The Bacterial Endotoxins Test – Back to the Future

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The fifteenth anniversary of American Pharmaceutical Review prompted my reflection on the 40+ years of evolution of the Limulus Amebocyte Lysate (LAL) test in our industry. This article will reflect on where the Bacterial Endotoxins Test (BET) was 15 years ago and predict what the future holds for the next 15.

The Past 15 Years

15 years ago, the test was referred to as the LAL test. Today, the compendial name for the assay is the Bacterial Endotoxins Test, or BET.

15 years ago, the gel clot test was the most commonly used test, although endpoint and kinetic assays were becoming widely accepted for routine release tests. Today, quantitative tests are used more often than gel clot across the industry, but the gel clot limits test still remains the referee test in the harmonized compendial chapter.

15 years ago, we had the choice of running quantitative assays on microtiter plate readers and tube readers. Today, these instruments have become more sophisticated and are supported by Part 11 compliant software that interfaces with laboratory information management systems (LIMS) and tracks and trends data. In addition, we have the option of a cartridge system, which is a convenient choice for many applications.

15 years ago, all reagents used for testing were derived directly from the circulating blood cells of the horseshoe crab. Today, we have alternatives. We can choose a reagent formulated with recombinant coagulogen, the clotting protein in the LAL reaction. This reagent, and future reagents that do not rely on bleeding horseshoe crabs will increase consistency in testing and will help ease concerns regarding pressure on the horseshoe crab population. In addition to Limulus-based reagents, we have the Monocyte Activation Test, an assay-based reaction of human blood to endotoxin that more closely mimics the action of endotoxin in humans.

15 years ago, the FDA's 1987 "Guideline on Validation of the *Limulus* Amebocyte Lysate Test as an End-Product Endotoxin Test for Human and Animal Parenteral Drugs, Biological Products, and Medical Devices" provided an Out of Specification (OOS) investigation scheme that included an immediate retest of twice the number of original replicates and a second retest of 10 units tested individually. Since then, FDA released its 2006, "Guidance for Industry: Investigating Out-of-Specification (OOS) Test Results for Pharmaceutical Production", which requires an investigation to justify a retest. However, footnote 3 in that Guidance indicates that the document is not intended to address biological assays. So, analysts and managers are left to decide: is the BET an analytical assay or a biological assay? There is still some debate, but the Agency's philosophy is clear – a retest without justification will be questioned.

15 years ago, we saw a different pattern of reagent consumption than we do today. Interestingly, when the LAL test was first introduced in the early 1970s, the focus was on raw material and in process testing, largely because it unclear that FDA would ever abandon the compendial Rabbit Pyrogen Test for release of finished pharmaceuticals and devices. After an exemplary collaboration between industry and the Agency, and the subsequent publication of the 1987 Guideline, the focus shifted to end product testing and replacement of the rabbit test. As test methods and data

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collection methods have matured, and consistent with the basic tenants of Good Manufacturing Practice, more reagent is currently used to monitor water systems, raw materials and processes than is used to test finished product. Today, we realize the benefits of thinking about product lifecycles so that we can identify critical control points for endotoxin in raw materials, equipment, and process during the development phase, well before the product goes commercial. We understand that we can't test quality into the product, but we can monitor quality of the product throughout the process.

This test has served patients remarkably well for the last 40 years, and we (including countless numbers of New Zealand White rabbits) are in debt to the scientists in FDA and industry who researched, realized, regulated and commercialized BET assays. Over the years, we have made the assay convenient for people to use so that we can continue to ensure patient safety. In spite of the many changes in the value of testing, the BET community is still faced with some challenges going forward.

"Food for Thought" – The Next 15 Years

With the advent of more complex drug product formulations, particularly in biologics, and with the increasing number of therapeutic and administration options (time release, nanoparticles, combination drugs), the BET community will be faced with new challenges to testing. We will likely find more formulations that interfere with the BET assay, meaning that we will have to not only understand the product formulation, but the science of the LAL reaction to determine the cause of the interference and the appropriate mitigations.

We know that the variability in microbiological assays is significantly higher than for standard analytical assays. Is the BET assay an analytical assay because it has a computer, a standard curve, and a result that reports out to 4-5 significant figures or is it a biological assay with a considerable error? We need to understand the scientific and mathematical limitations of the assay to understand the sources and implications of variability.

Until recently, the focus of the BET assay has been endotoxin as a Pyrogen (fever causing agent). The underlying studies to support the current threshold pyrogenic dose were conducted by looking at temperature rise after injection of metered levels of an endotoxin standard. We know, however, that endotoxin can result in a variety of clinical manifestations, not the least of which is inflammation. Should we be concerned about threshold inflammatory dose? If so, how does that impact on different routes of administration including inhalation, intraocular, intraperitoneal or topical application?

The use of an endotoxin standard has served us well for the last 40 years, yet endotoxin standards do not exist in nature. The endotoxin that contaminates our product is different than the Reference Standard Endotoxin (the primary standard, or RSE) or the Control Standard Endotoxin (secondary standard, or CSE) that we use to prepare positive product controls and standard curves. We know that the product matrix can affect endotoxin aggregation and recovery of purified endotoxin, so when is it appropriate to use a natural endotoxin in recovery studies? How do we justify such use, and how do we prepare those "natural" standards? Is an endotoxin standard necessarily the best to use for depyrogenation studies? Is the current requirement for a 3 log reduction of purified endotoxin standard the best indicator of depyrogenation, or is it more practical to validate the reduction of naturally occurring levels of endotoxin to levels that are safe for patients?

With endotoxin, as with all tests, we are challenged to make the best use of the data. Because of variability, the accuracy of and information provided by any single test result is not as great as the trend drawn from a series of data points. Process control is all about trends and connections to other systems or actions taken by the company. With the implementation of more formal process control programs in our industry, we need to continue to explore benefits of trends and assure that we use this valuable tool to our best advantage.

Indeed, we have come a long way technically, but the more difficult tasks of understanding the information that we get from assays and acting on it will continue to confront us. As an industry, we will meet the challenge to make the best of the tools that have been provided to assure process control and patient safety.

Congratulations and thank you to *American Pharmaceutical Review* for fifteen years of providing those of us who manufacture and test pharmaceutical products with information on the latest in technological innovation and compliance.