Low Endotoxin Recovery (LER) – Context and Resolution from a Broad Biologics Test Perspective

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Agenda

- What is Low Endotoxin Recovery?
  - Background
  - Current regulatory status
  - Practical considerations

- How to recognize LER in your process/product?
  - Hold-time studies
  - CSE/RSE versus NOE – What is the best approach?
  - What exactly is the worry?
  - De-masking – Theory and practice

- Endotoxin as an IIRMI – a potential shift of paradigm?
  - What other than ‘pyrogenicity’ should manufacturers keep in mind when a product exhibits LER?
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What is Low Endotoxin Recovery?

- Low Endotoxin Recovery (LER) is a controversial topic that has been circulating throughout the endotoxin detection community over the last three years. Brought to regulators attention by Dr. Joseph Chen (Genentech) in 2012.

- LER is theoretically described as:
  - “A masking effect” manifested in the biophysical formation of a complex that blocks the ability of Factor C, the main component in LAL detection, to bind endotoxin”
  - LER subjected samples also show poor (MAT) to variable (RPT) recovery in mammalian based tests
What is Low Endotoxin Recovery?

- A common drug excipient, polysorbate combined with a chelator such as citrate or phosphate buffers, has been identified as the cause of the masking effect more commonly referred to as LER.
  - Polysorbate is estimated to be used in more than 70% of protein formulations.\(^2\)
  - There is also some evidence that histidine-containing formulations may occasionally be involved in LER.

- LER is considered to be distinct from interference commonly seen with the LAL Assay that is normally overcome by dilution or other sample preparation methods.
What is the Current Regulatory Stance?

- The FDA included a stability screen requirement in their 2012 Q&A Guidance for Industry Pyrogen and Endotoxins Testing
  - Chen originally pointed to the phenomenon before the Q&A guidance came out but the hold time studies are where users have subsequently seen the LER phenomenon

- This guidance recommends that the industry performs hold-time studies to verify the amount of time a sample can be reasonably stored prior to final release testing
Why is the LER Focus Primarily Associated with Biologics?

Regulators require a **Biologic License Application (BLA)** to be submitted for each new biological drug product before it is released to the public.

- The BLA is a formal request for permission to introduce, or deliver for introduction, a biologic product into interstate commerce regulated under 21 CFR 601.20 – 680
- If the information provided meets FDA requirements, the application is approved and a license is issued allowing the firm to market the product
- FDA is currently requesting follow up studies post approval for drugs exhibiting LER
Why is the LER Focus Primarily Associated with Biologics?

- The rabbit pyrogen test (RPT) requirement applies to all of the BLA products regulated by CDER
  - For Non-Biologics, equivalence is assumed between RPT and BET

- This requirement may be waived if a method equivalent to the rabbit pyrogen test is demonstrated in accordance with 21 CFR 610.9 (Equivalent Methods and Processes)
  - Requires the applicant to provide evidence “demonstrating that the modification will provide assurances of the safety, purity, potency, and effectiveness of the biological product equal to or greater than the assurances provided by the method or processes specified in the general standards or additional standards for the biological product…”
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How to Recognize LER in a Product?

LER Screening Test / Hold-time Study

Direct spike into undiluted Drug Product, Drug Substance or In-Process Solution at a level ≤ the specification.

Hold product before testing according to pre-determined parameters (temperature, container type, # days).

Product should be tested at specific time-points throughout hold period (ex. 4hr, 24hr, 72hr, day 5, day 7- note day 7 is 8 days).

Determine recovery (%)* of spike compared to amount of endotoxin originally added to sample.

*Products exhibiting LER typically result in declining (≤50%) recovery at various or multiple time points throughout the hold period. Inability to recover spike CSE may occur in less than 4 hrs.
A simple **screening test** using CSE can demonstrate the presence or absence of LER for a given drug product, drug substance or in-process solution.

- **Does the product contain polysorbate?**
  - This is the “trigger” for LER potential. Hold time studies are being requested for all formulations containing Polysorbate surfactants.*

- **Spike undiluted drug product, drug substance, or in-process solution with endotoxin**
  - CSE is recommended for LER screening as the standard seems to be more affected by LER than Naturally Occurring Endotoxin (NOE) preparations (worst-case)

- **Carry out hold-time study to determine whether initial spike is recoverable**
  - Container type, storage conditions and reasonable sample hold-time should be taken into consideration (=process knowledge)
Hold-Time Studies – Should You Use CSE or NOE?

- Regulators have thus far discouraged the use of Naturally Occurring Endotoxin (NOE) in hold-time studies.
- Biological License Applications (BLA) using both CSE and NOE preparations display conflicting results between the LAL test and Rabbit Pyrogen Test (RPT).

<table>
<thead>
<tr>
<th>Spike Material</th>
<th>LAL Test</th>
<th>Rabbit Pyrogen Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSE</td>
<td>No LER</td>
<td>Pyrogenic</td>
</tr>
<tr>
<td>CSE</td>
<td>LER</td>
<td>Not pyrogenic</td>
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<tr>
<td>CSE</td>
<td>LER</td>
<td>Pyrogenic</td>
</tr>
<tr>
<td>NOE</td>
<td>No LER</td>
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</tr>
</tbody>
</table>
Hold-Time Studies – Should You Use CSE or NOE?

- Results so far have been confounding due to the variety of formulations, proteins, study designs, controls, test methods, standards and reagents used

Case Study – BLA for Therapeutic Protein

<table>
<thead>
<tr>
<th>Results</th>
<th>LPS Spike</th>
<th>NOE Spike</th>
</tr>
</thead>
<tbody>
<tr>
<td>LER exhibited within 24 hours</td>
<td>No LER for 23 days</td>
<td></td>
</tr>
<tr>
<td>Non-pyrogenic in rabbits</td>
<td>Non-pyrogenic in rabbits</td>
<td></td>
</tr>
</tbody>
</table>

- NOE data from rabbit is inconsistent with the results of the LAL method
Example BLA Post Approval Study Agreement

- Could already be outdated (from Feb. 2014)
- But shows FDA expectations in performing an LER study.

2. Describe the particular review issue and the goal of the study.

The drug product formulation contains excipients (e.g., polysorbate) that could result in low endotoxin recovery (LER). In Amendment the Sponsor provided data suggesting that the LER phenomenon does not occur with DP when measurement is conducted by the LAL method over time points ranging from 0 to 8 days. However, the studies were conducted with only one drug product lot and with one endotoxin spike level. The goal of the study will be to verify method reliability with 3 additional drug product lots using endotoxin spike concentrations closer to the acceptance criterion of ...

http://www.accessdata.fda.gov/drugsatfda_docs/nda/2014/125390Orig1s000OtherR.pdf
Hold-Time Studies –
Should You Use CSE or NOE?

Current FDA recommendations for performing hold-time studies – in summary

1. Test at least 3 different lots
2. Spike close to the drug specification (at or below)
3. Test for at least 8 days (finished product)
4. Use Control Standard Endotoxin (worst-case scenario and FDA recommendation)
What Exactly is the “Worry” with LER?

If LER obscures the ability to “see” endotoxin in a process, then the transient occurrence of microbial contamination cannot be known…

Concerns of alteration of product properties…
Endotoxin Aggregation – Do Monomers Matter?

To some, the LER issue seems to hinge on the “biological activity” of aggregated vs. disaggregated LPS (standard or natural endotoxin)...

- Endotoxin normally exists in an aggregated state and not as monomers
- Biological inactivity of disaggregated LPS in LER solutions easily demonstrated with LAL but is not so straightforward in mammals
Endotoxin (Dis)Aggregation – Do Monomers Matter?

- BLA submissions include user data showing that solutions subject to LER are in some cases pyrogenic.
- Fever typically accompanies Biologics infusion. Mabs are pre-dosed with steroid and anti-fever drugs.
- Lipid A is the well established “endotoxic principle” of LPS and fits into the human TLR4 architecture as a single molecule (not an aggregate).
Endotoxin (Dis)Aggregation – Do Monomers Matter?

- Do monomers matter?
  - Levin showed increasing LAL gelation time with increasing disaggregation via hemoglobin
  - There are >200 acute phase proteins (APP) in mammals responding to endotoxin
  - According to Mueller et al. only the aggregated forms of endotoxin are biologically active. However, many other studies point to the monomer’s activity *in vivo*
  - The body’s practice of partitioning of the endotoxin response complicates broad claims
  - The successful targeting and modulation of TLR4/MD-2 activation with small molecules that are not Lipid A is inconsistent with the need for an aggregated presentation

- Endotoxin can be immunogenic even with the loss of pyrogenicity (detoxified)

Levin: from his chapter “Effects of Human Hemoglobin on Bacterial Endotoxin in vitro and in vivo” in Endotoxin in Health & Disease.

Proteogenomics of selective susceptibility to endotoxin using circulating acute phase biomarkers and bioassay development in sheep, by Chemonges, Tung and Fraser, Proteome Science 2014, 12:12.


“serum free conditions”

Aggregation Behavior of an Ultra-Pure Lipopolysaccharide that Stimulates TLR-4 Receptors, Sasaki and White, Biophysical Journal Volume 95 July 2008 986–993. “LPS monomers and multimers are the active units for the immune system…”

Monomeric Re Lipopolysaccharide from Escherichia coli Is More Active Than the Aggregated Form in the Limulus Amebocyte Lysate Assay and in Inducing Egr-1 mRNA in Murine Peritoneal Macrophages, Takayarna et al.
The successful targeting and modulation of TLR4/MD-2 activation with small molecules that are not Lipid A seems inconsistent with the need for an aggregated presentation, at least in every case.

- Small molecule MW of ~390 versus ~1700-1800 for Lipid A.
- “A 17-residue peptide (MD2-I) was synthesized to reproduce the TLR4-binding region of the MD2 protein that contains all the critical interacting residues” and showed evidence that this sequence “targets TLR4 directly” as an antagonist.
Endotoxin Aggregation – LER Mechanism (Theory)

Theoretical Mechanism of LER

I = [LPS] pure
II = [LPS] + [surfactant] low
III = [LPS] + [surfactant] medium
IV = [LPS] + [surfactant] high

Loss of spike recovery over time

- If de-masking required for LER for BIOLOGICS
  - Could be viewed as first effort to arrive at “BIOLOGICS type” add-on test
  - Looking for detoxified or low pyrogenic endotoxins

*Picture courtesy of Hyglos*
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Endotoxin as an IIRMI – A Potential Shift of Paradigm?

- An *Emerging View of Biologics Testing* provides a broad scientific backdrop for LER
  - LPS subject to LER can be viewed as a “detoxified” form
  - Detoxification historically used to remove pro-inflammatory effects while retaining “adjuvant” effect for LPS use in vaccines
  - Thus endotoxin has a dual nature in mammalian systems:
    - *Pyrogenicity* is the historical impurity attribute of endotoxin
    - *Immunogenicity* is an impurity attribute of endotoxin when paired with TPs
    - And is an attribute of endotoxin separate from pyrogenicity
Endotoxin as an IIRMI – A Potential Shift of Paradigm?

- What is the risk associated with endotoxin contamination?
  Well, it’s “pyrogenicity” of course.
    Historically, yes
    Traditionally, yes
    Presently, yes

But no longer singularly from a Biologics perspective…
Endotoxin as an IIRMI – A Potential Shift of Paradigm?

With Biologics there is a newly recognized class of risk from endotoxin as an impurity

Immunogenicity – based upon “adjuvant” activity of endotoxin as an impurity… Stimulates immune system, promotes formation of ADAs toward TPs…

Whereas historically its only been about fever – RPT is fever; BET is based on calculations relative to the occurrence of fever (K)…

Immunogenicity is not new, but the relevance to endotoxin detection is an emerging view…
An emerging concept is that of endotoxin as an “Innate Immune Response Modulating Impurity” (IIRMI). The term “IIRMI” was first introduced by Verthelyi and Wang (2010) and is referenced in the FDA 2014 Guidance. This guidance document, titled "Guidance for Industry: Immunogenicity Assessment for Therapeutic Protein Products," is non-binding and was published in August 2014 by the U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research, Bethesda, Maryland, United States.

The article "Trace Levels of Innate Immune Response Modulating Impurities (IIRMIs) Synergize to Break Tolerance to Therapeutic Proteins" was published in PLOS One and lists the authors as Daniela Verthelyi and Vivian Wang. The authors are affiliated with the Division of Therapeutic Proteins, Office of Biotechnology Products, Center for Drug Evaluation and Research, FDA, Bethesda, MD.
5. **Impurities with Adjuvant Activity**

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 (IIRMI), 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.

**Recommendations**

It is very important for manufacturers to minimize the types and amounts of such microbial or host-cell-related impurities in therapeutic protein products.

Assays to evaluate the types of IIRMI present should be tailored to the relevant cell substrate. Because even trace levels of IIRMI can modify the immunogenicity of a therapeutic protein product, the assays used to detect them should have sensitivities to assess levels that may lead to clinically relevant immune responses.
Endotoxin as an IIRMI – A Potential Shift of Paradigm?

- So for the first time, officially, beginning with Biologics, endotoxin is recognized not only for its pyrogenic effect but for its immune stimulating effect on therapeutic protein drugs.

- This pairing phenomenon makes Biologics different.
Endotoxin as an IIRMI – A Potential Shift of Paradigm?

- Where can we see the IIRMI or “adjuvant” effect of endotoxin?
  - …in detoxified endotoxin used as an adjuvant - MPLA

- A subunit vaccine is a purified protein (=antigen) and needs an immune stimulator (=adjuvant)

- “A major complication of subunit vaccine development is that most recombinant proteins lack intrinsic immunostimulatory activity.”
LER can be viewed as one of many forms of LPS “detoxification”

Methods used historically to detoxify endotoxin in search for vaccine adjuvants include:

- Chemical
- Ionizing radiation
- **Surfactants** (reversible)
- Enzymatic (i.e. de-acylation)
- Mutation (pathway alteration, natural and induced)
- Antimicrobial peptides (host defense and synthetic)
- Antibody mediated
- Natural low pyrogenic forms

**Detoxification**

**Immunogenic and pyrogenic**

- LER Surfactants with chelator (almost irreversible)

**Immunogenic but not necessarily pyrogenic**

Detoxification is a change in endotoxin structure causing a loss of its proinflammatory capability…

By this view, LER not a “one-off” phenomenon
Endotoxin as an IIRMI – A Potential Shift of Paradigm?

IIRMIs


Triacylated lipoprotein Diacylated lipoprotein Flagellin Imidazoquinolines (anti-viral compounds) CpG DNA LPS dsRNA

TLR1 TLR2 TLR6 TLR2 TLR5 TLR7 TLR9 MD-2 TLR4 TLR3

Verthelyi and Wang\textsuperscript{7} showed IIRMI’s act synergistically to be active at lower levels than when present alone:

- Used r-Erythropoietin > transient change
- r-Erythropoietin with IIRMIs > long lasting anemia
- “Humans are likely to be much more sensitive to TLR agonists than rodents”
- TLR4 + TLR9 IIRMIs used together in vaccines

“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.”
Endotoxin as an IIRMI – A Potential Shift of Paradigm?

Various structural changes (LER = Detoxification)

Reveals the dual nature of endotoxin as pyrogenic and immunogenic

MPLA - acid hydrolysis removes a phosphate and makes MPLA 100 - 2,000X less pyrogenic

Some natural endotoxins “detoxified” by virtue of their non-proinflammatory structure …

“Detoxified” is an old word, studies go back to the 1930s
Endotoxin as an IIRMI – A Potential Shift of Paradigm?

What has the body evolved to do with various endotoxins?

- Secondary acyl groups prevalent in the most potent LPS types
- But potent types not likely contaminants…

Partitioning, i.e. spine is inches from the gut -100 trillion bacteria – contrasts HSC

Mammals ultimate worry: “mucosal types” (Enterobacteriaceae) – Keep IN

...body is not ignoring non-hexa-acyl types, just not in a panic…

Figure 1. Lipid A Structure, Bacterial Habitat, and Host Recognition
Some non-pyrogenic, naturally occurring, endotoxin types are expected to be immunogenic…

“Naturally occurring low biologically reactive lipopolysaccharide (LBR LPS) forms are known to function through TLR4 which directly activates B cells and indirectly activates naive T cells through APCs. Therefore, LBR LPS forms are attractive candidate molecules for future adjuvant study.”

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**Endotoxin as an IIRMI – A Potential Shift of Paradigm?**

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Endotoxin as an IIRMI – A Potential Shift of Paradigm?

“A-mAb” Dosing from Package Insert:

- Patients pre-medicated to minimize severity of infusion reactions:
- A-mAb - 1 or 4 mg/mL infusion, max. of 400 mg/hour
- in LVP of NaCl or dextrose
- So steroid is in one infusion solution and mAb is in another

This is a large volume of stuff going in at once… Shows the contrast of historic vs. current Biologics testing…

Co-administered

Steroid
Antihistamine
Antipyretic
Uriocostatic agent
Aggressive hydration

Infusion-1
Infusion-2
LVP1
LVP2
Mab
Endotoxin as an IIRMI – A Potential Shift of Paradigm?

- Historically based on TL calculations would be considered too much:
  - >1000 EU/dose potential with 350 EU/dose limit

**Then & Now**

- FDA Requires BIOLOGICS BET testing well below TL & Recommends testing below TL for all drugs:
  - Seen in BLA responses
  - Q&A Guideline: “…dilution just above the level that neutralized the interference.”
  - SVPs going in w/ Biologics have no BLA and likely historically validated (TL = K/M)
  - LVP has permissive limit, 0.5 EU/mL
  - IIRMI view may already be affecting BET in terms of pushing limits lower…
  - The very idea of the TL is eroding for Biologics… question now is: how far below TL to test?
Summary

LER continues to polarize industry participants

Emerging IIRMI view contrasts historic pyrogen view (<85>/<151>)

Hold time studies for BLA submissions have been defined...

...is an emerging view... but not without a strong foundation.

De-masking works but is still in developmental stage for use with LAL

Eventually, I believe TP manufacturers will want LPS artifact free capabilities...
Questions?

In case of further questions, please contact our Scientific Support Team:

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  scientific.support@lonza.com

- Visit our website:
  - www.lonza.com/lal
  - www.lonza.com/ler
Interested to Learn More?

- Join us for our second **Global Endotoxin Testing Summit** from 23 – 25 May 2016
  - Who should attend: QC Directors, lab managers, key opinion leaders, users and regulators
  - What will be discussed: industry hot topics ranging from endotoxin testing basics, regulatory guidance updates, and Low Endotoxin Recovery

Learn more on: [www.lonza.com/endosummit](http://www.lonza.com/endosummit)

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References

4. (Slide 13) P. Hughes, *Endotoxin Challenges – A Regulatory Perspective, PDA 9th Annual Global Conf. on Pharmaceutical Microbiology, October 2014*
7. (Slide 31) Division of Therapeutic Proteins, Office of Biotechnology Products, Center for Drug Evaluation and Research, FDA, Bethesda, MD