procedures. Most, however, employ a microplate reader. Depending on the reagent/manufacturer, a kinetic, endpoint, or diazo-endpoint assay can be performed. For the kinetic test, an incubating microplate reader or spectrophotometer is needed as well as software to analyze kinetic data. For all but the diazo-endpoint assay, the reader must be capable of measuring absorbance at 405 nm, the absorbance peak of the para-nitroaniline (pNA) chromophore. The diazo-endpoint assay requires a reader capable of measuring at 540 nm (diazo derivative of pNA).

The turbidimetric assay can be run kinetically or as an endpoint test, although the endpoint assay is seldom used today (Worthington Inc. marketed the first endpoint turbidimetric test). Dr. Levin first described the kinetics of LAL using turbidity. Today the kinetic turbidimetric test is run using an incubating microplate or tube reader in a fashion similar to that for the chromogenic test. Since turbidity blocks light, with increased absorbance at lower wavelengths, the test can be read at the 405 nm used in chromogenic assays or at lower wavelengths. White light, which is employed in the LAL-5000 Automatic Endotoxin Detection System, can also be used.

At this point in the life-cycle of the LAL product there is little that can be done to "improve" the reagent. Most recent advances have been made in machines to read and incubate the test, e.g. QCL-1000®, LAL-5000, and accompanying software, e.g. Pyros™ for Windows®. Our own research is looking at an improved version of the LAL-5000 based on what we learned from our LAL-6000 experience and more powerful and user-friendly software.

On the reagent side, there may be advantages to new substrates and chromophores. As fluorescent microplate readers become more available, fluorescent substrates may replace chromogenic substrates. Theoretically a gain in sensitivity and perhaps reduced sample interference is possible. Recently some articles have appeared employing various immunological means to reduce the amount of LAL needed. Unfortunately, the minor gains made in reducing LAL consumption are lost due to the complexity of the assay. The basis of the test, the LAL enzyme cascade, remains the same. Since most of the sample interference, i.e., pH, osmolarity, temperature, etc. acts directly on this cascade, nothing is gained in assay compatibility.

Although the endotoxin (vs. glucan) specificity issue surfaces now and then, endotoxin specific LAL seems only of value for the clinical diagnosis of endotoxemia. An excellent, albeit expensive product, Endospecy (Seikagaku Corporation) has been on the market for a number of years.

One large concern for the future of the LAL test is the continued availability of the horseshoe crab. Although we have not seen any decline in our local stocks, one only has to look at the Japanese situation to become concerned. Recent evidence indicates that even the US population, in the center of its range (Chesapeake/Delaware Bays) may be in decline. Future research dictates that we address this situation, not only through conservation, but through research for an alternative. Genetic engineering is a logical choice in a search for an alternative to the natural product. Most of the enzymes in the cascade have been described in detail and coagulogen has been produced synthetically. However, genetically engineered enzymes of the size and complexity of those in the LAL cascade will be very expensive to manufacture. Associates of Cape Cod continues to investigate this area. We hope to be the company that introduces LAL's replacement just as we commercialized LAL.

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LAL Methodology and Applications Seminar and Workshop

Associates of Cape Cod, Inc. continues to offer a two-day, in-house LAL seminar and workshop. The first day is a comprehensive lecture. Topics include: the structure, activity and control of endotoxins, the biochemistry of the LAL test, LAL methods and applications, and the regulatory issues for setting up an LAL laboratory and performing end-product release testing.

The second day consists of work in the laboratory under supervision. After demonstrations of the gel-clot, turbidimetric and chromogenic methods, you can work with the method of your choice. If arrangements are made in advance, you may bring a single (nonhazardous) sample to develop test protocols. Attendees are encouraged to ask questions pertaining to their own SOP's or products. Time is available for discussion in private as well as in the classroom.

Next classes will be July 9-10, August 6-7, September 10-11, October 15-16 and November 12-13, 1996.

If you are interested contact Robin McFarlin (800-848-3248 x 206) for registration details.