"Fit For Purpose" Endotoxin Analytes

Karen Zink McCullough

MMI Associates

One of the essential but as yet unresolved questions arising from USP's June 2019 "Workshop on the Future of Endotoxins and Pyrogen Testing: Reference Standards and Procedures" is the issue of an appropriate "fit for purpose" endotoxin analyte to be used in non compendial spike/recovery experiments. The opinions expressed by some follow traditional analytical thinking and require the use of the purest analyte possible, meaning the USP reference endotoxin standard (RSE) or secondary standards (CSE) both of which are extracted, purified and formulated lipopolysaccharide (LPS). However, the evolving understanding of the complexity of the Gram-negative (GN) cell membrane, the variety of fine structures of GN lipopolysaccharides, the role of GN outer membrane proteins in mammalian biological response mechanisms, and the very quick adaptation and LPS remodeling by GN organisms in response to changes in their environments (Bonnington and Keuhn, 2016) have renewed the "fit for use" discussion.

Gram-negative LPS is a highly anionic amphipathic molecule that comprises the majority of the outer leaflet of the outer cell membrane. The hydrophobic Lipid A segment of the LPS molecule, which is also the biologically active portion of LPS, anchors the molecule in the outer leaflet of the outer membrane. The hydrophilic, repeating polysaccharide O-antigen portion of the molecule extends into the cell's external environment. Figure 1 is a graphic representation of the GN cell envelope.

The USP Reference Endotoxin Standard (RSE) was originally prepared with the intent of providing a calibrator for reagents offered by various lysate manufacturers. RSE is carefully prepared from *E. coli* 0113:H10K with a well documented pedigree, a carefully detailed protocol, a well defined growth medium, a precise purification process using the hot phenol Westphal extraction method, and a strict formulation recipe for stability consisting of polyethylene glycol and lactose (Rudbach, 1976; Poole, 1997). *E. coli* was chosen, not because of its relevance to the pharmaceutical industry, but rather because it's a "typical" Gram-negative organism. The original strain remains the compendial Bacterial Endotoxins Test (BET) calibrator today, and is used to prepare standard curves/standard series and for preparing positive product controls.

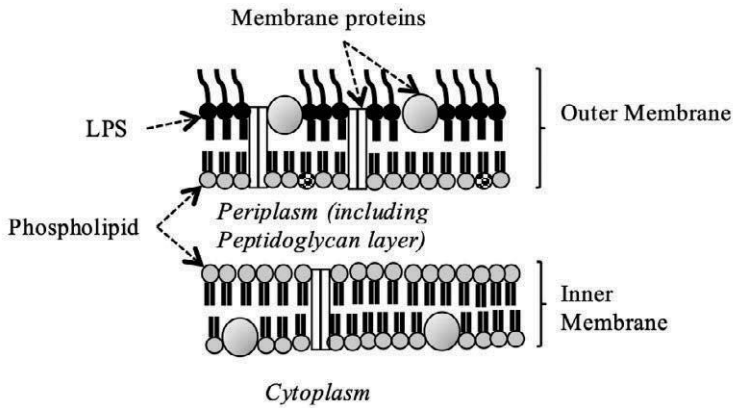

Figure 1. The Outer Membrane of the gram-negative Cell Envelope

Karen Zink McCullough *is principal consultant at MMI Associates, a consulting firm focusing on Quality System development and pharmaceutical microbiology. Ms McCullough is nationally and internationally known for her work in the Bacterial Endotoxins Test (BET), and is a frequent speaker, instructor author on such topics as BET, GMP, Metrics, Risk, and pharmaceutical microbiology. Her credits include editing two books on Microbiology and BET, authoring 26 book chapters and 19 published articles. Ms. McCullough received her BA degree in Bacteriology from Rutgers University and her MS in Molecular Biology from the University of Oregon.*

In our pharmaceutical manufacturing reality, we hopefully won't find *E. coli* in our facilities, water or raw materials, and we certainly won't find the USP RSE type strain *E. coli* 0113:H10K LPS in our products unless someone deliberately puts it there. Unlike bacterial exotoxins, pure LPS is not secreted from Gram-negative cells, so free floating/pure LPS is not seen in nature (Ellis and Keuhn, 2010). Rather, what we see in our products are whole cells, fragments of cell membrane that remain upon the lysis of the cells or Outer Membrane Vesicles (OMV). OMVs are naturally occurring structures that form on the surface of Gram-negative bacteria, are pinched off and released at all stages of the cell's lifecycle to make small free-floating vesicles that are about 25-250nm in diameter (see Figure 2 and Beveridge, 1999; Ellis, et al., 2010; Kulp and Keuhn, 2010; Schwechheimer and Keuhn, 2015). These vesicles are of the same composition as the outer membrane of the parent GN organism - integral LPS, membrane proteins, lipoproteins, phospholipids, and contain some of the cell's periplasm. Since they do not contain inner membrane or cytoplasmic structures, they are not considered to be the products of cell lysis. Rather, they are seen as the normal product of the "envelope stress response pathway" and the rate of their formation may be up regulated in response to a number of cell envelope stresses including exposure to the kinds of harsh environments found in pharmaceutical manufacturing (Kulp and Kuehn, 2010). OMVs cannot replicate and therefore are not viable, but they are stable and can act as foci for biofilm formation and as agents for the initiation of *in vivo* biological responses such as cytokine release during infection. They are not filterable by standard pharmaceutical 0.22µm aseptic filtration systems, but can be subject to concentration by ultrafiltration. According to Kulp and Keuhn (2010), "A gram-negative species, strain, or mutant that does not produce OMVs has never been identified." In Figure 2, the thick inner line represents the inner membrane. The thin outer line represents the cell's outer membrane. The space in between the two lines represents the cell's periplasmic space. Panels A-D represent the generation of an OMV.

Although purified LPS has been used for years as *in vitro* and *in vivo* analyte for bacterial endotoxins research, both historical and current research suggest that the activity of a highly purified standard, particularly in its ability to elicit a biological response in mammals or an LAL response may be subject to matrix effects (e.g. aggregation/disaggregation of purified LPS) and other experimental design challenges that may not impact their natural, adapted OMV analogs (Platco, 2014; Bolden, et al., 2015, Bolden, et al., 2016; Bolden et al., 2017). Of particular interest is current research which suggests that purified LPS from Gram-negative bacteria is not a particularly good initiator of the cytokine response, whereas the synergestic effect of LPS plus outer membrane proteins found both in viable GN bacteria and OMVs is much more effective (Post, et al., 2005; Ellis, et al. 2010; Turner, et al, 2016.)

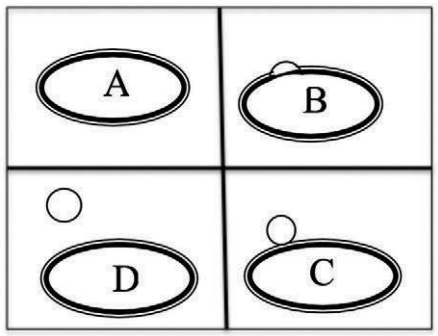

Figure 2. Formation of Gram-negative OMV.

The essential question remains: How can a carefully constructed, purified and formulated lipopolysaccharide preparation from a "typical" strain of *E. coli* that was originally meant only for assay calibration possibly represent the universe of Gram-negative organisms, never mind those organisms that have adapted to proliferate in pharmaceutical manufacturing environments and formulations? Is a purified standard meant for compendial assay calibration an optimum "fit for purpose" analyte for noncompendial spike/recovery studies?

- ICH Q2 (R1), "Validation of Analytical Procedures: Text and Methodology" suggests the following: *"Well-characterized reference materials, with documented purity, should be used throughout the validation study.* ***The degree of purity necessary depends on the intended use.****"* (emphasis added).
- The Certificate that accompanies the USP RSE makes no reference to characterization, molecular weight, molecular formula or purity of the LPS, but it does contain the following disclaimer, *"The suitability of this Reference Standard for use in non-compendial applications is solely the responsibility of the user."*
- Question 3 in FDA's 2012 Q&A Guidance for Endotoxin Testing cautions users to *"…consider the source of endotoxins used in the (hold time) study, bearing in mind that purified bacterial endotoxins might react differently from native sources of endotoxins."*
- ICH Q2 (R1), recommends the use of pure analytes for chemical studies but also reminds analysts that biological systems are complex, and thus analytical procedures for biological and biotechnological products may be approached differently, adding that these alternate approaches are both applicable and acceptable.

Each of these three documents reminds users that defining appropriate analytes, test matrices and experimental design in terms of "fitness for purpose" is important for developing a successful endotoxin spike/recovery strategy for noncompendial method development and data analysis.

That begs the question, what is the intended purpose of noncompendial hold time and depyrogenation studies? Simply stated, these studies are executed to answer the question, "If my drug product or product contact surfaces were naturally contaminated with endotoxins, would I be able to detect and quantitate the activity?" In the case of a hold time study, spike levels of endotoxins activity at T=0 are compared to the remaining activity after the hold to ascertain if the contamination is still assayable. For depyrogenation studies, the spike level of endotoxins activity upstream of the depyrogenating step is compared to the recovery of endotoxins activity downstream of the depyrogenating step to ascertain if the depyrogenation process is effective. Since we know that RSE/CSE can't be recovered from many, if not most undiluted products (Twohy, et al, 1984, PDA, 2019), if we

see negligible levels of endotoxins activity post depyrogenation of product streams, how do we know if the negative result was due to product interference or depyrogenation efficiency?

In 2016, the United States Pharmacopeia published an announcement entitled, "Early Input Sought on Proposed Naturally Occurring Endotoxin (NOE) Reference Standard." The point of this proposal was not to replace the calibration standard for its intended purpose of standardizing BET assays, but rather to provide for an alternate, well characterized, calibrated, consistently manufactured endotoxin analyte that is not a highly purified chemical entity but is "fit for purpose" as a representative of what a company might find in its product. The announcement was essentially a proposed User Requirement Specification (URS) for this new reference material. While the vast majority of the response to the proposal was positive, there were some valid concerns expressed that can be readily resolved.

1. How can we prepare this proposed standard in a way to assure that it's consistent and well characterized?

   It is suggested that the new analyte be manufactured under GMP conditions, which means that the process will be qualified and reproducible. The process will be subject to change control and include a batch record. Growth conditions (media, temperature, time) and OMV isolation will be well defined to assure reproducibility. It will be standardized against the USP RSE.

2. What is the best organism to use for the proposed standard?

   While there may be no one organism that is truly representative of the universe of Gram-negative bacteria, we can narrow the choices based on current knowledge and practice.

- Should the new preparation be made from *E. coli* 0113:H10K, which will link it to the current calibration standard?
- Should the new preparation be made from *E. coli* 055:B5, which will link it to the most often used *E. coli* strain for CSE?
- Given that *E. coli* is not routinely found in the pharmaceutical environment, is there another member of the family Enterobacteriaceae that would be more commonly isolated in the pharmaceutical environment yet still provide a genetic linkage to the current standard?
- Given that the most common source of endotoxins in pharmaceutical manufacturing is water, should the new analyte be prepared from an organism that has adapted to the minimal nutrient content of pharmaceutical waters and is commonly found in pharmaceutical water systems?

3. How can we assure availability of the new standard? Perhaps the standard can be distributed by USP.

   Which endotoxin preparation is the better "fit for purpose" analyte for the execution of noncompendial spike/recovery studies? Some see the use of purified LPS as "worst case" for product spike/hold studies or product stream depyrogenation. Others don't see it as "worst case", but rather an "impossible case" because of the highly purified state and unnatural conformation of the RSE/CSE. As we look to understand our products and processes, and reassure ourselves of the validity of our current and future test methods, we need to consider the unique chemistry and physiology of the complex biological contaminants that we might find in the product. It is important for industry, regulators, and reagent manufacturers to work together and consider an alternate analyte that can be used for non-calibration purposes and that is prepared, tested and "fit for purpose" according to the best science of the day.

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