LAL Update

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Letter From the President

This issue of the UPDATE is the third installment in a series on endotoxins Facts and Fiction. My original idea was for three parts. As the series progressed, however, I found there was too much information to adequately cover so I have expanded the series. Therefore, this issue Reaction of LAL with Different Species of Endotoxin will cover only the selection of a standard and species reactivity. The next UPDATE will conclude the series with Effect of LAL formulation on Reactivity and Environmental Endotoxin.

Perhaps the most important aspect of LAL testing following its discovery was the selection of a standard. Although this may seem trivial, the initial selection of a suitable standard spanned a number of years and elicited more than a little controversy. Even today, with a move toward harmonization of compendial tests, a suitable standard for the LAL test may be the last stumbling block.

Sincerely,

Thomas J. Novitsky, Ph.D.

Selection of the Standard

by Thomas J. Novitsky, Ph.D.

The FDA's first standard and the one that I was introduced to along with my introduction to LAL were prepared from Klebsiella puemoniae 1B under the auspices of the National Center for Antibiotic and Insulin Analysis. At the time, this was probably the best characterized and available purified LPS. Furthermore it had been examined expressly for use as a pyrogen standard.[1, 2] The source of this bacterium, a supposedly sterile solution of streptomycin, was somewhat surprising, as the genus Klebsiella was not a common contaminant of parenteral drugs. In fact, this was one of the criticisms for using 1B as a pyrogen standard for pharmaceutical preparations. Also at this time, the FDA's Bureau of Biologics (BoB) was investigating the use of LAL as an alternative to the Pyrogen Test. At this point in history, BoB was an integral part of the NIH which was very much research oriented. It was no surprise, therefore, that NIH experts on endotoxin and endotoxin assays, such as Ron Elin and K.C. Milner, were working closely with BoB's Don Hochstein to find an endotoxin standard. Although one could argue the merits of the 1B standard (it actually was a good pyrogen), the FDA had very little of this standard and was interested in supplying pharmaceutical manufacturers, researchers, and ultimately LAL manufacturers with enough material to standardize LAL and other bioassays for endotoxin. They also reasoned that a standard should have moderate activity with both LAL and the rabbit (Pyrogen Test), was relatively easy to produce consistently, and was well characterized and easy to analyze chemically. It could be said that the 1B standard was not ideal on all counts, especially since Klebsiella produced a capsule that could compound the purification of LPS. Thus BoB contracted with Ron Elin's colleague, Tony Rudbach at the University of Montana to produce a purified LPS standard from *Escherichia coli* O113:H10:K negative. Fortunately, Don Hochstein survived the winter trek (with skiing on the side)

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from Bethesda to Missoula to personally (with Elin) deliver the strain *E. coli* to Rudbach and initiate the contract. Two papers describe this standard in detail and also address the FDA s (and Rudbach's) reasoning for its selection.[3,4] Basically, an *E. coli* LPS falls in the middle of range of reactivity (LAL and Pyrogen) for a variety of LPS species. In addition, the O113:H10:K negative LPS's O antigen was well characterized and was found not to contain any dideoxyhexoses (e.g. colitose) as do some other LPS. This facilitated a strictly chemical assay for KDO (any dideoxyhexoses present in the O antigen would interfere with the thiobarbituric acid assay for KDO) as an independent marker for concentration/purity. Finally, the FDA chose to use the phenol-water extraction procedure for LPS as described by Westphal et al.[5] This ensured a highly purified, stable, relatively soluble LPS free of associated proteins and other bacterial components.

Although BoB was successful with the Rudbach contract and a well-characterized *E. coli* LPS standard resulted (the EC series is still used as the FDA's standard as well as the USP and EP standard), it was not readily accepted by industry or by other branches of the FDA. A competing standard adopted by the Health Industries Manufacturers Association (HIMA) and the FDA's Office of Medical Devices was *E. coli* O55:B5 manufactured in bulk by Difco Laboratories. This standard was also well characterized, and readily available. Probably because BoB was the lead agency for the manufacture of LAL and supplied the LPS standard used to set label claim on all released LAL products, the O113 standard was selected when the FDA issued a single guideline (all offices of FDA agreeing) for LAL use. To provide some degree of continuity, a large collaborative study, under the auspices of HIMA was undertaken to compare the original lot of O55:B5 to O113. Fortuitously, there was little difference found between the two.[6] Thus the EC standard, already chosen by the FDA and USP as the Reference Standard provided a good level of comfort for control of the LAL test.

During the debate on selection of a reference standard, the issue of using other standards for the day-to-day control of the test, the Control Standard Endotoxin (CSE), became an issue in its own right. After selection of O113 as the Reference Standard (RSE), O55:B5 became by default a CSE and continued to be used by device manufacturers. LAL manufacturers had also traditionally provided their customers with control standards. Some used O55:B5, others an LPS from the pathogenic *E. coli* O111:B4. In Germany, the highly purified LPS from Salmonella abortus equi prepared by Hermal Chemie GmbH (and later in the laboratory of Chris Galanos) was in common use and was actually used as the EP reference standard for many years (NP series). Associates of Cape Cod was extremely fortunate to obtain from Dr. Rudbach some of the excess O113 LPS produced for the FDA for use as their CSE. We still use this strain of LPS for most of its CSE products. We were also fortunate to obtain through our German subsidiary, Pyroquant Diagnostik, a supply of the S. abortus equi LPS. Actually it was Pyroquant that supplied the NP for use as the EP standard.

At one of the popular Endotoxin conferences held in Woods Hole, Massachusetts in September 1981, discussions on the most appropriate standard for pharmaceutical quality assurance applications became quite lively.[7] It was pointed out that early use and promise for the LAL test was as a clinical diagnostic. For that purpose of course an LPS from Klebsiella, Salmonella, or various *E. coli* would be very appropriate. Contaminants of pharmaceuticals however were more likely to be psuedomonads or some other common water system contaminant. It is interesting to note that the 1B standard, initially criticized as not representative of a common pharmaceutical contaminant was replaced with an *E. coli* standard. At the extreme end of reasoning, one participant even suggested the FDA use tap water as a standard. Fortunately no one took this seriously. Apart from the simple fact that tap water varies tremendously in space and time with respect to its bacteriology, the glucan and inorganic ion component would have wreaked havoc on existing and future LAL formulations. The better alternative would have been a purified pseudomonas LPS made up in WFI. As it is, the ideal of a chemically and biologically well characterized LPS of relatively modest reactivity with LAL and the rabbit (all indications are that LPS from pseudomonas species are generally less reactive on a weight basis than LPS from *E.coli*) is correct.

Relative Reactivity

One of the distinguishing features of gram-negative bacteria is that they generally possess some form of lipopolysaccharide in their outer membrane. These range from the very active (in the rabbit and LAL assay) lipooligosaccharides of Neiserria species,

to the essentially non-reactive LPS of cyanobacteria and some other photobacteria. *E. coli* falls somewhere in the middle. A very nice paper was presented by Rudbach that characterized the relative reactivity of a number of LPSs with the LAL assay 4. Others have also compared reactivity in other bioassays, especially the Pyrogen test, as well as compared the Pyrogen test to the LAL test.[8, 9] It is now generally agreed that the limits of endotoxin contamination selected based on pyrogenicity and LAL reactivity using the current *E. coli* standard or ones of similar (EU) reactivity, provide an adequate margin of safety. The fact that there have always been exceptions to a perfect correlation between LAL reactivity and pyrogenicity does not seem to arouse as much concern these days as it did in the 80s. This is not to say that this problem has been forgotten. We know a lot more now both about LPS and the LAL assay. For example, the biochemistry of the LAL reagent and its influence by physical and chemical factors is now so well known that it is unusual now to be stymied by an unexpected negative or positive result. For example, the role of glucans in activating the LAL cascade test is now well understood (although the FDA is still not sure how to handle glucan contamination of pharmaceuticals and endotoxin-specific LAL s are only research-only at the moment). The role of certain additives that affect LAL activity is also better understood. One compound in particular, Zwittergent, originally added to LAL to make the reagent more sensitive to standard endotoxin, is now known to inhibit factor G and also make Lipid A more difficult to detect. While lack of reactivity with glucans (i.e., fungal, plant, or other process contaminants) may be desirable to some pharmaceutical manufacturers, lack of reactivity with Lipid A, the portion of endotoxin responsible for toxicity, including pyrogenicity and LAL reactivity, cannot be considered desirable under any circumstance. That LAL would not react with pure Lipid A at first seems like a contradiction until it is understood that Lipid A by itself is rather insoluble. In the presence of Zwittergent, Lipid A is most likely made more insoluble and unreactive with LAL. On the other hand, standard endotoxin, rich in O-antigen, is extremely soluble to begin with and is probably further disagregated by Zwittergent becoming even more reactive with LAL. Since Lipid A and Lipid A-rich endotoxins are more likely to occur in naturally contaminated solutions, the chance of missing these when using Zwittergent containing LAL is high even though spike recovery (O-antigen rich endotoxin) appears normal.[10] Finally, physical and chemical factors that influence the LAL reagent per se (e.g. ions, salts, viscosity, agents that bind endotoxin, new therapeutic chemical entities, etc.), may also directly affect the confirmation, and hence the reactivity of the LPS molecule. The lesson here for pharmaceutical manufacturers is that what we see in the laboratory may not be what happens in a sick animal or human when treated with formulated drugs containing an unusual form of endotoxin or a combination of endotoxin and glucan. Fortunately, the research on new formulations of LAL and other endotoxin assays continues and with it an accompanying increase in the purity and safety of modern pharmaceuticals.

References

1. **Castor, G.B., Kantor, N., Knoll, E., Blakely, J., and Kirshbaum, A.** Characteristics of highly purified pyrogenic lipopolysaccharide. J.Pharm.Sci. **60:1578-1580, 1971**.

2. **Selzer, G.B.** Preparation of a purified lipopolysaccharide for pyrogen testing. Bull.Parenter.Drug Assoc. **24:153-156, 1970**.

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

4. **Firca, J.R. and Rudbach, J.A.** Reference Endotoxin: A practical rationale. In: Endotoxins and their detection with the limulus amebocyte lysate test, edited by Watson, S.W., Levin, J., and Novitsky, T.J. New York, NY: Alan R. Liss, Inc. **1982, p. 121-130**.

5. **Westphal, O., Lüderitz, O., and Bister, F. Über** die Extraktion von Bakterien mit Phenol / Wasser. Z. Naturforsch. **7:148-155. 1952**.

6. **Pearson, F.C., Weary, M., Sargent, H.E., Novitsky, T.J., Winegar, M.P., Lin, H., Lindsay, G., Berzofsky, R.N., Lane, A.L., Wilson, J.D., Cooper, J.F., Helme, E.J., Twohy, C.W., Basch, H.I., Rech, M., and Slade, J.W.** Comparison of several control standard endotoxins to the national reference standard endotoxin — an HIMA collaborative study. Appl.Environ.Microbiol. **50:91-93, 1985**.

7. **Progress in Clinical and Biological Research, Volume 93**: Endotoxins and their detection with the Limulus amebocyte lysate test, edited by Watson, S.W., Levin, J., and Novitsky, T.J. New York: Alan R. Liss, Inc. **1982**.

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8. **Weary, M., Pearson, F.C., Bohon, J., and Donohue, G.** The activity of various endotoxins in the USP rabbit test and in three different LAL tests. In: Endotoxins and their detection with the Limulus amebocyte lysate test, edited by Watson, S.W., Levin, J., and Novitsky, T.J. New York: Alan R. Liss, Inc. **1982, p. 365-379**.

9. **Rastogi, S.C.** Statistical procedure for standardizing control standard endotoxin against U.S. reference standard endotoxin using Limulus Amebocyte Lysate test. In: Endotoxins and their detection with the Limulus amebocyte lysate test, edited by Watson, S.W., Levin, J., and Novitsky, T.J. New York: Alan R. Liss, Inc. **1982, p. 49-54**.

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

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