Adaptation

Adapt (verb): to make fit (as for a new use) often by modification

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As parenteral manufacturers, we work hard to design facilities, maintain environments and design robust processes that reduce the risk of microbial contamination in our products to ensure patient safety. Though contamination in validated and well-controlled processes is rare, potential contamination by Gram negative microorganisms is of particular interest to pharmaceutical microbiologists because endotoxins produced as byproducts of the cell's normal growth cycle are pyrogens, meaning that they can elicit a fever response in patients.

The Gram negative cell envelope is a complex structure composed of inner and outer membranes that sandwich a rigid layer of peptidoglycan. While the focus of the current discussion is the outer membrane (OM), I urge the reader to take some time and learn a little about the remarkable Gram negative cell envelope (Beveridge, 1999; Silhavy, et al., 2010).

The OM is the cell's primary barrier to its external environment as well as a gatekeeper for nutrients and other materials that need to be transported in and out of the cell. The OM is an asymmetric lipid bilayer. The inner leaflet of the OM is largely phospholipids, and the outer leaflet is largely lipopolysaccharide (LPS) and outer membrane proteins, many of which assist in the transport of nutrients across the envelope. LPS molecules, though strongly anionic, are neutralized in the presence of membrane stabilizing cations such as Mg++ that are present in the external environment, allowing them to assemble in the OM to create a formidable barrier. In the absence of the neutralizing effect of the magnesium, the charge on the LPS molecules will destabilize the OM, threatening the organism's survival. (Bonnington and Keuhn, 2016).

As part of the normal growth cycle, Gram negatives create and release Outer Membrane Vesicles, or OMV. An OMV starts as a bud or bulge in the OM. The bud eventually gets pinched off from the cell and becomes a free floating minisphere that contains some peptidoglycan, surrounded by intact OM including LPS (Beveridge, 1999; Kulkarni, et al., 2014). Note that unlike bacterial exotoxins, LPS is not secreted into the environment as a distinct or purified chemical entity, but rather is found in nature as an integral part of an OMV or cell envelope fragment.

OMVs do not have a mechanism for replication, and therefore cannot be detected by normal bacterial growth based methods such as bioburden testing, sterility testing, or environmental monitoring. Of most interest to pharmaceutical microbiologists is that OMVs, and more particularly the Lipid A portion of the embedded LPS molecules, are capable of eliciting pyrogenic responses (Gu and Tsai, 1991). Routine detection of the active LPS contained in OMVs in pharmaceutical materials is accomplished by the use of the various compendial Bacterial Endotoxins Test (BET) assays (USP 2017a) or other assays that may be validated to detect active LPS.

When the Gram negative organism is stressed by a magnesium-depleted environment that destabilizes its OM, the organism can generate a "remodeled" LPS that is resistant to the destabilizing conditions (Bonnington and Keuhn, 2016). *How does the organism know to do that?*

Microorganisms are genetically wired with many "two component systems" that sense when environmental conditions are unfavorable and *respond* by initiating an adaptation to ensure the cell's survival (Capra and Laub, 2012). In the case of magnesium deprived Gram negatives, the PhoP/PhoQ two component system senses the low magnesium stress in the environment that could result in the organisms' demise and the organism responds by up regulating the generation of a "remodeled" LPS

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that can withstand the stress of a low magnesium environment, stabilize the OM and assure the cell's survival (Vescovi, et al, 1996; Capra and Laub, 2012; Bonnington and Keuhn, 2016). OMVs shuttle the "old" unstable LPS out of the cell while the cell generates/replaces it with remodeled LPS (Bonnington and Keuhn, 2016). *Could this biology help us to explain and understand "Low Endotoxin Recovery"?*

Low endotoxin recovery, or "LER", has been observed when one of the BET calibration standards (Reference Standard Endotoxin/RSE or Control Standard Endotoxin/CSE), which are extracted and highly purified preparations of LPS, are used as the analyte in hold time studies for a common biopharmaceutical formulation containing a citrate chelating buffer (binds divalent cations) and polysorbate (Chen and Vinther, 2013). We know that the organism from which the current RSE calibration standard was extracted was grown in a medium supplemented with magnesium (Rudbach, et al, 1987), suggesting that the LPS in RSE, and presumably CSE as well, have been stabilized by the presence of magnesium. *Could the loss of activity with the RSE analyte be due to the fact that it is a "non remodeled" LPS?*

Dubczak has reported on a native OMV preparation from *Enterobacter cloacae* ATCC 7256 that he "conditioned" to grow in 99% Water for Injection supplemented with 1% nutrient broth, which is a magnesium-depleted environment (Dubczak, 2014; Bolden, et al, 2015). When added to the "LER matrix" of a citrate buffer and polysorbate, Dubczak's conditioned native OMV preparation retained activity both in the USP Bacterial Endotoxins Test (USP 2017a) and the USP Rabbit Pyrogen Test (USP 2017b), whereas the activity of the RSE control was lost in both USP assays (Dubczak, 2014; Bolden, et al, 2015). Dubczak has also reported that if organisms can grow in the LER matrix, they can and do produce abundant levels of endotoxins that are readily detected by current compendial BET assays. *Could it be that Dubczak's conditioned organisms have adapted to their low magnesium environment and are making remodeled LPS that remains active in the "LER matrix"?*

There have been reports of native endotoxin preparations that act very differently than Dubczak's *E. cloacae* preparation. Reich (2015) describes a series of native endotoxin preparations that lose activity in the presence of a chelator, a finding consistent with the original "LER" observation of Chen and Vinther. But looking at Reich's data, it appears that these organisms were grown in a minimal medium that was supplemented with magnesium. *Could it be that Reich's native endotoxins did not contain remodeled LPS and were therefore susceptible to the LPS destabilizing effects of the chelating environment, rendering them inactive with respect to the compendial assays?*

Dubczak's data and Reich's data taken together with the recent results of a BPOG intralaboratory study (Bolden, et al, 2017), are consistent with the academic literature, suggesting that "remodeled" LPS generated by organisms that have adapted to low magnesium environments may be more resistant to the stresses of chelating environments than is "un-remodeled" LPS.

What does all of this mean to the real life world of biomanufacturing? More importantly, what does it mean to our ability to detect endotoxins by the compendial BET assays and ensure patient safety?

We can roughly divide a model bioprocessing sequence into two parts: cell cultivation (including harvest) and drug substance purification.

- The mammalian cell cultivation step utilizes growth medium that is typically supplemented with 1.5mM Mg++. Process control would dictate that the cell culture be monitored for bioburden and endotoxins at critical control points, but almost certainly at cell harvest where a decision can be made whether or not to reject the batch. The literature suggests that LPS generated by Gram negative contamination and growth during cell culture would be stabilized by the Mg++ in the growth medium. Depending on the ultimate growth of the organism, endotoxins would be readily detected by the BET assay.
- Post harvest, the buffers and other solutions used for elution and purification are typically not supplemented with Mg++. Any adventitious Gram negative contamination at this stage would either have to adapt to the magnesium-depleted environment presented by these buffers or die due to destabilization of the outer membrane LPS. Any OMV remaining from the cell culture effort would likely be destabilized in this new environment as well. The literature suggests that if a contaminating organism adapts to this stressful environment and grows, it will generate OMVs with "remodeled" LPS. If present in high enough titers, these "adapted" organisms along with their "remodeled" LPS would remain active, and would be detected by bioburden assays and BET assays at the designated critical control points during purification. Again, a decision can be made at that point whether or not to continue with the batch.

Summary

Current as well as legacy academic, peer reviewed, scientific research, augmented by data and observations by industry scientists, is providing an evolving picture of the biology that may help to explain the instability and loss of RSE activity in the "LER" matrix. Research has demonstrated that microorganisms sense and respond to external stress signals via any number of two component systems. These two component systems are among most prevalent genes in bacteria and have been found in the genomes of nearly all sequenced microorganisms (Capra and Laub, 2012). This gene expression repertoire allows organisms to survive disparate environmental conditions. The magnesium sensing PhoP/PhoQ system that allows Gram negative bacteria to remodel their LPS and adapt to a stressful environment is just one of hundreds of such systems. Two component systems explain the resistance of these microbes to OM destabilizing antibiotics such as Polymyxin B and Colistin. *Could PhoP/PhoQ and remodeled LPS also help to explain the inability to recover activity of the calibration standards in the "LER matrix" yet recover native endotoxins from organisms grown in a magnesium-depleted environment? Could "LER" be a manifestation of normal biological adaptation rather than an observation that calls into question the validity of the LAL test to detect potentially pyrogenic levels of endotoxins in biopharmaceutical products?*

The biopharmaceutical industry, after using the compendial BET assays for over three decades, has not had a single documented recall of an "LER" product for pyrogenic reactions. This is a credit to our industry's ever improving process control activities as well as the validity of the compendial BET assays for monitoring endotoxins produced by contaminants that may, in rare cases, find their way into and grow to sufficient levels to produce endotoxins in water, manufacturing materials, in process samples, and finished products. I would submit that public records indicate that "LER" is not a patient safety issue. It would appear, however, at least for "LER" products, that the long held assumption and current requirement to use the purified calibration standards as universally accepted and *de facto* substitute analytes for native endotoxins in pharmaceutical experiments may be unsupported by current biological research and industry findings.

The point of this discussion is not to suggest, even for a moment, that adaptation will result in the creation of pharmaceutical "super bugs" that will contaminate our products and produce copious levels of endotoxins. It won't. Nor is the intent to suggest, even for a moment, that any stray organism that finds its way into a process will pose a great risk to the process. It won't unless conditions are such that it can grow to numbers large enough where organisms and endotoxins become detectable. The point is simply to suggest that while our industry is spending resources trying to unwind the "why" and "how" of our inability to recover calibration standards that can't and don't exist in nature, legacy as well as current microbiological research and thirty years of safe biotherapeutics is providing us with a very good explanation for why we can recover activity of endotoxins that do exist in nature.

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