Part III, Endotoxin Test Concerns of Biologics: *LER From a Broad Biologics Test Perspective*

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Overview

Recent conferences have demonstrated the polarizing nature of the Low Endotoxin Recovery debate, including those in Berlin (PDA Europe), Iselin, NJ (PMF Bacterial Endotoxin Summit), and Bethesda, MD (PDA Global Micro). The debate has centered on the significance of samples subjected to LER conditions, which includes polysorbate and a chelator (citrate or phosphate buffer). Samples subjected to these conditions undergo a masking effect that renders undiluted spike into hold-time studies impossible to recover without special treatment. Industry participants are debating the potential for harm from such samples, in the unlikely event they were contaminated by biologics manufacturing processes. Three points outline a broad biologics-test-based endotoxin contamination control perspective:

1. Historical endotoxin detoxification studies
2. The emerging view of endotoxin as an IIRMI
3. A perspective on merging of the current pyrogen and emerging IIRMI view

Historical Endotoxin Detoxification Studies

There is a large volume of knowledge derived from the search for adjuvant candidates to aid the efficacy of vaccines that dates back over 80 years. These searches focused on the idea that one can treat purified lipopolysaccharide or natural endotoxin with harsh, or not so harsh conditions (see Figure 1) and produce a changed LPS structure that is no longer pyrogenic. However, while not pyrogenic, the endotoxin subjected to detoxifying conditions maintains its ability to stimulate the immune system. This immune stimulation is exactly what vaccine manufacturers want in order to direct the body's immune system against clean, recombinant proteins that do not, of

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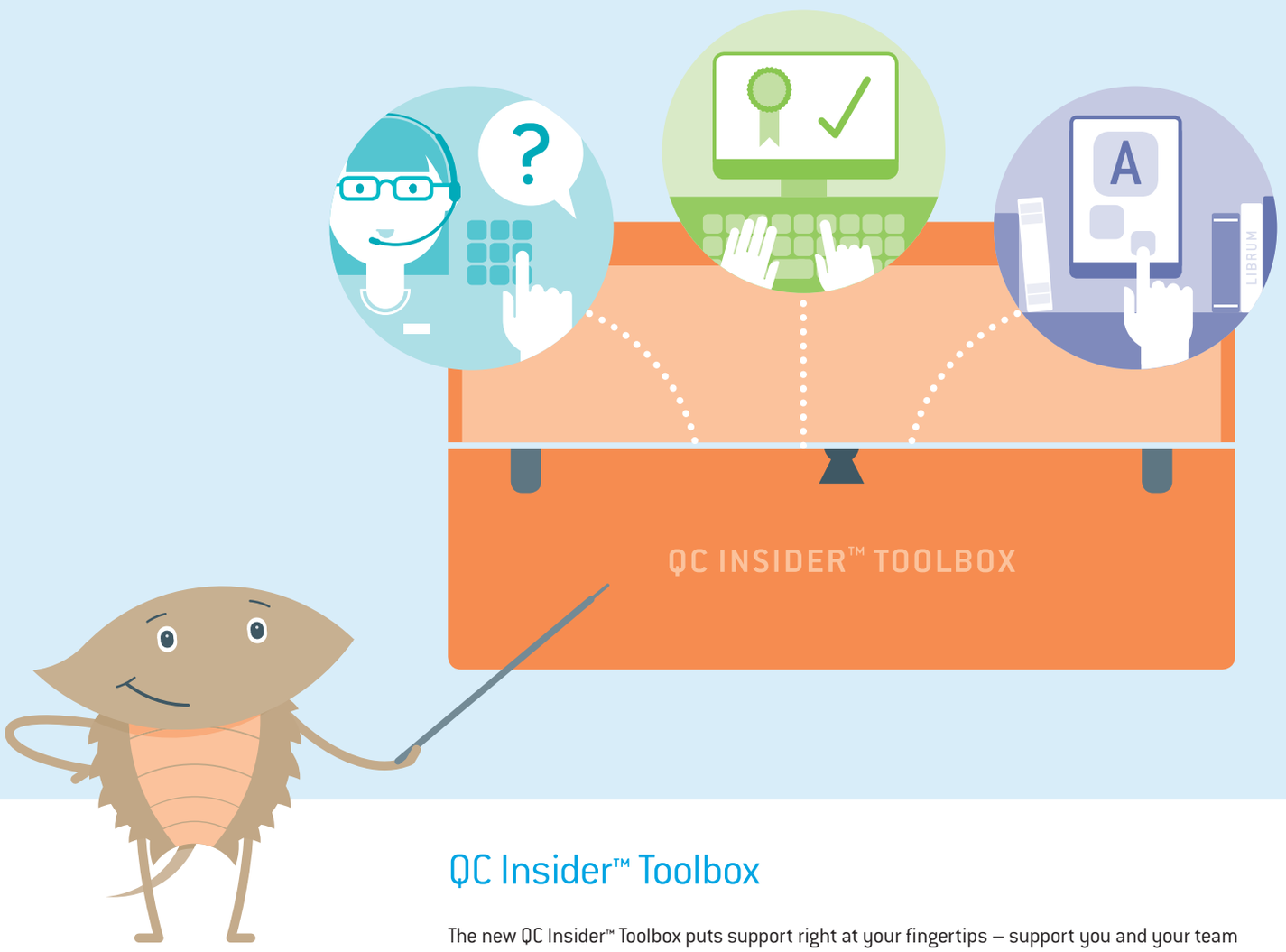

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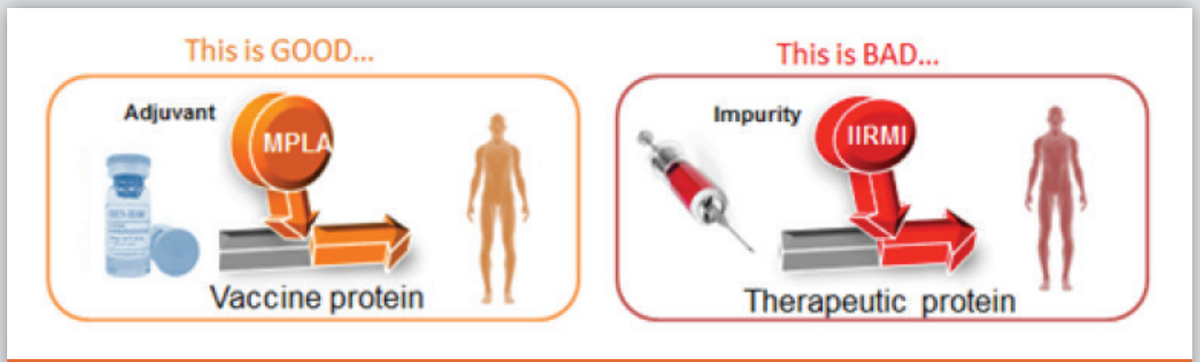

Figure 1. The basic mechanism of the IIRMI "adjuvant" effect. The immunogenicity phenomenon has been seen historically in mild to severe adverse reactions[8,9,10]. MPLA is Monophosphoryl Lipid A, a detoxified endotoxin, and is used as an adjuvant in vaccines.

themselves, elicit an immune response[1]. In this case, the stimulation of anti-drug antibodies (ADA) against recombinant drug proteins from bacterial and other antigens including Hepatitis B[2], Human papilloma virus[3], various cancer vaccines[4,5,6], and Malaria[7] is beneficial to the vaccine effort. The antigen-adjuvant effect forms the basis of vaccination, which is the most successful form of medical intervention ever employed. The downside of the so-called "adjuvant effect" involving clean proteins is that the same mechanism can serve to bring about ADAs toward life-saving therapeutic proteins via the inclusion of low levels of inadvertent microbial contaminants.

While the LAL reduction associated with O-deacylation and dephosphorylation has long been known, what is less recognized is that "detoxification" results in greatly muted LAL and rabbit pyrogen test activity ("when monophosphoryl lipid A's and lipid X were similarly tested, they showed very low pyrogenicity"[11]), however the adjuvant activity remains[12]. A wide variety of historically accumulated methods of detoxification are shown in Figure 2. These include: chemical,[13,14,15,16] ionizing radiation,[17,18] use of surfactants[19] (reversible), enzymatic[20,21] mutation[22,23,24] (natural and induced), antimicrobial peptides[25], antibody mediated[25] natural low pyrogenic forms[26] and LER. A review of practices that do not incinerate or completely remove the functional LPS PAMP might be in order from the "endotoxin as IIRMI" view for biologics processing. Detoxification does not remove the adjuvant effect of MPLA but rather significantly diminishes the pro-inflammatory effect[27]. This has been seen in methods used for depyrogenation as well, including Gamma irradiation, as seen in the treatment of *Salmonella* resulting in removal of its pyrogenicity, while allowing it to retain its immunogenicity-inducing capability[28]. Similarly, irradiated LPS "retains the adjuvant activity of LPS, and it serves as a good adjuvant for inactivated virus vaccines."

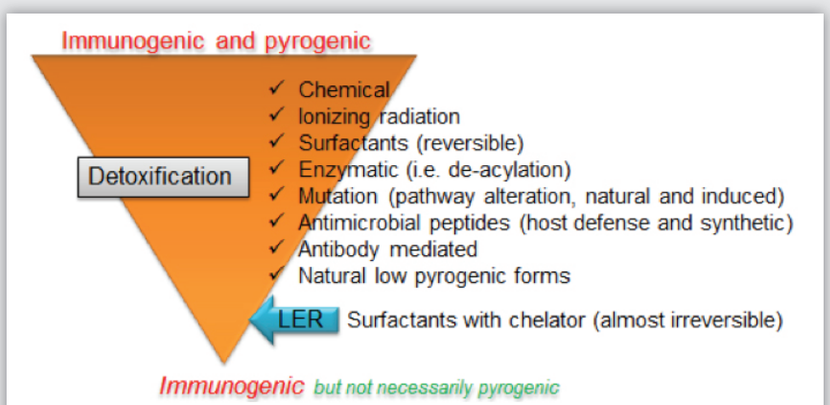

Figure 2. LER can be viewed as one of many historical methods of "detoxifying" LPS, historically performed for the purpose of adjuvant research.

LER solutions that are purposefully spiked or inadvertently contaminated could be considered a type of "detoxified" endotoxin. The search for compounds that utilize the immune stimulation properties of LPS without pro-inflammatory effects that include fever is ongoing, as many subunit vaccines do not have the ability to stimulate the immune system. In the realm of endotoxin testing, if one is singularly worried about the pyrogenicity of a sample, then it could come to play out that LER-subjected drug formulations are not particularly pyrogenic, although there is conflicting rabbit pyrogen data[29,30]. However, if one is worried that a given LER-prone protein formulation could increase therapeutic protein immunogenicity if such LPS monomers are present, then one would want to detect and preclude the presence of endotoxin monomers or otherwise "detoxified" endotoxin solutions that retain the potential to be recognized by mammalian immune systems.

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There is clearly concern here centered on a level of bacterial endotoxin that is below the level associated with the ability to bring about fever.

An Emerging View of Endotoxin as an IIRMI

An emerging view of endotoxin as a contaminant comes from FDA laboratory studies (Verthelyi and Wang[31]; Haile et al.[32]) that demonstrated that low levels of contaminants including LPS that they have termed IIMRIs or "innate immune response modulating impurities" can, either alone or synergistically to greater effect, stimulate the immune system against therapeutic proteins at a level that may be well below the level considered necessary for pyrogen or endotoxin preclusion testing (5 EU/kg). Studies in mice showed that LPS and bacterial DNA added to a recombinant protein (r-Erythropoietin) served to "break tolerance to self".

Researchers have shown both *in vitro* and *in vivo* that synergistically IIRMI's are active at lower levels than when present alone[31]:

> This synergistic effect was then confirmed *in vivo*, as studies showed that the combination of 10 ng of LPS and 500 ng of CpG ODN, which do not induce an immune response when present individually, were sufficient to promote the immunogenicity of proteins and contribute to a clinically relevant break in tolerance to self.

They showed that mice given r- Erythropoietin had mild transient side effects, while mice subject to r- Erythropoietin with low levels of LPS and bacterial DNA contaminant developed long-lasting anemia. Thus, the attack on the r- Erythropoietin turned into a full-fledged attack on the mice's own natural proteins. This is among the worst forms of undesirable immune reactions. Furthermore, either of the components acting alone (LPS or DNA) at very low levels did not show an adverse effect. The levels involved for mice do not translate directly to humans, as mice are notoriously less affected by endotoxin relative to humans[33].

The IIRMI view is referred to as "emerging" here, but it is already included in the 2014 FDA Guidance Document: *Immunogenicity Assessment for Therapeutic Protein Products* in section 5, page 18, "*Impurities with Adjuvant Activity*". While considered "non-binding," the non-binding designation also applies to the current Q&A Guidance document. An excerpt from the former is shown below:

> Adjuvant activity can arise through multiple mechanisms, including the presence of microbial or host-cell-related impurities in therapeutic protein products (Verthelyi and Wang 2010; Rhee et al. 2011; Eon-Duval et al. 2012; Kwissa et al. 2012). These innate immune response modulating impurities (IIRMIs), including lipopolysaccharide, β-glucan and flagellin, high-mobility group protein B1 (HMGB1), and nucleic acids exert immune-enhancing activity by binding to and signaling through toll-like receptors or other pattern-recognition receptors present on B-cells, dendritic cells, and other antigen-presenting cell populations (Iwasaki and Medzhitov 2010; Verthelyi and Wang 2010). This signaling prompts maturation of antigen-presenting cells and/or serves to directly stimulate B-cell antibody production.

The researchers quoted by the Immunogenicity Guidance document (Verthelyi and Wang) describe the current conundrum in endotoxin detection. They discuss the relevant levels of endotoxin viewed as an IIRMI to those standardized for testing of pyrogens, by either *Limulus*-based methods (LAL and rFC) or rabbit pyrogen tests (RPT).

> Of note, the current guidelines for setting limits on these impurities are not based upon their potential impact on product immunogenicity. For example, the current recommendations for endotoxin content in parenteral products (0.5 EU/kg/hr)[34] is based on its pyrogenic potential, while WHO recommendations for DNA content (<10 ng DNA/dose) are based on minimizing the risk of DNA integration (USP <85> and [45])[35]…the levels of agonists sufficient to stimulate an innate immune response can be lower when multiple receptors are engaged.

In terms of immunogenicity[36], biologics have become increasingly safe over time with the realization that natural animal-derived proteins may be recognized as non-self, and improved manufacturing methods for recombinant proteins has lessened potential protein aggregation to further limit immunogenic reactions. The "humanization" of previously animal-based and chimeric monoclonal antibodies has also lowered immunogenicity rates. But a low level (and sometimes not so low level) of persistent proclivity toward immunogenicity remains and can be seen in clinical studies and marketed package inserts.

A Perspective on Merging of the Current Pyrogen and Emerging IIRMI View

There is a "trend" that could be interpreted as a rudimentary, perhaps utilitarian effort to merge the pyrogen and IIRMI preclusion concepts. How else is one to interpret the continued reduction of endotoxin tolerance limits (TL) associated with biologics? This can be seen in (a) Biologic License Application (BLA) requests to lower limits calculated by the TL = K/M method, (b) hold-time study recoveries from undiluted drug process and final solutions, and (c) the Q&A verbiage that makes it clear that current expectations are that testing be performed as low as possible after interference has been overcome. There is clearly concern here centered on a level of bacterial endotoxin that is below the level associated with the ability to bring about fever. Historically, one would calculate the tolerance limit (TL) by dividing the threshold pyrogenic response constant (K=5 EU/kg/hr) by the drug dose in

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active dosage units per kilograms of body weight. The dose of a given biologic drug appears to be less and less a consideration since the advent of biologics. Today, the question becomes "How far below the TL should be validated and routinely tested?"

Alternatively, the concerns could also stem from the large number of additional solutions going in with biologics such as pre-medications for expected fever reactions (steroids, antipyretic, antihistamine, etc.), which are themselves (the biologic and pre-medications) typically each contained within large volume parenteral solutions (LVP). Note that the limit for LVPs is less stringent than other drug types (0.5 EU/mL). According to the IIRMI or adjuvant view, a lesser quality standard for a drug to be co-administered or subsequently administered with a biologics could contribute IIRMIs. One wonders if a quality designation of "*For use in Biologics*" might serve to safeguard against emerging concerns associated with the adjuvant effect that can come from non-biologic sources when administered with biologics. This concern extends to potential contaminants added by compound pharmacy activities[37], which have recently come under fire and to which the FDA has responded with a new draft guideline[38].

Historically, there has been a singular focus on precluding the bacteria that produce pro-inflammatory, "endotoxic" endotoxins (*Enterobacteriaceae*). The revelation that non-pyrogenic endotoxin can be recognized for other effects[39] fits an underlying, longstanding theme that microbial artifacts injected into the blood stream may have significant effects that do not necessarily correlate with our ability to "see" them, analytically speaking, or correlate with their ability to produce fever. The mammalian physiological view of endotoxin is ultra-sophisticated when it comes to the detection of microbe invaders and their artifacts, given that it has accrued over a billion of years of evolution. The basic Lipid A PAMP should be viewed as a set of dials (phosphate, sugar, number and types of acyl chains-symmetrical/asymmetrical, substitutions, etc.) rather than an "on-off" button (pyrogenic or non-pyrogenic)[40]. The activity of LPS at low levels is being borne out in studies of the low dose effect of endotoxin in various disease states (i.e. sepsis[41], inflammation[42], cancer[43], and cardiovascular disease[44]).

In LER discussions, test users have wondered if endotoxins disassociated by LER conditions could pass along any harm to patients. The adjuvant model seems to answer this question as a detoxified, non-pyrogenic endotoxin such as FDA-approved adjuvant monophosphoryl lipid A (MPLA) is added to recombinant vaccine proteins to stimulate the immune system against vaccine proteins. While the detoxified form is not pyrogenic (detoxified), and users are testing for pyrogenic effects via rabbit pyrogen and bacterial endotoxin test methods instead of immunogenicity, it answers the question as to whether it is possible for such forms to potentially harm patients. It is not only possible, it is an effect routinely taken advantage of in the vaccine realm.

The difficulty LER raises for regulators and drug manufacturers is that the endotoxin test currently provides a multifunctional duty as a process control to exclude endotoxins. There is not another method or a better method to control endotoxins in processes other than by recognizing their presence by bacterial endotoxin testing. If the BET vantage is lost due to a common masking phenomenon (LER or other biologics' inherent process conditions), then the presence of pyrogen-causing and immunogenicity-causing endotoxins cannot be known. There have been developmental efforts to restore the activity lost by the LER phenomenon as shown by J. Chen of Genentech, J. Reich of Hyglos and here at Lonza. While demasking takes a little more time and labor and may not be perfect from the perspective of process control, it does allow a way forward that does not completely ignore the potential harm from endotoxin that would, if present as a masked contaminant, remain masked right up to the point of patient administration. Disaggregated endotoxin consists of the entire microbial PAMP and active endotoxic principle (lipid A) that has served for a billion years (plants) of metazoan recognition of prokaryotic invasion. There is now ample evidence that even non-pyrogenic endotoxin is immunogenic, and that this activity proceeds by a pathway separate from the pro-inflammatory pathway after the initial recognition via TLR4/MD-2 dimer complex[45,46].

Conclusion

The last thing biologics manufacturers intend is to include adjuvants in the use of therapeutic proteins by way of impurities. As we have seen, endotoxin adjuvants administered with vaccine proteins do indeed elicit effective, non-pyrogenic endotoxin responses. The need for an updated view is well stated by Haile, et al: "*It is only the more recent understanding of the innate immune system's biology that dictates the need of assessing a broader spectrum of known and unknown IIRMIs in order to control or reduce the risk of unwanted immunogenicity by therapeutic proteins*[47]." Modern biologics manufacturing concerns contrast with historic, purely pyrogen-centric activities that have represented an important but more minimal standard.

In conclusion, three posits may help form a working model towards a broader goal of containing endotoxin contamination by including the IIRMI perspective:

1. Endotoxin detection from a multipurpose perspective should be performed at levels lower than those dictated solely by the TL calculation.
2. Processes known to employ detoxifying conditions (such as LER) should pursue orthogonal test methods that supplement conventional methods.
3. The philosophical view that non-biologics cannot practically contribute to biologics contamination should be challenged given the adjuvant activity they can contribute to biologics via co-administration.

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