LAL Update

ASSOCIATES OF CAPE COD, INCORPORATED • DECEMBER 2002 • VOLUME 20, NO.5

Letter From the President

Dear LAL User:

Since beginning my research with Limulus Amebocyte Lysate (LAL) over 27 years ago, my major interest has been in assay development. I am especially proud of the fact that this interest resulted in the successful commercial introduction of the first kinetic LAL assay. While this achievement was notable, it was simply a result of providing a machine and an LAL formulation to bring Levin and Bang's [(1)] original observation to the commercial level. My real interest, however, lay in replacing LAL with a synthetic or chemical alternative. Beginning with research begun in the laboratory of Dr. Stanley Watson at the Woods Hole Oceanographic Institution, I became interested in an LAL component that had an extremely high affinity for endotoxin and which prevented the recovery of active endotoxin from the LAL reaction. Once this substance, which we termed Limulus endotoxin-binding (or neutralizing) protein (ENP) was isolated and characterized, it became apparent that it could form the basis of numerous products and applications. Their novelty led to a series of US and foreign patents (see ACC patents on page 5). Notable among these was a device for removing endotoxin from solution and an endotoxin assay. Since ENP was even more difficult to obtain from horseshoe crabs than LAL itself, it was readily apparent that any commercialization of this product, especially for therapeutic applications, depended on the ability to make it synthetically. In our first attempt, we were able to construct a synthetic gene and express a recombinant form (rENP) in yeast. Subsequent development led to secreted rENP at high levels of expression. With our current system, gram amounts of highly purified rENP can be rapidly produced. These developments led to a series of products - END®-X, an affinity endotoxin removal device; MicroQuikChek®, a latex-agglutination assay for gram-negative bacteria in industrial fluids; and most recently, EndoFluor®, a fluorescence polarization assay for the detection of endotoxin in solution. Under the research leadership of Dr. Jack Sloyer, the EndoFluor reagent and accompanying reader, PolarScan®, were introduced earlier this year for the detection and quantitation of endotoxin in metalworking fluids and air. These applications were relatively straightforward, since endotoxins collected in air and those occurring in contaminated industrial solutions are in relatively high concentration. Thus the product for these applications does not need to be especially sensitive (≤ 1.0 EU/ml). Additional work on both the reagent and the machine were required however to improve the sensitivity to > 0.01 EU/ml for pharmaceutical applications. Jack Sloyer and his team have now achieved this goal. This UPDATE then will serve to introduce LAL users to the "Next Generation in Endotoxin Testing" --- EndoFluor and PolarScan.

Sincerely,

Thomas J. Novitsky, Ph.D.

(1) Levin, J. and F.P. Bang. 1968. Clottable protein in Limulus: Its localization and kinetics of its coagulation by endotoxin. Thromb. Diath. Haemorrh. 19:186-197.

Endotoxin Determination with EndoFluor® Reagent and the PolarScan® Reader - The "Next Generation in Endotoxin Testing"

By Jack Sloyer, Ph.D. and Thomas J. Novitsky, Ph.D.

Limulus Endotoxin Neutralizing Protein (ENP) was discovered, isolated, purified, and characterized by a team from Associates of Cape Cod, Inc. (ACC) in the 1980's. This discovery led to US patent No. 5,594,113 in 1997. Japanese investigators independently discovered *Limulus* Anti-LPS Factor (LALF) at about the same time. Naturally derived ENP is essentially similar to LALF with expected differences in purity and some minor amino acid heterogeneity. Since the blood from one season's collection of over 70,000 horseshoe crabs was required to produce the first 20 grams of 65% pure ENP, it was obvious to us that the natural product, which we now refer to as LALF, would never be a viable commercial entity. Thus, immediately following characterization, ACC set out to make a recombinant product. LALF is a small peptide consisting of 100 amino acids. Based on a consensus amino acid sequence, we were able to construct a synthetic gene and achieve expression in yeast for an active molecule. Today, Erik Paus and his fermentation group can produce over thirty grams of 95% purity in a week's time at our Cape Cod facility. With characterization of the crystal structure of rENP, we were able to show its three dimensional structure and confirm our ideas of endotoxin specificity and binding. Further work indicated that rENP was highly specific for a wide variety of endotoxin species. In addition, rENP had an extremely high affinity for endotoxins. Not only was rENP competitive with *Limulus* factor C and human bactericidal permeability increasing protein (BPI), once bound to endotoxin, it could not be disassociated without denaturation. Further characterization showed that rENP was stable in solution to 80°C and could be labeled with fluorescent tags without compromising its stability or its ability to bind endotoxin. Although we had earlier discovered and patented a fluorescent assay based on the inherent fluorescence of rENP, this assay was relatively insensitive and required a UV/VIS fluorometer. Subsequent work by Jack Sloyer, Richard Ridge, and I, showed that fluorescently labeled rENP could measure low concentrations of endotoxin by fluorescence polarization (see Box 1). In 1998 when we did our initial work with fluorescence polarization (FP), there were few available research FP readers. We therefore bought the rights to a small reader and reengineered it to meet our requirements. The result was a patented machine, PolarScan® (see Box 1). The EndoFluor® reagent and PolarScan instrument were introduced earlier this year to assay endotoxin in metal cutting solutions and air. Work continued on both the reagent and the machine with the ultimate goal of achieving a sensitivity equivalent to LAL. Currently, our FP "system" has a sensitivity of 0.01 EU/ml, with a coefficient of variation (C.V.) of < 4%. A typical assay requires no dilution and a test can be read in a mere **10 seconds** at room temperature! Moreover, the chemical and physical nature of this assay will allow true automation in the near future (see Box 2).

Table 1 and Figure 1 below show the reproducibility and sensitivity (standard curve) of the EndoFluor reagent measuring Reference Standard Endotoxin in the PolarScan instrument. To perform the test, EndoFluor reagent is added to 1 ml of a standard solution in a glass 10 x 75 mm test tube. After appropriate blanking, the tube is inserted and the read command executed. Fluorescence emission is measured in the horizontal and vertical planes and the resulting mP (milli rho) recorded. Plotting mP against endotoxin concentration produces a standard regression line. In order to show reproducibility, multiple readings (n=10) of individual endotoxin concentrations were measured and their mP values used to calculate a standard deviation and coefficient of variation (Table 1). A limit of detection (LOD) and limit of quantitation (LOQ) were calculated from the standard curve (Figure 1).

TABLE 1

Detection of Endotoxin using Fluorescence Polarization and Endfluor®

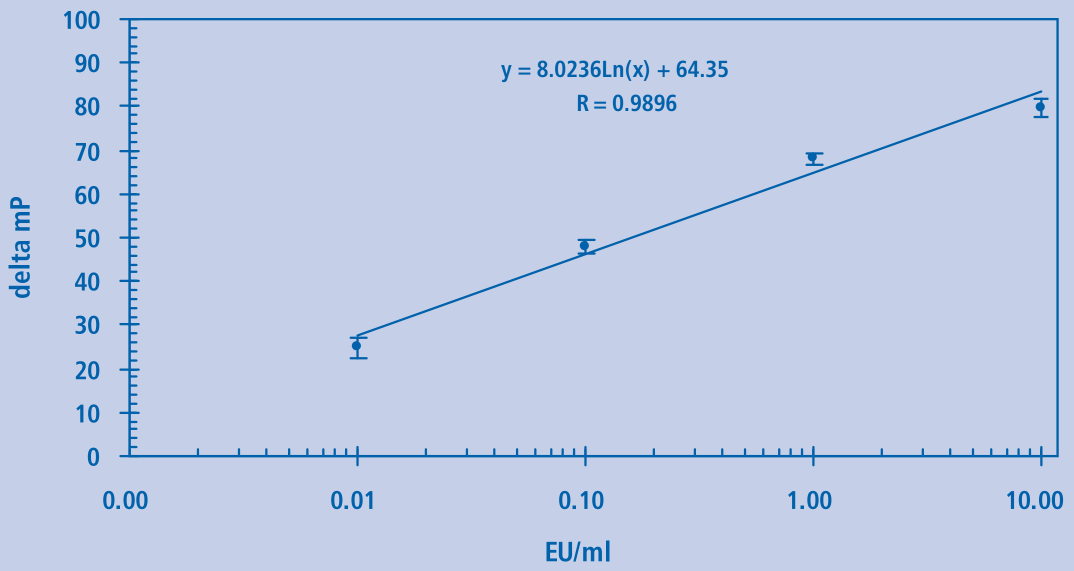
	EU/ml
Mean	0.0091
Standard Deviation	0.0019
LOD	0.015
LOQ	0.028

Results obtained by calculating the mean of 10 determinations from each of 10 different tubes

LOD = mean + 3.3 Standard deviation

LOQ = mean + 10 Standard deviation

FIGURE 1: DETECTION OF ENDOTOXIN USING FLUORESCENCE POLARIZATION



BOX 1: FLUORESCENCE POLARIZATION AND POLARSCAN

The property of a fluorescent molecule to emit light in a different plane after excitation with plane-polarized light is called **fluorescence polarization** (FP). To employ this technique, an instrument requires a wavelength specific light source, a polarizer, and a photomultiplier. There are a number of FP instruments currently on the market that include plate and tube readers. Most notably, an FP technology using antibodies (fluorescence polarization immunoassay, FPIA) for monitoring therapeutic drugs has been in use since 1981 (TDx® System, Abbott Diagnostics Division). Newer devices, e.g. Abbott Diagnostic's IMx® and ADx® systems also employ FPIA to detect various diseases and drugs of abuse. Although our system employs a non-antibody binding protein, the principle is the same, i.e. the molecular properties that allow FP to be used as an assay relate to molecular volume and molecular rotation. Molecules emit light in a different plane only if they are rotating. The degree to which emission intensity moves from the vertical to the horizontal plane is related to the mobility (rotational ability) of a molecule. Thus, under constant conditions, a larger molecule will rotate more slowly than a smaller molecule. Our assay works in this system

since EndoFluor is a relatively small fluorescent molecule. When it binds to a relatively large endotoxin molecule, molecular size (combined EndoFluor plus endotoxin) increases and rotation slows. The EndoFluor polarization assay for endotoxin is therefore a direct assay in that polarization is directly proportional to the endotoxin concentration in the sample being tested. The PolarScan calculates the average rotation of all the fluorescing molecular species in the sample, I.e., the EndoFluor, both complexed with endotoxin and free

BOX 1: (CONTINUED)

EndoFluor reagent. Therefore, the more endotoxin that is added, the more complexed molecules exist in solution. This is the basis for relating polarization to endotoxin concentration.

In the Polarscan reader, wavelength-specific polarized light (photons) captured by the photomultiplier from different planes is measured and used to calculate P (the Greek rho). Since P is very small, the result is multiplied by 1000 to give milliP (mP) according to the following equation:

$$mP = \frac{\text{parallel plane photons minus perpendicular plane photons}}{\text{parallel plane photons plus perpendicular plane photons}} \times 10^3$$

The patented PolarScan FP tube reader was initially designed to measure and calculate endotoxin concentrations using EndoFluor, but because of its fluorescein wavelength specification, it can be used to measure antigen-antibody reactions as well as PCR reactions. PolarScan consists of an LED light source, wavelength filters, a liquid crystal polarizer, and a photomultiplier tube. Accompanying software performs all necessary calculations and reports results in EU/ml.

To perform an endotoxin assay, EndoFluor reagent is added to 1 ml of sample in a depyrogenated 10 x 75 mm glass tube and mixed. After appropriate blanking, the tube containing mixed sample plus EndoFluor is placed in the machine and the read command entered. Following reading in the vertical and horizontal planes, the accompanying software calculates the mP, and calculates endotoxin concentration from a stored standard curve. Reading and calculation takes about 10 seconds.

BOX 2: AUTOMATION OF ENDOTOXIN TESTING WITH ENDOFLUOR

True automation of endotoxin tests using LAL has been elusive. Many claims have been made for automated LAL tests employing sophisticated robots and autodiluters. At best these amount to expensive "pipetting/diluting aids". The nature of existing LAL formulations (poor reconstituted stability and propensity for contamination); the requirements for sample dilutions and standard curves; and long temperature-sensitive incubation times required for a valid test; render the LAL assay difficult, if not impossible to automate at a reasonable cost. ACC envisions true endotoxin test automation as either an in-line device similar to the TOC analyzers currently available, or an "auto sampler" device, which can be loaded with samples and reagent and left unattended. The FP assay, which ACC developed, lends itself to both applications. Since the FP reagent is extremely stable, requires no reconstitution, and reacts with endotoxin almost instantaneously, a simple auto sampler arrangement with reagent injection is possible. Likewise, the basic design of the PolarScan reader can be adapted to a flow-through (in-line) application. Engineering studies of both these applications, including a patented biosensor design are currently underway at ACC.

ACC Patents Related to ENP:

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