MATERIAL MEDIATED PYROGENICITY AND ISO/TC 194 STANDARDS

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ASTM

ENDOTOXINS AND PYROGENS

- Pyrogens and endotoxins are not synonymous
- Pyrogens are substances that induce fever; they may be:
 - <u>Exogenous</u> (external origin) pyrogens:
 - Are of microbial origin: cell wall components from certain gram negative bacteria (andotoxin/ LPS) or gram positive bacteria or fungi
 - <u>Endogenous</u> pyrogens (produced by leukocytes in response to exogenous pyrogens): cytokines-IL1,IL2, TNF, interferon
- Endotoxins are by far the most prevalent and potent pyrogen, unique to gram negative bacteria
 Stable and withstands most sterilization procedures.



The lipopolysaccharide is part of the outer wall of Gramnegative bacteria and is released when the bacteria die or grow

HISTORY OF LAL TESTING

- First official rabbit pyrogen test published by the USP in 1942 to assess the pyrogenicity of parenteral solutions
- 1964-1968- Levin and Bang discovered a clotting mechanism in amoebocytes in presence of bacteria
- 1971- Cooper, Levin and Wagner proposed use of LAL test as an <u>alternative to rabbit pyrogen test</u>
- 1977- Use of LAL test for medical devices first appeared in the Federal Register; First federal license to LAL test manufacturer
- 1979 Mascoli and Weary reported "no LAL false negatives occurred":
 - >28,000 rabbit tests and >143,000 LAL tests
 - all pyrogens in fluids and devices were endotoxins
 - only 37 LAL failures, of which 4 also failed rabbit test
 - LAL test >100 more sensitive, accurate and specific.



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LAL TEST INVENTORS

- In 1956 Fred Bang reported that gram negative bacteria, even if killed, cause blood of the horseshoe crab (e.g. Limulus polyphemus) to become a semi-solid mass. (keen observation!)
- Later Jack Levin discovered that crab's blood cells, called amoebocytes, contain coagulogen, that is released when endotoxin is present.



Drs. Frederick B. Bang and Jack Levin

 Collecting blue blood from *Limulus* sp.
 (Atlantic horseshoe crab)

Blood cells are separated from serum by centrifugation and then placed in DH2O which causes them to swell and burst ("lyse"). The released chemicals inside the cell ("lysate") are then purified and freezedried.

Afterward crabs are returned to the ocean. It has been estimated that \sim 6% of those returned don't survive.







- Endotoxin produces an opacity and gelation that is easily recognized.
- The simplicity and economy to the LAL test encourages the testing of in-process solutions and raw materials as well as end-product drugs, devices and biologics.

RABBIT PYROGEN TEST

- Why rabbit is used:
 - Other species are not as predictable
 - Rabbit provides a reproducible pyrogenic response
 - The pyrogenic response is similar to the human response
- Detects all types of pyrogens (microbial-derived and material-mediated)

Note: rabbits used for pyrogen testing are reused for additional pyrogen tests; and then often for other endpoint tests, e.g. efficacy.

>RABBIT PYROGEN TEST

- United States Pharmacopeia (USP), European Pharmacopoeia (EP), and Japanese Pharmacopoeia (JP) methods are generally equivalent, and recommended in ISO 10993-11 Annex G (formerly Annex F)
- Brief description:
 - the test solution is prepared by extraction from test sample with physiological saline
 - an intravenous injection is administered to three rabbits¹
 - their rectal temperature is measured² for <u>3 hours</u> after injection
 - this temperature is compared with the temperature shortly before injection.

¹ Rabbits are typically restrained "with light-fitting neck stocks that allow the rabbits to assume a natural resting posture" [USP <151>] (non-invasive and with minimal discomfort to rabbits.)
 ² Today most facilities use automatic measurement via thermistor temperature sensors connected to computer software that automatically records temperatures.

^o PYROGENS

- Broadly classified:
 - Endotoxin (lipopolysaccharide, LPS)
 - Non-Endotoxin pyrogens:
 - Material-Mediated Pyrogens (MMPs) chemicals
 - Microbial components other than endotoxin (e.g. Gram-positive (lipoteichoic acid, LTA), and fungal zymosan)
- "While testing in rabbits can detect the presence of basically any pyrogen, an endotoxin test can detect endotoxins, but not other pyrogens. However, in the case of the contamination of medical devices or their materials by microorganisms, contamination by Gram-negative bacteria and other microorganisms usually occur together. Therefore, the result of an endotoxin test can predict contamination with other microorganism-derived components." [Haishima, Japanese Guidance, Part 7: Pyrogen Test]

NOTE: term "non-endotoxin pyrogens" consistently used incorrectly – should be:

- <u>non-endotoxin microbial pyrogens</u>: those originating from gram positive bacteria (LTA, lipoteichoic acid) and fungi; and
- <u>material-mediated pyrogens</u>: chemicals which may leach from medical materials during use and directly initiate a pyrogenic response.

>BACKGROUND ON THE ISO 10993-11 ANNEX ON PYROGENICITY

- Several years ago a group of us ISO/TC 194 WG 7 delegates discussed having never
 seen a non-endotoxin related positive Rabbit Pyrogen Test
 - We wanted to eliminate the rabbit test if possible
 - A Task Group was formed headed by me to investigate the existence of known materialmediated pyrogens within each of our companies. A list of known MMPs was assimilated
 - The WG decided to create an annex to ISO 10993-11 to 1) explain what MMPs are not, and 2) provide the list of known MMPs and state that if your device does not contain any on the list, your would not need to perform the Rabbit Pyrogen Test
 - However, the FDA member countered stating that these are the known MMPs at this time.
 If there are new polymers created, there could be additional MMPs
 - ISO 10993-11Annex F was published in 2006 and slightly revised in 2017

ISO 10993-11 ANNEX G (INFORMATIVE) INFORMATION ON MATERIAL-MEDIATED PYROGENS:

Pyrogenic responses may be: material-mediated, endotoxin-mediated, or mediated by other substances, such as components of gram-positive bacteria and fungi or chemicals.

ISO 10993-11 is concerned only with <u>material-mediated pyrogenicity</u>, whereby the material itself elicits a pyrogenic response.

Only the Rabbit Pyrogen Test is suitable for the detection of material-mediated pyrogenicity.

It is not necessary to test all new medical devices for *in-vivo* (rabbit) pyrogenicity. However, materials containing <u>new</u> chemical entities, and/or those which have previously elicited a pyrogenic response, should be evaluated for material-mediated pyrogenicity. Also for medical devices which may be used in combination products, testing of product pyrogenicity should be considered.

Endotoxin contamination may be a source of a pyrogenic response, and should not be confused with a material-mediated pyrogenicity.

For endotoxin-mediated pyrogenicity BET/LAL testing/ not the rabbit test is performed.

>ISO 10993-11, ANNEX G: Material-mediated pyrogenicity:

The following is a list of substances which are known (and verified) to generate a pyrogenic response, without being endotoxins:

- endogenous pyrogens (e.g. IL-1, IL-6, TNFα, INF-γ);
- prostaglandin;
- inducers (e.g. polyadenylic, polyuridylic, polybionosinic and polyribocytidylic acids);
- substances disrupting the function of thermoregulatory centers (e.g. LSD, cocaine, morphine);
- uncoupling agents of oxidative phosphorylation (e.g. 4, 6-dinitro-o-cresol, dinitrophenol, picric acid);
- N-phenyl- β -naphthylamine and aldo- α -naphthylamine (the febrile mechanism is unknown);
- bacterial exotoxins (e.g. TSST-1, SEA, Spe F, Spe C);
- neurotransmitters (e.g. noradrenaline, serotonin)
- Metals such as nickel salts, in some instances

DR. YUJI HAISHIMA'S RECENT COMMENTS ON MMPs IN ISO 10993-11 ANNEX G:

- All chemicals listed other than uncoupling agents and naphthylamines categorized into "Material-mediated pyrogenicity" are pyrogens that directly stimulate the thermoregulatory center in the brain through the hypothalamus. Not aware of any new additional chemicals.
- These chemicals are not currently used as materials for medical devices. Naphthylamines had been used in rubber (ex. esophageal catheter) as anti-aging agents, but the chemicals are no longer used because of their potential carcinogenicity. Cytokines are involved in regenerative medical products consisting of living cells, but the products are not medical devices.
- Listed MMPs other than cytokines and exotoxins are not likely to be detected by the MAT, because the substances do not act as agonists of TLRs, notch or other signaling pathways for inducing immune response. However, cytokines are also induced by immune cells by other stimulation such as stress or cell toxicity, and hence it may be meaningful to verify the reactivity of MMP in MAT.

BACKGROUND ON ISO WG 16 AND Draft TR 21582, Pyrogenicity — PRINCIPLE AND METHOD FOR PYROGEN TESTING OF MEDICAL DEVICES

- ISO established WG 16 in 2007 to develop a Technical Report on all types of pyrogenicity testing of medical devices.
- The WG was headed by Dr Wendel, co-inventor of the MAT test. Dr. Wendel wrote the first draft.
- Dr. Vicki Hitchins replaced Dr. Wendel as Convener.
- The second draft in 2008 based on a proposal by Dr. Yuji Haishima and 2 other Japanese experts, was balloted and approved for publication in 2010.
- ISO Secretariat, despite several inquiries from we WG members never forwarded it within ISO for publication.

BACKGROUND ON ISO WG 16 AND Draft TR 21582, Pyrogenicity — PRINCIPLE AND METHOD FOR PYROGEN TESTING OF MEDICAL DEVICES, CONT.

- In 2016 we were told that it had "dropped off" the ISO work plan
- To rectify we re-balloted it (including some edits made by Dr. Hitchns in 2012) in May 2016, ballot was approved.
- Vicki Hitchins passed away in June 2016
- Kim Darnell was appointed new WG Convener
- US WG 16 held meeting June, 27, 2016 and resolved US comments
- Planned WebEX meeting to address international comments (5 non-US countries) was never held
- WG 16 will meeting at the ISO/ TC 194 meeting in December 2018 to finalize TR 21582

CONTENTS OF DTR 21582:

- Characterization of pyrogen
 - Bacterial endotoxin
 - Microbial components other than endotoxin
 - Pro-inflammatory cytokines
 - Chemical agents and other pyrogens
 - Principle of febrile reaction
- Assessment of pyrogenicity
 - Bacterial endotoxin test
 - Principle of LAL reaction
 - General procedure of LAL test
 - Properties of the LAL test
 - Rabbit pyrogen test
 - Principle of the rabbit test
 - Procedure of the rabbit test
 - Characteristic of the rabbit test
 - Human cell-based pyrogen test (HCPT)
 - Principle of the HCPT
 - Selection of human cells
 - Selection of marker cytokine
 - Procedure of HCPT
 - Characteristic of the HCPT
 - Validation study
 - Conclusion

^oISO DRAFT TECHNICAL REPORT 21582, INTRODUCTION:

- Material-mediated pyrogenicity represents a systemic effect that is included in ANNEX-F-G of ISO 10993-11, but efforts have been taken to generally address pyrogenicity testing in a technical report.
- A pyrogenic response is the adverse effect of a chemical agent or other substance, such as microbial component to produce a febrile response. Tests for a pyrogenic response have been required to evaluate the safety of products that have direct or indirect contact to blood circulation and the lymphatic system, and interact systemically with human body.
- At present, the in vivo rabbit pyrogenicity test and the in vitro bacterial endotoxin test are available as accepted methods for evaluating the pyrogenicity of medical devices and their materials. Basic procedures, including sample preparation of each test article, are already established, internationally harmonized, and mentioned in the related guidelines and pharmacopoeia of each country such as USP, EP, and JP.
- Recently, a new in vitro pyrogen test using human immune cells, the so-called human cell-based pyrogen test (HCPT), has been developed and applied to pyrogen testing of parenteral drugs in Europe.
- Therefore, the concept of the application of pyrogen testing for medical devices is being considered due to the direct immersion of the material in human blood (HCPT). Hence, the advantages and disadvantages of the various pyrogen tests and their applications to date are systematically described in
 this document.

^oSTATEMENTS INCLUDED IN DTR 21582;

- Since febrile responses induced by TLR agonists are mediated by pro-inflammatory cytokines such as TNFa, IL-1b, IL-6, and INF-g produced by human immune cells, the endogenous mediator itself naturally acts as pyrogen
- Pyrogenicity of chemicals or natural substances other than microbial components is not well known. In addition, over 1,000 new compounds are discovered or synthesized each year world wide, but the biological properties of each compound are not well understood.
- Most chemicals currently used as biomaterials for medical devices, are safe and are nonpyrogenic to humans. However, it can be possible that some new biomaterials and chemicals can cause febrile reaction to human.
- This possibility holds also true for non-autologous cellluar products which can evoke immunological recognition and activation of immune-competent cells.

DTR 21582 LIST OF MMPs SAME AS THOSE IN ISO PART 11, ANNEX G:

- Without cytokines and exotoxins:
- enclogenous pyrogens (e.g. IL-1, IL-6, TNFα, INF-γ);
- prostaglandin;
- inducers (e.g. polyadenylic, polyuridylic, polybionosinic and polyribocytidylic acids);
- substances disrupting the function of thermoregulatory centres (e.g. LSD, cocaine, morphine);
- uncoupling agents of oxidative phosphorylation (e.g. 4, 6-dinitro-o-cresol, dinitrophenol, picric acid);
- N-phenyl- β -naphthylamine and aldo- α -naphthylamine (the febrile mechanism is unknown);
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- neurotransmitters (e.g. noradrenaline, serotonin)
- Metals such as nickel salts, in some instances

DTR 21582: HCPT ADVANTAGES COMPARED WITH RPT & BET:

- HCPT does not use animals, and is able to predict the potency of febrile reaction directly in human body with high sensitivity.
- HCPT has a wide spectrum of pyrogen detection as compared with the bacterial endotoxin test.
- HCPT is available for the detection of pyrogenic contaminants in certain products that are not evaluated by the rabbit test or the bacterial endotoxin test (for example, inhibitors and enhancers to the LAL reaction, drugs that influence the central or peripheral mechanisms of body temperature regulation and cause immunological responses in the rabbit, and to solid materials).
- HCPT is able to test solid materials directly as test sample without any extraction, because immune cells recognize both pyrogens eluted from the test sample into a culture medium and those bound to the surface. Pyrogens are effectively detected without any pre treatment of the materials adsorbing endotoxin.

Sold DTR 21582, HCPT DISADVANTAGES:

- Material-mediated pyrogens that are chemical agents do not operate through the cytokine network to induce a febrile reaction and most likely will not be detected on the HCPT.
- Drugs that interact with monocytes or macrophages (for example, cytokine receptor antagonists, non-physiological solutions, cytotoxic agents, recombinant proteins with cytokine activity) or the detection system (for example, rheumatic factors), may not be tested with HCPT.
- HCPT may be not applicable to tissue-engineered products containing living cells that release cytokines and chemokines.
- The response to pyrogen in this test can be dependent on the donor of the blood sample or cell conditions. Particularly, whole human blood may vary due to differences in donors age, gender, genetic background (genetic polymorphisms in genes coding for Toll-like receptors cytokine receptors, etc.), safety issues with infected donors, diurnal variation, influence of diet, and other factors which may influence the sensitivity and specificity of the whole blood in vitro tests.
- The whole blood supply system can be a problem.
- HCPT using solid samples directly may be unavailable for routine quality control testing of batches of large finished, sterilized products/devices for presence of pyrogen contamination.
- HCPT using human myelomonocytic cell lines has the disadvantages of time, cost and technical complication for preculture and priming of the cells.
- NOTE The only reliable positive control is LPS; controls for assessing interference for other pyrogens is generally unknown.

ISO DTR 21582 VALIDATION STUDY AND CONCLUSION:

Validation Study

 A further validation study can be required to determine whether the HCPT can detect other pyrogens that induce a febrile reaction by a different mechanism other than the TLR signaling pathway and phagocytosis, including material-mediated pyrogens.

Conclusion

 In some cases, the HCPT can be a useful alternative to traditional pyrogenicity test methods (rabbit and LAL); however, the rabbit test will need to be retained for detection of pyrogens not detected by the HCPT, including material-mediated pyrogens. Therefore, it is very important that the appropriate method is selected based on the purpose of pyrogen test of medical devices and their materials.

² JAPANESE GUIDELINE*, PART 7, PYROGEN TEST:

- HCPT* can detect any pyrogen that is recognized by receptors involved in the host defense mechanism such as TLRs, which are expressed on the cytoplasmic membrane of immunocompetent cells including monocytes and macrophages.
- In HCPT, ELISA is used for detection and quantification of proinflammatory cytokines (e.g., IL-1b, IL-6 and TNFa), which are produced by immunocompetent cells such as macrophages when they are activated by various TLR agonists, as markers of pyrogenic activity
- <u>It may be possible to use HCPT to evaluate the effect of microparticles</u> on the body, such as wear debris, which are removed by macrophage phagocytosis.
- However, <u>because of their characteristics</u>, HCPT is highly unlikely to detect materials that induce fever independently of cytokine networks; namely, material-mediated pyrogens.
- <u>Furthermore, HCPT has certain disadvantages</u>. For example, they cannot be used to evaluate the pyrogenicity of specimens containing materials that affect cells or regenerative medicine products consisting of live cells. They also have limitations in terms of the size of specimens that can be examined using direct method.

C*HCPT: Human Cell based Pyrogen Test

CASES OF FEVER INDUCED BY CHEMICAL SUBSTANCES

[JAPANESE GUIDANCE *, PART 7]

- Only a few cases of febrile reaction induced by chemical substances have been reported for medical devices, e.g:
 - Shimohira, et al. reported that N-phenyl-b-naphthylamine and aldol-a-naphthylamine used in rubber as anti-aging agents were both pyrogenic in rabbits and the peak of increase in body temperature was 1 to 2 hours after injection¹³⁾.
 - They also reported that N-phenyl-b-naphthylamine was actually detected from the rubber of esophageal catheters. However, these naphthylamines are no longer used because of their potential carcinogenicity.
- Other chemical substances that induce an increase in body temperature are as follows¹⁴):
 - 4,6-Dinitro-o-cresol used as a pesticide
 - dinitrophenols used as intermediates of black sulfur dyes
 - o-,m-,and p-Nitrophenols, used as intermediates in organic synthesis and for antifungal agents and insecticides
 - picric acid used for production of insecticides and dyes
 - Neurotropic substances such as LSD and morphine are known to increase body temperature by directly acting on the CNS and disturbing the thermoregulatory mechanisms.

JAPANESE GUIDELINE*, PART 7, PYROGEN TEST, CONT.:

• "In the case of so-called synthetic polymers, although extremely rare, the possibility that pyrogenicity may result from chemical substances as well as natural pyrogens cannot be excluded. Therefore, in order to determine if material-mediated pyrogens are also present, the rabbit pyrogen test must be conducted."

*MHLW OMDE Yakushokuki-hatsu 0301 No.20, Guidance on Test Methods for Biological Safety Evaluation of Medical Devices (2012)

>MY CONCLUSIONS:

- In this talk I have tried to present the information currently contained in ISO (and JP) standards/drafts on medical device pyrogenicity testing.
- It appears that mechanistically MAT/HCPT tests are unlikely to detect the rare chemicals (MMPs) which directly stimulate the thermoregulatory center in the brain without going through the cytokine/ monocyte – macrophage pathway.
- Therefore, unless proven otherwise with a validation study using known MMPs, the replacement of the Rabbit Pyrogen Test with the MAT test can not be made.
- The MAT test appears to be a valid alternative to the LAL test, with the added ability to detect non-endotoxin microbial pyrogens (e.g. LTA, fungi components).