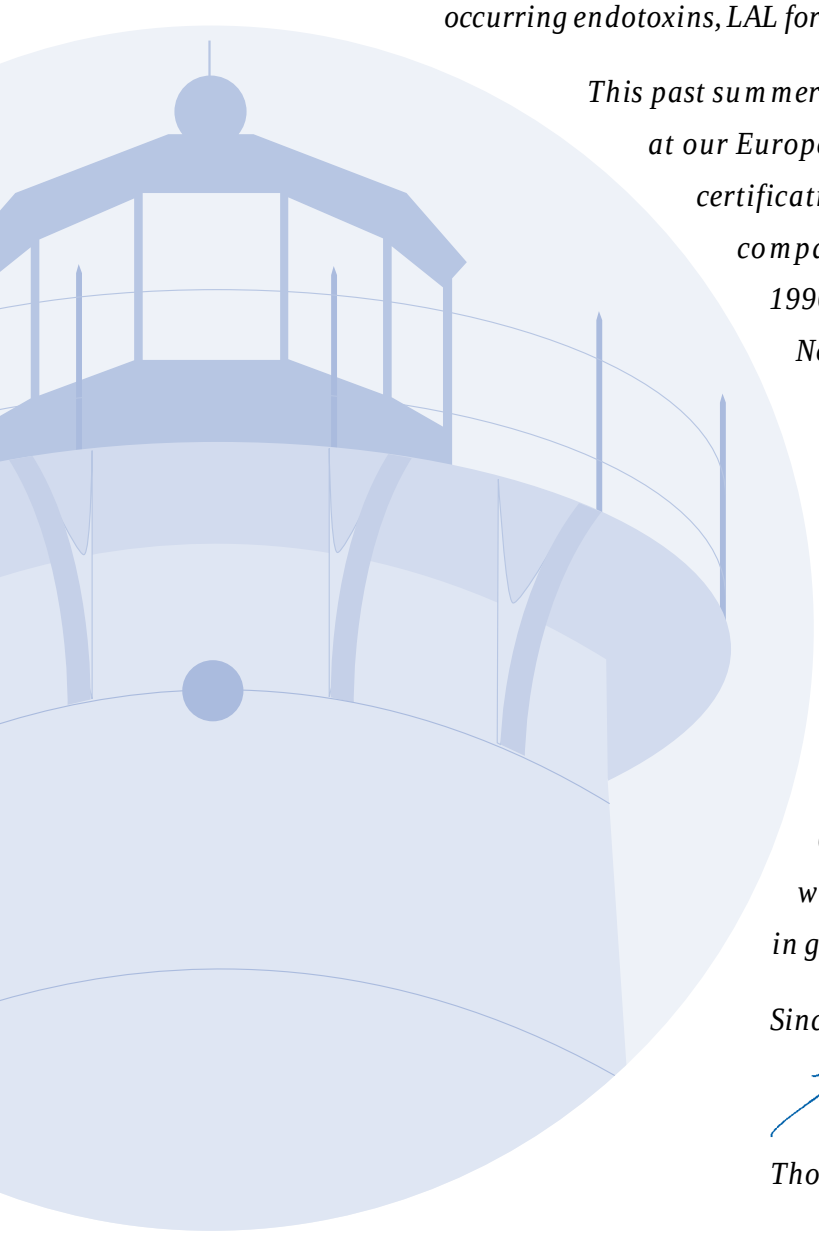
LAL Update®

ASSOCIATES OF CAPE COD INCORPORATED SEPTEMBER 1998 / VOL. 16, NO. 3

Dear LAL User,

This LAL UPDATE begins a three-part series entitled "Endotoxins—Facts and Fiction." The intention of this series is to define the limits of variability of endotoxin so as to better understand LAL results. To accomplish this, I will describe the physical and chemical properties of endotoxin which affect its reactivity with the LAL reagent. In the first installment, ***Endotoxin as a Component of the Bacterial Cell****, I will address the properties of endotoxin related to its production by and association with bacteria in the natural environment. The second installment,* ***The Standard Curve****, will cover the properties of standard endotoxin and the kinetics of LAL. Finally, in* ***Reactivity of LAL with Different Species of Endotoxin****, I will attempt to explain the correlation between standards, naturally occurring endotoxins, LAL formulations, and the pyrogen test.*



This past summer has been a busy one at ACC both here on Cape Cod and at our European offices. On July 3, our UK branch received ISO 9002 certification. This is the final ISO certification for our expanded company as the US facility achieved ISO 9001 on August 30, 1996 and our European Branch was granted ISO 9003 on November 8, 1995.

I am also happy to announce the appointment of Mr. Jack Driscoll as National Accounts Manager for ACC. Jack is a long-time Cape Cod resident who brings to ACC many years of sales and management experience. Jack will be working closely with our Marketing, Technical and Customer Services departments to expand our sales staff and level of service. ACC also welcomes Ms. Gillian Kutcher to our Technical Services staff. Gillian has a strong background in biochemistry and has worked in the pharmaceutical industry. She will specialize in gel-clot applications.

Sincerely,

Thomas J. Novitsky, Ph.D.

Endotoxins
Facts and Fiction

Endotoxin as a Component of the Bacterial Cell

By Thomas J. Novitsky, Ph.D.

TRUE OR FALSE?

Only free endotoxin, i.e. that shed or released from bacteria during growth or after bacterial death (lysis), can react with LAL.

At first glance this looks like an easy question. As students in introductory microbiology learn, endotoxin, as lipopolysaccharide (LPS), is a structural component of the outer membrane of gram-negative bacteria. In its integration into the outer membrane, the lipid A, hydrophobic portion of the LPS is buried in the membrane, while the O-polysaccharide, hydrophilic portion is oriented toward the cell surface and aqueous environment. As students of LAL, we know that it is the lipid A portion of endotoxin which activates the LAL cascade. Thus, LAL should not react with intact bacteria. Even before the discovery of LAL, researchers knew that old, lysed cultures of gram-negative bacteria were a good source of endotoxin. Later it was found that even young cultures of intact bacteria released endotoxin into the culture medium. The term "free endotoxin" was introduced to describe this released LPS. Later it was found that growth conditions or chemical treatment, including antibiotics could affect release. Furthermore, whether endotoxin was released or not and the amount released was also dependent on the species or strain of bacteria being studied.

In 1974, James H. Jorgensen, a pioneer in the study of endotoxin using the LAL assay, published a paper entitled "Measurement of Bound and Free Endotoxin by the Limulus Assay"(1). In this study, Jorgensen attempted to answer a number of questions regarding the activity of endotoxin, but two of his findings are of particular concern here. First, "that a coating of bound endotoxin persists on the cell surface." Second, this coating is relatively constant and allows "approximate quantitation of viable bacilli in fluids using the Limulus assay." Jorgensen could not answer the question of whether the bound endotoxin he measured was necessary for bacterial survival, i.e. still a structural component. He also did not compare LAL measurements of bound endotoxin with those of bound endotoxin that had been mechanically released. We still do not know the answer regarding the nature of the "bound" endotoxin, although it is possible that the LAL reagent acts to release or solubilize endotoxin, since antibiotic activity has been ascribed to LAL and some of its components. We do know however, that cells that are mechanically ruptured release additional endotoxin that cannot be entirely accounted for as "bound."

So, the question was, after all, a trick question. Endotoxin associated with intact bacteria can be measured but the nature of that endotoxin, i.e. whether it is still a structural component or has been released and is just "stuck" to the cell surface, has not yet been determined. The quality assurance analyst therefore should keep in mind: **an LAL test on an unknown solution will measure combined free and bound endotoxin.**

QUESTION:

If the LAL test can measure bound endotoxin, why hasn't the LAL test become a diagnostic for bacterial diseases or a rapid test for bacterial contamination of water?

The answer to this question does not necessarily relate to the fact that LAL can measure bacterial associated endotoxin as much as LAL cannot detect small numbers of bacteria or differentiate between species of endotoxin.

The results of my early studies employing the LAL test as an indirect method for determining bacterial number and biomass yielded excellent results (2). The application of the LAL test in this study however, was well controlled. First, essentially all of the bacteria in the marine environment are gram-negative. Second, the numbers present are quite high, resulting in endotoxin concentrations of 0.04 to 17.8 ng/ml (approximately 0.4 to 178 EU/ml). Third, seawater is an excellent medium for the LAL test. In a later paper, Evans and his colleagues showed good correlation between bacterial numbers in contaminated river water and bound LAL. The fact that coliform counts also

correlated with the LAL results was most likely coincidence (3). Although high total heterotrophic plate counts are often associated with fecal coliforms, this is not always the case (4). On the other hand, where low numbers of bacteria are present, an LAL test would most likely be negative. In these cases, total endotoxin, comprising mostly free LPS, does often correlate with total organic carbon (TOC)(5). Finally, a number of studies have shown that LAL is a good predictor of certain gram-negative infections in humans. Bacteriuria is a good example. Again, this is fortuitous as bacteriureas are mainly due to gram-negative organisms that are present in the urine in high numbers (~ 5 x 10^5 cells/ml). Although in this case the LAL test may seem ideal for screening urine samples, the relatively high cost of the test (where the majority of tests are negative), and its propensity to be easily contaminated have not attracted commercial interest. Other clinical applications for LAL have fared just as poorly. For example, LAL has been proposed as a potential rapid means of indicating sepsis, or at least endotoxemia. Unfortunately, there are often only a few viable circulating bacteria in a septic patient's blood, far too few for the LAL test to detect (the most sensitive LAL can detect about 10 *E. coli*-like bacteria per ml under "ideal" conditions). Second, it would be extremely difficult to separate these bacteria from circulating "free" endotoxin or even from the cellular components of blood itself. Third, circulating endotoxin is often present in blood without evidence of sepsis and therefore "endotoxemia" is poorly defined and may not have clinical utility (6).

Thus, to answer this question, yes the LAL test has been used as a rapid method to detect and enumerate bacteria but it suffers from lack of sensitivity (best level of detection about 10 cells per ml under ideal conditions), and lack of specificity (can't differentiate between species of bacteria). The important implication for the supervisor of water production however is that endotoxin is the result of bacterial growth somewhere along the line. This could be at the water source (well, reservoir, etc), in the piping (biofouling), overloaded deionizers, etc. At any one of these locations, intact, viable bacteria could account for much of the endotoxin measured by LAL, while at others only free endotoxin or bacterial fragments will be present. The value of the LAL test is in understanding and appreciating the nature and associations of the endotoxin measured.

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Additional Reading

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CALENDAR

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Session on "Choosing an

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