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Pyrogenicity — Principles and methods for pyrogen testing of medical devices

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Contents

Page

Foreword

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This document was prepared by Technical Committee ISO/TC 194, *Biological and clinical evaluation of medical devices*.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

At present, safety assessments of medical devices are guided by the toxicological and other studies recommended in the ISO 10993 series of standards.

Material-mediated pyrogenicity represents a systemic effect that is included in of ISO 10993-11:2017, Annex G, but efforts have been taken to generally address pyrogenicity testing in this document.

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, cerebrospinal fluid (CSF) 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 pharmacopoeias.

Recently, an *in vitro* pyrogen test using human immune cells, the human cell-based pyrogen test (HCPT), has been developed and applied for pyrogen testing of parenteral drugs. The concept of the application of pyrogen testing for medical devices is being considered due to the direct or indirect exposure to human blood cells (HCPT).

Pyrogenicity — Principles and methods for pyrogen testing of medical devices

1 Scope

This document specifies the principles and methods for pyrogen testing of medical devices and their materials.

2 Normative references

There are no normative references in this document.

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— IEC Electropedia: available at https://www.electropedia.org/

— ISO Online browsing platform: available at https://www.iso.org/obp

3.1
medical device
instrument, apparatus, implement, machine, appliance, implant, *in vitro* reagent or calibrator, software, material or other similar or related article, intended by the manufacturer to be used, alone or in combination, for human beings for one or more of the specific purpose(s) of

— diagnosis, prevention, monitoring, treatment or alleviation of disease;

— diagnosis, monitoring, treatment, alleviation of or compensation for an injury;

— investigation, replacement, modification, or support of the anatomy or of a physiological process;

— supporting or sustaining life;

— control of conception;

— disinfection of medical devices;

— providing information by means of *in vitro* examination of specimens derived from the human body;

and does not achieve its primary intended action by pharmacological, immunological or metabolic means, in or on the human body, but which may be assisted in its function by such means.

Note 1 to entry: Products which may be considered to be medical devices in some jurisdictions but not in others include:

— disinfection substances;

— aids for persons with disabilities;

— devices incorporating animal and/or human tissues;

— devices for *in vitro* fertilization or assisted reproduction technologies.

[SOURCE: GHTF/SG1/N071:2012, 5.1]

3.2
pyrogen
substance that causes fever

3.3
pyrogenicity
ability of a chemical agent or other substance to produce a febrile response

3.4
febrile response
temperature above the normal range due to an increase in the body's temperature set point

Note 1 to entry: It is also referred to as fever or pyrexia.

3.5
oxidative phosphorylation
metabolic pathway in most aerobic organisms, which uses enzymes to oxidise nutrients to release energy

4 Abbreviated terms

COX	Enzyme cyclooxygenase
CpG	Cytosine (C) next to guanine (G) in the DNA sequence, with the p indicating that C and G are connected by a phosphodiester bond.methyl group to the 5 position of the cytosine pyrimdine ring
ELISA	Enzyme llinked immunosorbent assay HCPT, e.g. monocyte activation test (MAT)
IKK	IkB kinase, an enzyme complex involved in propagating cellular response to inflammation
IRAK	Interleukin-1 receptor-associated kinase
LAL	Limulus amebocyte lysate
LPS	Lipopolysaccharide
MD-2	Molecule secreted glycoprotein that binds to extracellular domain of TLR4
MCP	Macrophage chemotactic protein
MIP	Macrophage inflammatory protein
NOD	Nucleotide-binding oligomerization domain
PGE_2	Prostaglandin E_2
RANTES	Regulated on activation, normal T-expressed and Secreted
RNA	Ribonucleic acid
SEA	Staphylococcal enterotoxin A
Spe C	Streptococcal pyrogenic exotoxin C
Spe F	Streptococcal pyrogenic exotoxin F
TBK	TANK binding kinase

TLR Toll-like receptor

TNF Tumour necrosis factor

TSST Toxic shock syndrome toxin

5 Characterization of pyrogen

5.1 General

On the basis of pyrogen origin, febrile response can be divided into three groups:

a) material-mediated pyrogenicity caused by chemical agents;

b) endotoxin-mediated pyrogenicity;

c) pyrogenicity mediated by microbial components other than endotoxin.

Non-endotoxin-mediated pyrogenicity corresponds to a generic name of febrile responses originating from a) and c) above. However, the latter can be clearly distinguished from material-mediated pyrogenicity, because the febrile reaction is originated from microbial contamination.

TLRs are proteins that constitute an important part of the immune system against microbial infections, closely relate to pyrogenicity of microbial components. Thirteen kinds of human TLRs from TLR1 to TLR13 and the agonists to some of them have been identified to date. Most pyrogens that can be assessed in the field of medical devices can be bioactive substances derived from microorganisms present as contaminants of the device manufacturing process or present in materials. Since the components are TLR agonists and act as pyrogen to human, the knowledge for TLRs is very significant for understanding pyrogens.

5.2 Bacterial endotoxin

Bacterial endotoxin, an important component of the outer membrane of Gram-negative bacteria, is the most powerful pyrogen recognized by TLR4. Endotoxin is a modulator of the host immune response and exhibits a variety of biological activities, for example, activation of macrophages, mitogenicity and adjuvanticity, causing Schwartzman reactions in addition to pyrogenicity. From the clinical standpoint, endotoxin causes sepsis, septic shock and multiple organ failure, which are systemic disorders with a high mortality rate.

Endotoxin generally consists of a heteropolysaccharide part subdivided into an O-specific chain, a core oligosaccharide, and a lipid component called lipid A that is a biologically active centre of endotoxin. The potency of endotoxin is influenced by acylation and phosphorylation patterns, and the presence/absence of polar-head group bound to phosphate residue in lipid A molecule. In addition, endotoxin has species-specificity for the expression of its bioactivity.

In the natural world, Gram-negative bacteria are widely distributed in water (rivers and sea), air, soil, and also human body. It is likely therefore that biomaterials made of natural substances are contaminated with the bacteria and their components. Autoclaving, irradiation and gas sterilization during the manufacturing process are able to kill the bacteria. However, microbial components, particularly endotoxin cannot be inactivated by such ordinary sterilization methods, and once contaminated it is quite difficult to remove the endotoxin during the manufacturing process. During the manufacturing process, endotoxin contamination can be reduced or eliminated by depyrogenization (e.g. 250 °C for 30 min, use of chemicals to inactivate endotoxin such as polymyxin-B[50][66] or by using endotoxin-free water in the washing and manufacturing processes.

5.3 Microbial components other than endotoxin

Microorganisms produce various bioactive substances other than endotoxins. Lipoteichoic acid, an important component of the outer membrane of Gram-positive bacteria, represents a counterpart to endotoxin and acts as a pyrogen recognized by TLR2 that interacts and forms a heterodimer with TLR1 or TLR6. Lipoproteins, lipopeptides, and lipoarabinomannan that are the cellular components of various microorganisms are also known to act as TLR2 agonists. Although peptidoglycan that constructs cell wall of Gram-positive and Gram-negative bacteria was considered as TLR2 agonist, it is recently suggested that NOD1 and NOD2 proteins can play a role of mediating the expression of its bioactivity rather than TLR2. In addition, viral double-stranded RNA, bacterial flagella, and bacterial and viral CpG DNA have been identified as the agonists of TLR3, TLR5, and TLR9, respectively, and all of them acts as pyrogens to human. Although pyrogenicity has not been reported for any kind of (1,3)-β -D-glucan preparation, it can be noted that certain kinds of (1,3)-β -D-glucan can enhance endotoxin toxicity.

It has been reported that exotoxins and enterotoxins such as TSST-1, SEA, Spe F, and Spe C produced by various pathogenic microorganisms cause febrile response in the human body by the toxin-specific manner that can be different from TLR signal transduction. There was an outbreak of inflammation, fever and peritonitis in some patients due to contamination of solution with peptidoglycan during dialysis[52][76].

5.4 Pro-inflammatory cytokines

Since febrile responses induced by TLR agonists are mediated by pro-inflammatory cytokines such as TNFα, IL-1β, IL-6, and INF-γ produced by human immune cells, the endogenous mediator itself naturally acts as pyrogen. Each cytokine further activates immune cells through the cytokine network, because receptors specific to the cytokines are located on the cell surface of monocytes and macrophages in addition to TLRs.

5.5 Chemical agents and other pyrogens

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 worldwide, but the biological properties of each compound are not well understood. Most chemicals currently used as biomaterials for medical devices, are safe and are non-pyrogenic to humans. However, it is possible that some new biomaterials and chemicals can cause febrile reaction to human.

This possibility also holds true for non-autologous cellular products which can evoke immunological recognition and activation of immune-competent cells.

As an example, chemicals that are known to induce febrile reaction in humans are listed below. These chemicals can be divided mainly into three groups according to principle for inducing a febrile response:

a) agents that directly stimulate thermoregulatory centres of the brain and nervous system,

b) uncoupling agents of oxidative phosphorylation, and

c) pyrogens with mechanisms that are not well known.

The chemicals listed below are known to cause a febrile response in humans:

— prostaglandins;

— inducers (e.g. polyadenylic, polyuridylic, polybionosinic, and polyribocytidylic acids);

— substances disrupting the function of thermoregulatory centres (e.g. lysergic acid diethylamide, cocaine, morphine);

— neurotransmitters (e.g. noradrenaline, serotonin);

— 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);

— metals such as nickel salts, in some instances.

In addition to these chemicals, there is a possibility that microspheres[23] and nanoparticles,[61] including implant-derived wear debris,[7] can act as pyrogens. Microspheres, particles[23] and nanoparticles[61] consisting of specific sizes could be phagocytosed by macrophages and activate macrophage-released pro-inflammatory cytokines such as TNFα. TNFα is one of the endogenous pyrogens.

5.6 Principle of febrile reaction

TLRs are a class of single membrane-spanning non-catalytic receptors that recognize structurally conserved molecules derived from microbes once they have breached physical barriers such as the skin or intestinal tract mucosa and activate immune cells. They are believed to play a key role in the innate immune system and are known to function as dimers. Although most TLRs appear to act as homodimers, TLR2 forms heterodimers with TLR1 or TLR6, each dimer having different ligand specificity. TLRs can also depend on other co-receptors for full ligand sensitivity, such as in the case of TLR4's recognition of endotoxin, which requires a MD-2 molecule. CD14 and LPS binding protein are known to facilitate the presentation of endotoxin to MD-2. When activated, TLRs recruit adapter molecules within the cytoplasm of cells in order to propagate a signal. Four adapter molecules are known to be involved in signalling. These proteins are known as MyD88, Tirap (also called Mal), Trif, and Tram. The adapters activate other molecules within the cell, including certain protein kinases (IRAK1, IRAK4, TBK1, and IKKi) that amplify the signal, and ultimately lead to the induction or suppression of genes (NF-κB, AP-1, and IRP3) that orchestrate the inflammatory response.

Following activation by ligands of microbial origin, several reactions are possible. Immune cells can produce cytokines that trigger inflammation. Particularly, IL-1β is closely associated with induction of febrile reaction. IL-6 and TNFα were isolated later and found to be pyrogenic cytokines as well, although at much higher doses[28],[29]. The current understanding of the mechanism of fever in mammals is that these proinflammatory cytokines result in the expression of the COX-2 which mediates PGE_2 synthesis.[30] Mice deficient in COX-2 do not develop fever in response to LPS, IL-1 or IL-6[47], and[48] PGE_2 triggers an intracellular signalling cascade that changes the set point of body temperature. Thus, IL-1β, IL-6 and TNFα are the mediators released by immune cells upon contact with pyrogens that are responsible for triggering the fever reaction in the brain. Substance P is known to induce fever through the production of TNF-α, IL-6 and PGE2[17].

TLRs seem to be involved in the cytokine production and cellular activation as well as in the adhesion and phagocytosis of microorganisms and other potential pyrogens.

Independent of TLR signalling pathway and subsequent cytokine production, body temperature could be increased by agents that directly stimulate thermoregulatory centres. Also, uncoupling agents of oxidative phosphorylation can increase body temperature as a result of activating the electron transport chain in mitochondria.

6 Assessment of pyrogenicity

6.1 General

There are three methods used for pyrogenicity testing, which are described below. The *in vivo* rabbit pyrogen test is the only test that directly measures the febrile response in the body as an end point, in accordance with the definition of a pyrogen, which the other two methods do not. Instead the *in vitro* methods detect pyrogens using different end points, such as cytokine production and protein coagulation.