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Out of the Endotoxin Box: Rethinking Pyrogens

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PURPOSE

Biological responses in the Monocyte Activation Test (MAT) follow non-linear patterns, either sigmoidal (Lipopolysaccharide or LPS-type) or biphasic (LTA-type).

The MAT requires two-fold serial dilutions of samples to establish the secretory pattern of the pro-inflammatory cytokine biomarker. Measuring a single data point or sample dilution cannot provide an accurate quantification of sample pyrogen content.

Optimal sample dilution corresponds to the inflection point of the secretory pattern, is unique, non-ambiguous, and specific. Its interpolated values result in valid quantification of the sample's pyrogen concentration. All other dilutions result in equivocal paired concentrations, which should be considered invalid. This algorithm allows the determination of pyrogen contents with ineluctable or foolproof accuracy.

OBJECTIVE(S)

- Rules applied to bacterial endotoxin tests are non-applicable to biological responses, especially the validation requirement of linearity.
- To show alternative quantification methods specific to the Monocyte Activation Test (MAT), due to the fact that *biological responses to pyrogens are not linear.*

METHOD(S)

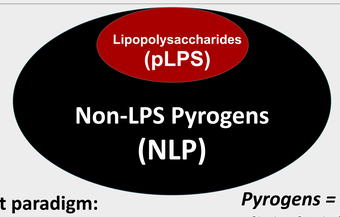
Methods: Iterative exhaustive dilutions of different ligands of pattern recognition receptors, including endotoxin and non-endotoxin pyrogens, result in two distinctive non-linear biological responses reflecting the product pyrogenicity profiles. Pooled peripheral blood mononuclear cells (PBMC) from eight healthy donors released cytokines in response to reference standard endotoxin and lipoteichoic acid (LTA).

Results: The endotoxin-induced secretory pattern of interleukin-6 (IL-6) used for pyrogen detection is consistently sigmoidal, independently of cell density, defining a characteristic biological response type designated ***"Lipopolysaccharide or LPS-type response."***

The LTA-induced secretory pattern, ***"LTA-type monocytic response",*** is characteristically biphasic (maximal response between the high and low dilutions). The pattern showed one unique bifurcation/inflection point at the optimal dilution.

Other dilutions corresponding to symmetric or asymmetric paired concentrations are considered equivocal and invalid, and cannot be used for quantification of pyrogen contents. The inflection point corresponds to the unique valid dilution/optimal concentration.

RESULT(S)



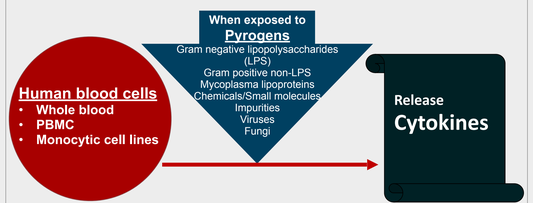
Current paradigm:
Endotoxin = Lipopolysaccharide (LPS)

Proposed new definition:
Endotoxin =
Pyrogenic lipopolysaccharide **(pLPS)**
+ **DAMP** (damage-associated molecular patterns
+ **PAMP** (pathogen-associated molecular patterns)

Pyrogens = killers of clinical trials

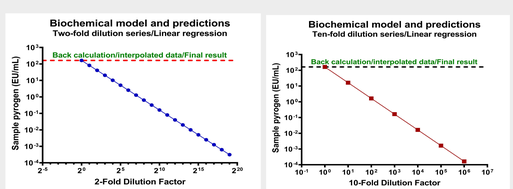
Pyrogens are substances that cause severe adverse events including:
- Fever
- Sepsis
- Multiple organ failure
- Death

The right tool for pyrogen detection:
The Monocyte Activation Test (M.A.T)



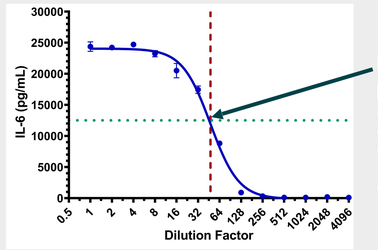
Detection and quantification of cytokines by ELISA

Conventional linear biochemical models in LAL-based detection methods



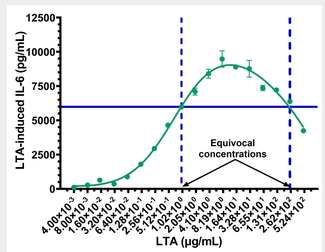
In the linear biochemical methods, 1-3 dilutions are sufficient for the quantification of the pyrogen content. For the conventional LPS detection, Limulus amebocyte lysates (LAL) are used for this detection.

Iterative two-fold serial dilutions of test article
Sigmoidal pattern: *LPS-type response*



Bifurcation or inflection point
- Unique
- Non-ambiguous
- Representative of the pyrogen content of the sample
- Valid concentration

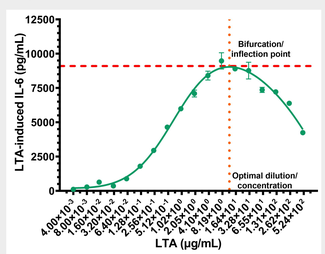
LTA-induced biphasic response pattern:
Equivocal coordinates



Existence of a unique pyrogen concentration per sample
- Inflection point
- Optimal dilution
- Valid concentration

Equivocal concentrations
- Invalid dilution pairs:
- Cannot be used for interpolation

LTA-induced biphasic PBMC response:
Optimal dilution and unambiguous concentration



- There exists a **unique pyrogen concentration** for each product, whether this pyrogen is simple or complex molecular entity.
- The bifurcation or inflection point represents a ***non-ambiguous concentration***, representative of the pyrogen content.
- This defines what we call ***"LTA-type pattern or profile"***

PyroDex Acceptance Criteria for a Valid MAT

Acceptance criterion	Specifications
Cell viability	• ≥90%
Adjusted live cell density for activation*	• >2.5 x 10^5 cells/mL
Coefficient of determination of standard curve [IL-6 *vs reference standard endotoxin* (RSE)] R^2	• ≥0.980
Iterative exhaustive serial dilutions (IESD) of sample	• ≥12 serial 2-fold dilutions
Definitive product pyrogenicity profile (DPPP or D3P) by IL-6 release from IESD	• Biphasic or • Sigmoidal

1. The pyrogen world is larger than we thought
2. **Pyrogens are "killers" of clinical trials**
3. The right tool for pyrogen detection is the Monocyte Activation Test.
4. The establishment of definitive product pyrogenicity profiles is required for an accurate evaluation of raw material pyrogenicity and of the safety status of finished products prior to their release for clinical trials or commercialization.
5. Results suggest that *iterative exhaustive serial dilutions* of parenteral drugs are required to establish this ***definitive product pyrogenicity profile*** (DPPP or D3P) characterizing the specific test article, for foolproof accuracy.

FUNDING/GRANT

FRIENDS AND FAMILY
SALES
HARBOR BANK
TEDCO

PyroDex

Our expertise: The Monocyte Activation Test. Safer drugs. Better treatments. More lives saved