LER Frequently Asked Questions

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Since the initial reports of Low Endotoxin Recovery (LER) in 2013 drug manufacturers and regulators have voiced concern that there may be conditions under which the Bacterial Endotoxins Test (BET) could under-report endotoxin activity in biological drug products (Hughes, et al, 2015). Clearly, this potential risk to patient safety merits further investigation and discussion.

What is LER?

While conducting a hold time study on a monoclonal antibody formulated in a chelating buffer and polysorbate ("LER matrix"), Joseph Chen and Anders Vinther from Genentech/Roche reported a temperature dependent, irreversible, almost instantaneous and reproducible decline in the Limulus Amebocyte Lysate (LAL) reactivity to Control Standard Endotoxin (CSE) after its addition to the test sample (Chen and Vinther, 2013.) They coined the phrase, "Low Endotoxin Recovery" or LER to describe this phenomenon. LER has been replicated by a number of researchers including Bolden, et al (2014), Platco (2014), Burgenson (2014) and Dubczak (2014). No one disputes that low recovery of CSE activity in this type of formulation is a real phenomenon.

Interestingly, these same researchers all also report that there is a lesser decline or in some cases, no decline in recovery when endotoxin is used instead of CSE as the analyte "spike" in the hold time experiments using LAL reagent for detection of activity. A summary of these data may be found in Bolden, et al (2015).

I'm confused. Isn't CSE the same as Endotoxin?

Unfortunately, the terms, "endotoxin" and "CSE" are often used interchangeably, but they technically are not the same.

Endotoxins are components of the outer cell membranes of Gram-negative bacteria. Endotoxins exist as vesicles containing lipopolysaccharide (LPS) molecules surrounded by surface proteins, lipoproteins and phospholipids. Cell wall fragments (dead bug parts) that are shed either as part of the normal bacterial life cycle or by other processes that disrupt cells, are the real-life contaminants in pharmaceutical raw materials, water systems, in process samples, and finished drug products. Some researchers use the term, "naturally occurring endotoxin" or NOE to distinguish endotoxin from LPS.

Lipopolysaccharide (LPS) is the biologically active component of the endotoxin complex. The nature of this amphipathic molecule is such that the hydrophobic Lipid A portion is embedded within the outer cell membrane, and the hydrophilic O-specific polysaccharide is exposed to the extra-cellular environment. Purification of LPS from the Gram-negative cell membrane is a harsh process, requiring extraction in phenol/water or phenol/chloroform/petroleum ether. As a purified preparation, the amphipathic "naked" LPS will form micelles, ribbons, and other aggregate forms in solution. The extent of the aggregation is largely dependent on the chemistry of the matrix in which the LPS resides.

What adds to the confusion in the LER conversation is that the compendial Reference Standard Endotoxin (RSE) and purchased Control Standard Endotoxins (CSE) are, in fact, **NOT** endotoxin, but rather purified LPS. The RSE and most CSEs are further formulated with stabilizers and other excipients to enhance their shelf life (Poole, 1997). RSE and CSE preparations are used as **calibration and test standards** in BET tests and assays for assigning label claim sensitivity, creating standard

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curves, performing compendial suitability (inhibition/enhancement) studies, and preparing positive product controls (PPC) for routine testing.

While RSE and CSE are convenient for use as analytes in a variety of experiments including hold time studies, the use of LPS does not mimic a naturally contaminated product, because formulated LPS does not exist in nature. LPS and endotoxin are physically, structurally and chemically very different, and we cannot reasonably expect that they will act or react identically, particularly in complex pharmaceutical formulations. Many current publications describing LER experiments do not make the distinction between LPS and endotoxin, leading the reader to surmise that the phenomenon equally applies to both endotoxin and LPS.

I've conducted suitability (inhibition/enhancement) testing on all of my products with no problems. Does that mean that my product doesn't exhibit LER?

Not necessarily. USP <85>, "Bacterial Endotoxins Test" requires that the analyst prepare the sample to a non-interfering state, generally through dilution in Water for BET, and demonstrate that a nominal level of CSE activity can be quantitatively recovered from the **prepared** test sample. There is no requirement or expectation that CSE be recovered in undiluted product (USP, 2015a). In fact, published studies by FDA and other laboratories have consistently shown that CSE is generally not quantitatively recovered from undiluted product (Twohy, et al., 1983; Twohy, et al., 1984; McCullough, 1990; McCullough and Weidner-Loeven, 1992.) If your formulation is consistent with the LER matrix, it is likely to exhibit a typical LER response in a hold time study with a CSE or RSE spike.

So, is LER really another type of test interference?

Yes. There are two types of test interferences in the BET: enhancement and inhibition. Enhancement describes the potentiating effect that some substances have on the assay such that the activity of an added CSE spike is over-estimated; activity added < activity detected. For example, 1-3 β D glucan is a material that can enhance the activity detected in an LAL test.

By far the more common form of interference is inhibition, where the activity of an added CSE spike is under-estimated; activity added > activity detected. There are many sources of inhibition, but they generally fall into two classes: conditions that reduce the efficiency of the LAL reagent to detect activity, and conditions that affect the CSE/LPS spike such that activity of the LPS itself is masked or diminished (Cooper, 1980; Dubczak, 2011).

What causes LER?

Although the precise mechanism of the LER matrix interference is unknown, there are a number of theories advanced by industry researchers:

- Because LPS is negatively charged at pH >2, one possibility is that binding between the CSE and a cationic (positively charged) protein could "mask" the effect of the LPS and reduce it's detectability. (Chen and Vinther, 2013; Williams, 2014)
- The LAL reagent needs divalent cations to proceed through the reaction cascade. Sequestration of these divalent cations by chelators will reduce the reagent's ability to detect activity. However, Chen reported that the addition of Mg^{++} did little to restore activity in his LER condition, so clearly there is something else that also contributes to the LER effect (Chen and Vinther, 2013).
- The literature has shown that the use of a detergent or dispersing agent such as polysorbate, sodium desoxycholate (DOC) or sodium lauryl sulfate (SLS) will disassociate LPS micelles into smaller complexes or even monomers, and once the monomeric state is achieved, LPS biological activity diminishes to the point where it may become undetectable. Depending on the detergent or dispersing agent and the formulation matrix, micelles may re-form after the agent is removed, restoring activity. (Ribi, et al, 1966; Hannecart-Pokorni, et al, 1973; Tsuchiya, 2014; Reich, et al, 2014)
- Petsch and Anspach (2000) demonstrated that the combination of removal of divalent cations by chelating agents and the addition of detergents drove the aggregation of the LPS micelles toward a biologically non-reactive monomeric state.

The ultimate LER mechanism is most likely a combination of factors, with some dependence on the type and concentration of the chelator, the type and concentration of the polysorbate, and the ionic nature of the protein.

You have suggested that many researchers see LER with CSE but not with endotoxin. How is that possible?

The association of endotoxin's LPS with other cell wall components may make it less inclined to form the same types of aggregates and conformations as well as monomer generation typically seen with CSE. This difference in structure and chemistry could help to shield the LPS in endotoxin from effects of the LER matrix.

Can I make my own endotoxin in the laboratory?

Yes. However, there is no standard way to prepare laboratory-derived endotoxin, so analysts need to be clear and consistent in their methodologies.

- Bowers and Tran (2011) prepared laboratory-derived endotoxin from a number of species by growing the organisms in Tryptic Soy Broth (TSB), centrifuging the cells down and filtering the supernatant through a 0.45µm filter.

Given the rate with which the LER effect can happen, experimental design must be very precise in timing, as the LER effect continues until the sample is tested.

- Dubczak utilizes an ATCC strain of *Enterobacter* cloacae and subcultures it in decreasing concentrations of nutrient broth until it's starved (1.0% nutrient broth). At that point, endotoxin is harvested by filtering through a series of two 0.22µm filters (Dubczak, 2014)

In both cases, the researchers report that their preparations are remarkably stable, much more so than LPS in water. Differences in organisms, culture conditions and whole cell removal methods will likely impact yield. As with any process, it's important to develop the methodology, properly document and control the method, and characterize the product of that method.

How would one characterize a laboratory-derived endotoxin?

Some guidance for characterization is provided in USP XXI, <85>, "Bacterial Endotoxins Test" that was referenced in the withdrawn 1987 FDA Guideline (USP 1980). USP said,

"If a CSE is a preparation not already adequately characterized, its evaluation should include characterizing parameters both for endotoxin quality and performance (such as reaction in the rabbit), and for suitability of the material to serve as a reference (such as uniformity and stability). Detailed procedures for its weighing and/or constitution and use to assure consistency in performance should also be included."

USP spoke to assessing the preparation's stability, performance, uniformity and consistency, and while it described performance using the USP Pyrogen Test (USP 2015a) as an example, we understand now that the variability in the rabbit test such that it may not be the best predictor of performance. A more current and technologically controllable performance assessment might require that the laboratory-derived endotoxin exhibit the same standard curve attributes (onset times, slope, y-intercept) as the RSE or CSE standard.

What's the difference between activity and potency?

The difference between activity and potency also references back to USP XXI and the 1987 FDA Guideline. The Endotoxin Unit (EU) is the unit of measure of biological **activity** in BET (ability to initiate the LAL cascade) and rabbit assays (ability to elicit a fever response). Activity is expressed as a concentration, typically EU/mL., or in the case or RSE, EU/vial.

Purchased CSEs are provided as lyophilized preparations that are filled by weight, typically ng/vial. **Potency** is defined as the **specific activity** of the CSE, meaning activity/unit weight, typically EU/ng. Determination of potency as reported on the Certificate of Analysis provided by the lysate vendor, is a way to relate units of **weight** of the reconstituted CSE (ng/mL) to units of **activity** of the reconstituted RSE (EU/mL) to derive a **potency** of the CSE in EU/ng. Since laboratory-derived endotoxins are not powders or lyophilized preparations, we cannot calculate a potency as currently defined, but they do have activity as quantitated by reference to an RSE standard curve or standard series. Although CSEs may have different **potencies**, neither the LAL chemistry nor the rabbit pyrogen test is capable of discriminating between the **activity** of LPS or endotoxins prepared by different methods or from different organisms. 1 EU = 1 EU.

Does LER affect pyrogenicity in rabbits?

Concurrent rabbit pyrogen tests and LAL assays conducted by Dubczak using laboratory derived endotoxin from various Gram-negative bacteria and LPS in the form or RSE or CSE demonstrate that under LER conditions there is a correlation between LAL reactivity and pyrogenicity using both CSE and endotoxin under LER conditions (Dubczak, 2014). However, these data are in conflict with Chen's rabbit test data using CSE that suggest a positive rabbit test and a negative LAL test – a false negative (Chen and Vinther, 2013). Conflicting data are always troublesome, but variability in terms of rabbit colonies, individual rabbits, and test conditions may contribute to these results and call into question the utility of a three rabbit Pyrogen test as an arbiter of conflicting results (Dabbah, et al, 1980; Hochstein, et al, 1983; Pearson, et al., 1985).

Can experimental design influence my data?

As with any laboratory experiment, the test parameters and acceptance criteria must be clearly defined.

- Bolden (2014) reported that variability in the y-intercept of the laboratory's standard curves impacted test results and enhanced the observed LER effect.
- Design of the spiking method and subsequent calculation can also impact results. For example, if a study requires a nominal (assumed) spike of 5 EU/mL, yet **measures** 1 EU post hold, is there really an 80% reduction? Since the initial spike was not quantitated, the calculation may be inaccurate. Always measure, never assume.
- Given the rate with which the LER effect can happen, experimental design must be very precise in timing, as the LER effect continues until the sample is tested. For example, when conducting concurrent rabbit and BET assays, the animal injection and BET inoculation must happen simultaneously, as the test results may be biased if the BET assay is inoculated either before or after the injection.
- Dubczak's data suggest that there is some variability in the ability of different methodologies (LAL, ligand, Monocyte Activation Test) to detect endotoxin or LPS activity (Dubczak 2014). Understanding the limitations of any endotoxin or CSE detection method is essential to good experimental design.

Does LER impact the safety of the drug supply? Is LAL a good predictor of endotoxin contamination?

The BET has been the safety test of choice since the early 1980s, replacing the rabbit pyrogen test for drugs, biologics, and devices. The same LER

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matrix has been in use in biologics since at least 1987, 28 years ago. FDA's recall data from 2004-2015 for non-device biologics indicate that there have been nineteen (19) recalls due to OOS test results, and five (5) recalls due to safety issues. Further examination of the actual recall notices confirms that none of these recalls was associated with assumed (reported ADE fever responses) or confirmed endotoxin contamination (FDA, 2015).

Conclusion

A good outcome of the investigation of the LER phenomenon is a greater appreciation amongst industry researchers and regulators of the differences between LPS (RSE and CSE) and endotoxin. While the lack of recovery of LPS under LER conditions is a problem that clearly merits further investigation, FDA's data on biologics recalls coupled with published experimental data collected to date indicate that there is no current evidence of related safety issues in these formulations. Published data to date suggest that when an LER condition is experienced, it is reasonable to execute experiments with a well characterized laboratory derived endotoxin before reverting into highly variable rabbit testing and/or requiring mitigation the LER/CSE issue in every biologic formulated with platform buffering solutions. For next steps, we need to better understand the implications of the LER environment, devise a prudent path forward, and endorse the use of a laboratory derived endotoxin as a pragmatic and scientifically appropriate option for testing, as it is a more representative surrogate for contaminated product.

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