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Pyrogenicity — Principles and methods for pyrogen testing of medical devices

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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.

6.2 Bacterial endotoxin test (BET)

6.2.1 General

The bacterial endotoxin test is harmonized with several pharmacopaeia. This test is to detect or quantify bacterial endotoxin of Gram-negative bacterial origin using a lysate reagent that is an aqueous extract of circulating amebocytes of the horseshoe crab (*Limulus polyphemus* or *Tachypleus tridentatus*). The bacterial endotoxin test is technically divided into two methods; one is the gel-clot technique based on gel formation by the reaction of the lysate reagent with endotoxins, and other is the photometric technique originating from endotoxin-induced optical changes of the lysate reagent. The latter is further subdivided into two methods: one is turbidimetric technique measuring the endotoxin concentrations of sample solutions based on the measurement of turbidity change accompanying the gel formation, and other is chromogenic technique estimating the endotoxin concentrations by measuring optical density of the colour of chromophore released from a synthetic chromogenic substrate that is a substituent for the final step of the enzymatic cascade reaction described below. Each photometric technique is classified as either end point or kinetic method.

The bacterial endotoxin test can be used to monitor endotoxin contamination in manufacturing processes of medical devices and the final products from the viewpoint of routine quality control. Before starting use of the lysate reagent extracted from *Tachypleus tridentatus*, this test was termed Limulus amoebocyte lysate (LAL) test in the past.

NOTE Other methods are available for the detection of bacterial endotoxins, for example, the fluorescent method using recombinant Factor C, see Reference [6].

6.2.2 Principle of LAL reaction

The BET is an enzymatic cascade reaction that has the highest sensitivity to detect and quantify Gram-negative bacterial endotoxins. First, factor C, endotoxin-sensitive serine protease zymogen present in the lysate reagent, is activated by endotoxin. The activated factor C converts factor B from the inactive form to the active form that further converts proclotting enzyme to clotting enzyme. Finally, the clotting enzyme converts coagulogen to coagulin that leads to gel formation. In addition to factor C, original lysate reagent contains factor G that is activated by (1,3)-β-D-glucans and subsequently converts proclotting enzyme to clotting enzyme. Endotoxin-specific LAL reagent has been developed by removing factor G or saturating its function.

Since the BET is based on an enzymatic reaction, it is influenced by the temperature and pH of sample solution, and it is also enhanced or inhibited by various compounds such as protease, protease inhibitors, metal ions, surfactants, chelates, salts and sugars if they are present in sufficient concentrations. Therefore, tests for interfering factors can be performed to check the presence of inhibitors and enhancers of the reaction in sample solution. The effect of the interfering factors can be avoided by the dilution of the sample solution.

6.2.3 General procedure of BET

General methods for detection and quantification of endotoxin have been discussed in pharmacopoeias in several countries and AAMI ST 72. The details are referred to in these documents.

It is noted that pyrogen-free water can be used as an extraction medium for preparing sample solution unless otherwise specified. Endotoxin present in medical devices and their materials typically are extracted at the ambient temperature.

6.2.4 Properties of the BET

The LAL assay is available as a simple and quick test with high-sensitively detect to endotoxin, indicating the presence of Gram-negative bacterial contamination on medical devices. Endotoxin has species-specificity for expression of the toxicity depending upon chemical structure of lipid A portion. Since the structural requirement of endotoxin on LAL activity is not strict compared with other biological

properties of endotoxin such as cytokine release from inflammatory cells, the BET exhibits a wider spectrum of endotoxin detection than HCPT[16].

Endotoxin contamination on medical devices can be indicative of more general microbiological contamination. However, there is no correlation between the amount of endotoxin on a device and the number of other microorganisms on a device. The BET is very specific for Gram-negative bacteria or endotoxin contamination. Historically, the predominant and most potent pyrogenic contaminants in the manufacturing of medical devices that can be controlled are bacterial endotoxins. Bacterial endotoxin contamination is difficult to prevent, because it is ubiquitous in nature, stable, and small enough to pass through conventional sterilizing filters.

However, it could be considered that the property of BET described above provides not only merit, but also a disadvantage. The most important disadvantage is that the BET cannot detect pyrogens other than endotoxin. In addition, since the knowledge of the kind of active endotoxin to human is limited due to the highly structural requirement for expression of the toxicity, then the BET is not always reflective of the human pyrogenic response.

Device manufacturers extract endotoxin from medical devices and know that the recovery extraction efficiency of endotoxins from devices is less than 100 %. In some cases, endotoxin adsorbed on to certain plastic, ceramic, or metal and certain natural products are not efficiently recovered by general extraction methods using water. However, the extraction parameters recommended by both the US. Food and Drug Administration and other national Pharmacopeia, according to regulatory evidences proven through years of use, have been sufficient in assuring the non-pyrogenicity of medical devices at the specified limits for the intended use of the device[20].

6.3 Rabbit pyrogen test

6.3.1 General

The rabbit pyrogen test is the only known test to detect material-mediated pyrogens, as well as pyrogens caused by microorganisms.

The positive pyrogenicity of the high-temperature extract in this test suggests the presence of non-endotoxin pyrogens in the products. The rabbit test does not cover methods to quantify endotoxin in the final products as a routine quality control test.

As stated in ISO 10993-11, medical devices containing new substances that have previously elicited a pyrogenic response, and/or new chemical entities where the pyrogenic potential is unknown can be evaluated for material-mediated pyrogenicity.

6.3.2 Principle of the rabbit test

After intravenous injection of a physiological saline extract from medical devices or their materials, core body temperature is kinetically measured to evaluate the *in vivo* mammalian febrile response to pyrogens.

6.3.3 Procedure of the rabbit test

The protocol for testing is addressed in the related guidelines and pharmacopoeias. Although the different country guidelines/pharmacopeias vary in restriction for body weight of rabbits, determination of baseline temperature, injection dose of sample solution, and reuse of rabbits, most parts of the testing protocols are very similar. Sample/solvent ratios and extraction conditions applicable to prepare the extract from medical devices and the materials are also defined in ISO 10993-12.

It is noted that only pyrogen-free physiological saline can be used as an extraction medium for preparing sample solution. The highest temperature among the conditions of ISO 10993-12 that does not lead to significant deterioration of the material can be used in extraction.

6.3.4 Characteristic of the rabbit test

Basically, the rabbit test can detect all kinds of pyrogens, and the real potency of the febrile response. This response induced by each pyrogen is estimated as *in vivo* core body temperature increase in the rabbit. To this end, although species-specificity to pyrogens is different between humans and rabbits, the rabbit *in vivo* test seems to have an advantage to HCPT. However, the rabbit test has disadvantages such as low sensitivity, use of animals and requires a large amount of test article.

The rabbit pyrogen test is the only known and validated method for the detection of material mediated pyrogens, chemical leachables from a material capable of causing a pyrogenic (febrile) reaction in a patient. New blood-contacting or implantation materials/ devices can be screened for material-mediated pyrogens.

However, in most cases the rabbit pyrogen test is not used in lieu of the BET.

6.4 Human cell-based pyrogen test (HCPT)

6.4.1 General

HCPT is based on immunological reaction at the cellular level and is *in vitro* method for detecting and quantifying pyrogens inducing febrile reaction through activation of human immune cells such as monocytes and macrophages[13]. This test is designed to evaluate endotoxin-mediated pyrogenicity and pyrogenicity mediated by other microbial components corresponding to a febrile response induced by TLR agonists. In addition to TLR agonists, there is a possibility that HCPT can quantify the potency of the inflammatory response cascade initiated by phagocytosis of microspheres and wear particles.

Monocytes and macrophages are *in vivo* primary targets of endotoxin, and a number of investigations have been performed to elucidate the relationship between chemical structure and biological activity of endotoxin by using macrophage cells to date. The basic technique itself used in HCPT is based on traditional methods in endotoxin research. Since this test exhibits a wider spectrum compared with endotoxin test, it can be useful as a method to bridge the gap between endotoxin test and rabbit test if appropriately validated.

6.4.2 Principle of the HCPT

Macrophages are cells within the tissues that originate from specific white blood cells called monocytes. Monocytes and macrophages are phagocytic, acting in non-specific defence (or innate immunity) as well as helping to initiate specific defence mechanisms (or cell-mediated immunity) of vertebrate animals. Their role is to phagocytize cellular debris and pathogens either as stationary or mobile cells and to recognize soluble pathogens through TLRs located on the cell surface and to stimulate lymphocytes and other immune cells to respond to the pathogens. As secretory cells, monocytes and macrophages are vital to the regulation of immune responses and the development of inflammation. These cells are activated by phagocytosis or TLR agonists (see 4.2 and 4.3) to produce powerful chemical substances including cytokines. Cytokines such as TNFα, IL-1β, and IL-6 as regulatory factors induce a febrile response according to the mechanism described in 4.6. In the HCPT, cytokines released from these cells are detected and quantified by an ELISA method as a measure for evaluating the potency of the febrile reaction.

6.4.3 Selection of human cells

In addition to human whole blood, human myelomonocytic cell lines such as, MM6[1)], and human mononuclear cells[2)] [72] are also available as indicator cells for HCPT. From the point of laboratory use

1) German Collection of Microorganisms and Cell Cultures (DSMZ; Cat. No. ACC 124), Braunschweig, Germany

2) The Monocyte Activation Test, CTL-MAT LLC, 20521 Chagrin Boulevard, Shaker Heights, Cleveland, OH 44122-5350 The information given lists examples of suitable products available commercially. This information is given for the convenience of the user of this document and does not constitute an endorsement by ISO of the product.

(supply system and safety), these cell lines seem to be more useful than whole blood. However, whole blood has the following advantages:

— that all blood components are present in physiological proportions that are needed for the interaction of pyrogen with leukocytes;

— immersion in whole blood is possible, which might not be possible for other cell systems.

On the other hand, the cell lines have the following disadvantages:

— the time (usually weeks) consumed to establish the cell line starting from a frozen aliquot. A sufficient number of cells of sufficient sensitivity (to pyrogens) are needed to use in a test;

— inconsistencies in the sensitivities of cells among different passage numbers;

— the requirement for co-stimulation with agents such as phorbol ester or calcitriol;

— verification of the continued presence on the cell line of the various receptor elements necessary for the transduction of responses to pyrogens.

It has been reported that sensitivity to endotoxin of the cell lines is different from each other, and among the cell lines, only MM6 cells produce a large amount of TNFα in response to relatively small amount of endotoxin after priming with calcitriol. Whole blood cells exhibit higher sensitivity to endotoxin compared with these cell lines, but MM6-CA8 cells[3] a subclone of MM6 cells,[58] exhibit almost identical sensitivity to whole blood cells in the detection of endotoxin and peptidoglycan.

6.4.4 Selection of marker cytokine

Although the amount of cytokine and chemokine produced from human whole blood cells by stimulation of TLR agonists and microspheres is different according to kind of the stimulators, in all cases, the cells induce a significant amount of MCP-1, IL-1βra, IL-8, MIP-1α, and/or MIP-1β in addition to IL-1β, IL-6, and TNFα that are traditional marker cytokines.

Among these cytokines and chemokines, IL-1β or IL-6 becomes good markers because of the highest signal to noise (S/N) ratio. Also, MIP-1α and MCP-1 exhibit relatively higher S/N ratio, but the ratio of MCP-1 for detecting TLR3 agonist and particles and microspheres is relatively low. See References [35] and [36].

MM6-CA8 cells exhibit different pattern of cytokine and chemokine production from whole blood cells. The cells induce a significant amount of IL-8, RANTES, IL-1βra, MCP-1, MIP-1α, and MIP-1β in addition to traditional marker cytokines. IL-6 exhibits the highest S/N ratio for detecting all TLR agonists and microsphere among these cytokines and chemokines. MIP-1α, MIP-1β, and IL-1β also exhibit higher S/N ratio than TNFα.

Taking these things into consideration, an appropriate marker cytokine or chemokine can be selected according to the cells used in HCPT.

6.4.5 Procedure of HCPT

Freshly drawn heparinized human whole blood from a healthy donor or cryopreserved human whole blood from a pool of donors is diluted in physiological, pyrogen-free clinical grade saline or RMPI1640 medium and brought together with a piece of the sample device in direct method[63] or suitable amount of test solution extracted from the device in indirect method. After incubation for 8 h to 24 h at 37 °C in an atmosphere appropriate for the selected culture medium as required by the test method, the amount of marker cytokine such as IL-1β reflecting the amount of pyrogen in the respective sample is measured by ELISA.

3) Dr. Yuji Haishima, Division of Medical Devices, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan, haishima@hihs.go.jp
The information given lists examples of suitable products available commercially. This information is given for the convenience of the user of this document and does not constitute an endorsement by ISO of the product.

In case of human myelomonocytic cell lines, the cells are maintained in RPMI 1640 medium containing 10 % heat-inactivated fetal bovine serum and appropriate supplements such as 2-mercaptoethanol, HEPES, glutamine, non-essential amino acids, sodium pyruvate, bovine insulin, and antibiotics. The cells are primed with phorbol myristate acetate or calcitriol (1a, 25-dihydroxyvitamin D3). After incubation for 72 h with the priming reagent, the cells are plated in a 24-well plate at 1×10^6 cells/ml/well with a piece of the sample device in direct method or suitable amount of test solution extracted from the device in indirect method. After incubation for 8 h to 24 h at 37 °C in an atmosphere appropriate for the selected culture medium as required in the test method, marker cytokine released from the cells into culture medium is quantified by ELISA.

The ELISA is a standard immunological test that is very sensitive and specific. A marker cytokine, such as IL-1β in the test article sample, is sandwiched between a monoclonal coating antibody and a labelled polyclonal detection antibody. Unbound materials are removed by a washing step. The bound detection antibody is linked directly or indirectly to the enzyme horseradish peroxidase, which metabolizes a substrate, TMB (tetramethylbenzidine) inducing a change in colour from colourless to blue. The reaction is stopped by addition of acid which causes a colour change from blue to yellow. This is measured photometrically at 450 nm with a reference wavelength of 690 nm.

A dose-response curve for the amount of produced marker cytokine and endotoxin from *E. coli* O111:B4 or other suitable standard such as *E.coli* UKT-B endotoxin calibrated to the international WHO reference standard from *E.coli* O113:H10, is prepared in each assay.[64] This dose-response curve needs to contain a concentration of 0,5 EU/ml corresponding to 50 ρg/ml of the international WHO reference standard. The value is considered to be the threshold endotoxin concentration which causes fever in the rabbit. This threshold has been confirmed by a study performed in 2005 which included 171 rabbits[40].

In HCPT, marker cytokine is measured as the total amount released from immune cells by stimulation of all pyrogens present in test sample. However, the result yielded in HCPT is evaluated by converting the amount of produced marker cytokine into the amount of endotoxin, because endotoxin is the strongest pyrogen and the critical amount of TLR agonists other than endotoxin for inducing febrile reaction is not authorized until now. Thus, the concept for detecting the pyrogenicity of test sample is the same with bacterial endotoxin test. The HCPT has the capacity to detect targeted cytokine releasing pyrogens which is not possible with a bacterial endotoxin test.

It can be noted that the HCPT tests cannot be used instead of the bacterial endotoxin test (LAL) for routine quality control testing of batches of large finished products/devices for presence of bacterial endotoxin contamination unless validated with a medical device extract. Generally, *in vitro* HCPT methods require a small flat sample of the material to be placed in a small holder and tested directly with the whole human blood. These methods only assay a very small defined area of material (2 cm^2 if in a 24-well plate)[35], or in a 1,5 ml polypropylene reaction vials for pyrogenicity.[53] There are also extraction based methods available. Bacterial endotoxin contamination can occur for many devices if they contain ingredients/components of biological origin, contain water other than water for injection or inhalation, use of water for manufacturing (e.g. aqueous leaching or soaking, extrusion operations) of the device, or are processed using water (e.g. steam autoclaving, washing). For routine quality control of batches of large, finished, sterilized devices, an extraction study is recommended to ensure that all potential sources of bacterial endotoxin are fully investigated by the endotoxin-based pyrogenicity test. The actual finished device can be very large, encompassing a very large surface area that could cause material-mediated pyrogenicity (e.g. additive) in the patient.

Human blood products present particular hazards for which adequate training on the necessary safety precautions is indicated for all technical staff.

6.4.6 Characteristic of the HCPT

HCPT has the following advantages compared with rabbit pyrogen test and bacterial endotoxin test.

— HCPT does not use animals. The cells are derived from human tissues and thus avoid the need for cross-species extrapolation.

— HCPT has a wider spectrum of pyrogen detection as compared with the bacterial endotoxin test[75].

— 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 (e.g. 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).

— 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 bound to the surface[75]. Pyrogens are effectively detected without any pre-treatment of the materials adsorbing endotoxin[75].

HCPT has the following disadvantages.

— Material-mediated pyrogens are chemical agents that typically 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 (e.g. cytokine receptor antagonists, non-physiological solutions, cytotoxic agents, recombinant proteins with cytokine activity) or the detection system (e.g. rheumatic factors), might not be tested with HCPT.

— HCPT might not be 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 can vary due to differences in donors age, gender, genetic background (e.g. genetic polymorphisms in genes coding for toll-like receptors cytokine receptors), safety issues with infected donors, diurnal variation, influence of diet, and other factors which can 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 can 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 pre-culture and priming of the cells.

6.4.7 Validation study

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

7 Conclusion

In some cases, the HCPT can be a useful alternative to traditional pyrogenicity test methods (rabbit and LAL); however, the rabbit test is needed to detect 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.

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