LAL Update

ASSOCIATES OF CAPE COD, INCORPORATED • VOLUME 17, NO. 2

Letter From the President

In the final installment of series "Endotoxins—Facts and Fiction," I will cover the effect of formulation and environmental or "natural" endotoxin on LAL reactivity. It is fairly obvious the formulation, i.e., non-LAL components of gel-clot and chromogenic LAL reagents are different, but what about formulations of gel-clot LAL from different manufacturers? After all, the US Food and Drug Administration regulates this product and uses a "standard" LAL to assess the suitability of all licensed LAL manufacture's products. Therefore one might think of LAL as "Ageneric" in the sense of compendial pharmaceuticals. This is not the case however, and LAL products from different manufacturers, even though designed for a particular method, e.g., the gel-clot test, may vary widely with respect to formulation. Does this variability affect reactivity of the various LAL with RSE in water? No, not really. Does variability affect reactivity with naturally occurring endotoxin in water or pharmaceuticals? Unfortunately, yes. This edition of the LAL Update will discuss the issues surrounding LAL formulations and reactivity with naturally occurring endotoxin so the user can make informed decisions regarding the purity of their products under test.

Sincerely,

Thomas J. Novitsky, Ph.D.

LAL Formulation and Environmental Endotoxin

by Thomas J. Novitsky, Ph.D

Even before LAL was licensed by the Food and Drug Administration for use as an alternative to the Pyrogen Test, studies on the non-enzymatic components, i.e., chemicals not directly involved in the LAL cascade, were found to affect not only the sensitivity of the LAL reagent, but also its compatibility with certain products (inhibition/enhancement).[1, 2, 3, 4, 5, 6] In addition, ways of treating the initial LAL extract, either before or after final compounding, also affected sensitivity and compatibility.[7, 8] A good example of the later is the solvent extraction methodology invented by Dr. Stanley Watson, founder of Associates of Cape Cod, Inc. (ACC), and ACC's first director, Dr. James Sullivan.[9] This procedure is still the cornerstone for highly sensitive LAL production at ACC and has been copied by several competitors. Likewise, the addition of mono- and divalent ions plus protein still serves as the basis for ACC's gel-clot products.[3] More recently, formulations have appeared containing buffers to help overcome inhibition when testing samples outside the LAL pH optimum. In at least one published case, the addition of buffer to LAL actually lead to an activation delay (or an increase in sensitivity of the LAL with time after reconstitution).[5] In our experience, most common buffers added to gel-clot LAL formulations prior to lyophilization tend to interfere with LAL activity, solubility, and stability if added in sufficient concentration to effectively control pH in the sample/LAL reaction. We, therefore, provide buffer (PYROSOLTM) as a "reconstitution solution" in order to avoid these problems. **With turbidimetric and chromogenic formulations, added buffers do not seem to cause the same problem and are currently part of these formulations or will appear in newer ones.** A truly buffered gel-clot

continued page 2

reagent, i.e., one with a buffer concentration of 0.1 M, may also be in the offing. It is interesting to note that standardized endotoxin came along after ACC developed its LAL formulation.[10] Thus our development was mainly concerned with detecting naturally occurring endotoxin in products and environmental samples and fortuitously was not constrained by the somewhat artificial "recovery" of standard endotoxin (spikes) from products. This spike recovery of standard from "pure" samples during the validation phase of the LAL test is now de rigeur. During our preliminary reagent development, test samples were biological products and seawater. Since these samples contained plenty of endotoxin, external spikes of "standard" endotoxin were not needed to "see" inhibition/enhancement.[11] Formulations of LAL (not ACC's) using only recovery of CSE or RSE as an indication of a valid test can sometimes miss naturally occurring endotoxin and Lipid A since the formulation was selected for its reactivity to the standard.[12, 13] In addition, since the RSE is not pure endotoxin but also has been formulated by the addition of lactose and PEG, the RSE in a sense becomes a "sample of lactose and PEG."[14] It is difficult to say how these formulation components affect the recovery of spiked RSE in a sample. I suspect in general that they make recovery easier since they were added to the RSE originally to make it more uniform in solution. Interestingly, an earlier RSE, EC-2, which was formulated with serum albumin, was found to be more difficult to recover from saline solutions than pure endotoxin (from the same bulk used to prepare EC-2).

While the variation in LAL formulation may be cause for some concern among users, if the limitations of the reagent are understood, variability should not pose too much of a problem. It is doubtful that the FDA will ever mandate a single formula, since no one could ever agree on which formulation is the best even though the FDA uses a "standard" LAL to assess various manufacturer's batches.[17] For now multiple formulations actually provide a choice to users which, in some cases, can make testing easier. There are several good examples in the literature to illustrate this.[12, 13, 15, 16] While most LAL brands (i.e., formulations) are able to test most compounds, some brands are more compatible with certain products than others. This concept can also be extended to different methods (e.g., gel-clot vs. chromogenic). Thus the user has a opportunity to choose the brand/method which is most compatible (i.e., shows the least inhibition/enhancement) with their product. Compatibility however, should not be confused with always providing a negative result. Since recovery of RSE can be independent of a LAL formulation's ability to detect naturally occurring endotoxin, part of the preliminary evaluation of an LAL should include the reagent's ability to detect low levels of contamination in the product, regardless of the MVD and the ability to recover RSE. If one obtains contradictory results when two different LAL formulations are used to test a sample, it should not be assumed that one of the reagents is giving a false positive. It is quite possible that one reagent is not detecting natural, or environmental, endotoxins. False positives are quite easy to distinguish form the real thing since simple, specific tests are now available for glucans and/or endotoxins.[13, 18, 19] Although the endotoxin and glucan-specific reagents and chemical treatments to differentiate between endotoxin and glucan are not yet approved for routine use by the FDA, they can and should be used to verify results. And regardless of the outcome, LAL-reactive material, including true endotoxin, unless part of the formulation of the product under test, should be regarded as a contaminant and treated accordingly.

References

1. **Sullivan, J. D., Jr., and S.W. Watson.** 1974. Factors affecting the sensitivity of *Limulus* lysate. Appl. Microbiol. **28:1023-1026.**

2. **Valois, F.W.** 1979. Quantitative method for determining less than a pg/ml of LPS, p. 415-422. In E. Cohen (ed.), Biomedical Applications of the Horseshoe Crab (Limulidae),

Progress in Clinical and Biological Research, Vol. 29. Alan R. Liss, Inc. New York.

3. **Sullivan, J.D., Jr., and S.W. Watson.** 1975. Inhibitory effect of heparin on the *Limulus* test for endotoxin. J. Clin. Micobiol. **2:151.**

4. **Tsuji, K., and K.A. Steindler.** 1983. Use of magnesium to increase the sensitivity of *Limulus* amoebocyte lysate for detection of endotoxin. Appl. Environ. Microbiol. **45:1342-1350.**

5. **Bussey, D.M., and K. Tsuji.** 1984. Optimization of a chromogenic *Limulus* amebocyte lysate (LAL) assay for automated endotoxin detection. J. Parenter. Sci. Technol. **38:228-233.**

6. **Dikeman, R.N.** 1982. Process for preparing *Limulus* lysate. United States Patent 4,322,217.

7. **Guilfoyle, D.E., and T. Munson.** 1982. Procedures for improving detection of endotoxin in products found incompatible for direct analysis with *Limulus* amebocyte lysate, p. 79-90. In Endotoxins and their detection with the *Limulus* amebocyte lysate test. Alan R. Liss, Inc. New York.

8. **Steindler, K.A, K. Tsuji, and R.M. Enzinger.** 1981. Potentiating effect of calcium gluconate on the *Limulus* amebocyte lysate (LAL) gelation-endpoint assay for endotoxin. J. Parenter. Sci. Technol. **35:242-247.**

9. **Sullivan, J.D. Jr., and S.W. Watson.** 1978. *Limulus* lysate of improved sensitivity and preparing the same. United States Patent 4,107,077.

10. **Rudbach, J.A. F.I. Akiya, R.J. Elin, H.D. Hochstein, K.R. Thomas, M.K. Luoma, and E.C.B. Milner.** 1976. Preparation and properties of a national reference endotoxin. J. Clin.Microbiol. **3:21-25.**

11. **Watson, S.W., T.J. Novitsky, H.L. Quinby, and F.W. Valois.** 1977. Determination of bacterial number and biomass in the marine environment. Appl. Environ. Microbiol. **33:940-946.**

12. **Takayama, K., N. Quereshi, C.R.H. Raetz, E. Ribi, A.G. Johnson, J. Peterson, J.L. Cantrell, F.C. Pearson, and J. Wiggins.** 1984. Influence of fine structure of lipid A on *Limulus* amebocyte lysate clotting and toxic activities. Infect. Immun. **45:350-355.**

13. **Roslansky, P.F, and T.J. Novitsky**. 1991. Sensitivity of *Limulus* amebocyte lysate (LAL) to LAL-reactive glucans. J. Clin. Microbiol. **29:2477-2483.**

14. **Hochstein, H.D., D.F. Mills, A.S. Outschoorn, and S.C. Rastogi.** 1983. The processing and collaborative assay of a reference endotoxin. J. Biol. Stand. **11:251-260.**

15. **Wachtel, R.E., and K. Tsuji.** 1977. Comparison of *Limulus* amebocyte lysates and correlation with the United States Pharmacopeial pyrogen test. Appl. Environ. Microbiol. **33:1265-1269.**

16. **Twohy, C.W., M.L. Nierman, A.P. Duran, and T.E. Munson.** 1983. Comparison of *Limulus* amebocyte lysates from different manufacturers. J. Parenter. Sci. Technol. **37:93-96.**

17. **Rastogi, S.C., E.B. Seligmann, Jr., H.D. Hochstein, J.H. Dawson, L.G. Farag, and R.E. Marquina.** 1979. Appl. Environ. Microbiol. **38:911-915.**

18. **Obayashi, T., H. Tamura, S. Tanaka, M. Ohki, S. Takahashi, M. Arai, M. Masuda, and T. Kawai.** 1985. A new chromogenic endotoxin-specific assay using recombined *Limulus* coagulation enzymes and its clinical applications. Clinica. Chimica. Acta. **149:55-65.**

19. **Aketagawa, J., H. Tamura, and S. Tanaka.** 1994. Measurement of (1-->)-b-D-Glucan using Gluspecy (G-Test). published from Third Glucan Inhalation Toxicity Workshop. Rylander, R. and Goto, H. Kompendiet, Goteberg:Committee on Organic Dusts, ICOH. **1/94:4-17.**

CALENDAR OF EVENTS

OCTOBER

October 24 – October 27
Society for Neuroscience
Miami Beach Convention Center
Booth #860

October 27 – October 28
ASM–NE Branch
Worcester Centrum Center

NOVEMBER

November 30 – December 2
PDA
Marriott Wardman Park Hotel, Washington DC
Booth #209

DECEMBER

December 11 – December 15
ASCB
Washington Convention Center
Booth #537

For customer service:
call (800) LAL–TEST or (508) 540–3444.

For technical service:
call (800) 848–3248 or (508) 540–3444.

Please visit our website! www.acciusa.com

Printed on recycled paper.

ASSOCIATES OF CAPE COD INCORPORATED
Creating New Horizons in Endotoxin Testing
704 Main Street ▪ Falmouth, MA 02540

LAL Update®
VOLUME 17, NO. 2