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Research paper

Inflammatory response to pyrogens determined by a novel ELISA method using human whole blood

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ABSTRACT

Presence of pyrogens on implants, medical devices, drugs and biological materials compromise on the biosafety and poses a major health hazard in therapeutics. Detection of pyrogenic contamination has so far been done with either *in vivo* rabbit pyrogen assay or Limulus Amoebocyte Lysate (LAL) methods, each of which having their distinct advantages and disadvantages. An indigenously developed ELISA method quantifying the pro-inflammatory response triggered by pyrogens on human whole blood is demonstrated for its versatility to detect the pyrogenic response to gram-negative, gram-positive bacteria, chemical and biological pyrogens. The method was used to test and quantitate the pyrogen levels in polymeric biomaterials. Unlike the existing pyrogen test procedures, this assay is adapted to detect all pyrogens, besides yielding faster, sensitive and quantifiable data, thereby reduce/replace animal experimentation. The method also provided insight into the possible correlation between variable blood profile among individuals and their role in determining inflammatory response to different pyrogenic stimuli.

1. Introduction

One of the inevitable criteria for toxicological safety of any biomaterial, parenteral drugs, cell therapies, recombinant proteins, medical devices and implants is the conformity for non-pyrogenicity. A multitude of microbial and inorganic substances of varying origin and chemical composition can cause fever response. While lipopolysaccharide (LPS) endotoxin from gram-negative bacteria are the ubiquitously found pyrogens, lipoteichoic acid (LTA) and peptidoglycan from gram-positive bacteria and other chemical pyrogens are being increasingly recognized as potent immune stimuli (Morath et al., 2001; Schindler et al., 2003; Martis et al., 2005). In view of the plethora of pharmaceutical strategies involving cellular therapies, recombinant proteins, parenteral drugs, implants and medical devices, the need to look out for pyrogens from non-endotoxin sources is gaining precedence (Ochiai et al., 2001; Yamamoto et al., 2000; Kawasaki et al., 1987; Fujiwara et al., 1999; Dinarello, 2004).

Pyrogens from gram-positive bacteria, especially of Bacillus species, are particularly problematic in biotechnological manufacturing processes, since many of the steps involved are prone to contamination from environmental spore-forming species. The clinical significance of non-endotoxin chemical pyrogens is probably under-estimated and under-reported. Febrile responses following few hours after administration of contaminated drugs is deemed as side-effects rather than their pyrogenic nature. Similarly, pyrogenic nature of several biological samples including viruses is not well-studied and their mechanism of inducing fever is not established.

The Pharmacoepoeia lists the *in vivo* rabbit pyrogen test and the *in vitro* Limulus Amoebocyte Lysate (LAL) test and monocyte-activation test (MAT) for pyrogenicity testing (European Pharmacopoeia, 1997; European Pharmacopoeia, 2010; USP 31 NF 26, 2008). The rabbit pyrogen test measures the change in body temperature after injection of test substance but has limitations in its utility due to insufficient

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accuracy and requirement of large number of animals (van Dijck and van de Voorde, 1977; Grant, 1950). Meanwhile, LAL test detects only LPS endotoxins from the cell wall components of gram-negative bacteria (Levin and Bang, 1968; Tanaka and Iwanaga, 1993) and does not necessarily correlate with *in vivo* biological activities of endotoxins (Pearson et al., 1982; Takayama et al., 1984). The pyrogen response in a rabbit or arthropod does not necessarily reflect the actual human immune response to pyrogenic challenges (Schindler et al., 2003; Hartung, 2002). Henceforth, efforts to develop a rapid, accurate and cost effective ELISA method to detect all classes of pyrogens utilizing human whole blood is gaining credence. Exogenous pyrogens stimulate the mononuclear phagocytes in the blood to release cytokines such as IL-1, IL-6 and TNF-α, which then transmit the fever signal to thermoregulatory centers of the brain, thereby inducing cycloxygenase (COX)-2-dependent prostaglandin (PG)E_2, the putative final mediator of the febrile response (Dinarello and Wolff, 1982; Dinarello et al., 1988). The cytokine release in response to pyrogen stimulation is detectable and quantifiable and gives a human-relevant perspective towards any source of pyrogens.

The current state-of-the-art guidelines for testing biomaterials and medical devices, ISO 10993, stipulates the pyrogen safety of implant devices by obtaining the liquid extract or wash fluid of the test biomaterial and employed in the rabbit pyrogen test or LAL test (Weary and Wallin, 1973; Devleeschouwer et al., 1985; ISO, 10993-12, 2007). It is known that the release of cytokines such as IL-1β and TNF-α have definite impact of the biocompatibility of medical devices and biomaterials (Shaldon and Dinarello, 1987; Cardona et al., 1992; Guth et al., 2000). Therefore, a quantitative assessment of the cytokine-inducing contaminations present on the biomaterial, by way of detecting cytokine release levels in the blood, will help in marking of biocompatibility of biomaterials in a more sensitive way.

This study makes use of the innate immune response of human blood in *in vitro* conditions towards various pyrogenic stimulations to develop a highly sensitive ELISA technique to evaluate the pyrogenic response towards compounds including gram-negative bacterial LPS, gram-positive bacterial LTA and a chemical pyrogen, 2,4,6-trinitrophenol and a mitogenic biological, phytohaemagglutinin (PHA). We also demonstrate the application of the developed test method to detect as well as evaluate the pyrogen level in two different polymer biomaterials. The ELISA method was further used to understand the differential cytokine release in response to different pyrogenic stimuli observed in different individuals with distinct blood profile.

2. Materials and methods

2.1. Materials

Purified human recombinant interleukin 1β protein (Sigma Aldrich) and Freunds' complete and incomplete adjuvants (Sigma Aldrich) were used for immunization. Pyrogen-free 0.9% saline (Bendtt, India) and sterile filtered pyrogen-free RPMI1640 media (Himedia, India) were used for setting up *in vitro* pyrogen test reaction. Endotoxin standards from *E. coli* strain 055:B5 lipopolysaccharide standardized against USP reference standard endotoxin (Sigma Aldrich) was resuspended in pyrogen-free ultrapure water to 4000 EU/ml. Lipoteichoic acid from *Bacillus subtilis* (Sigma Aldrich) was resuspended in pyrogen-free water to 1 mg/ml. Reagent-grade 2,4,6-trinitrophenol (Sigma Aldrich) was diluted to 10 ng/μl using pyrogen-free ultrapure water. Phytohaemagglutinin lectin from *Phaseolus vulgaris* (Sigma Aldrich) was similarly resuspended in pyrogen-free ultrapure water to 1 mg/ml. Endotoxin-free certified pyrogens were used for the assay. The glasswares used for the study were depyrogenated at 250 °C for 30 min. Non-pyrogenic microfuges, syringes and tips were utilized for the test. Sterile pyrogen-free 96-well flat-bottom microplates (Nunc Maxisorp) were used for ELISA. Two polymeric biomaterials (Sample 1 and Sample 2) were made available to the lab for pyrogenic evaluation.

2.2. Animals

Healthy male New Zealand white rabbit weighing not less than 2 kg was selected and acclimatized in anodized steel cages placed in climate-controlled room provided in accredited in-house animal house. The animals were received from the Division of Laboratory of Animal Sciences, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India. All animal experimentations were carried out in accordance with approved Institutional protocols, CPCSEA Guidelines for the Care and Use of Laboratory Animals and Institutional Animal ethics Committee.

3. Method

3.1. Collection of wash fluid (liquid extract) of material

Collection of wash fluid (liquid extract) was carried out as per USP and ISO guidelines (USP 31, 2008; ISO, 10993-12, 2007). All extraction, by fast rinsing was carried out in depyrogenised glasswares using pyrogen-free saline under aseptic condition. Two grams of the polymer samples 1 and 2 were incubated in 10 ml of physiological saline at 37 °C for 1 h followed by vigorous agitation. The wash fluid was collected and used for pyrogen evaluation.

3.2. Antibody development

3.2.1. Immunization of rabbit

Anti-human IL1β antibodies were raised in rabbit by immunizing the animal with purified human recombinant interleukin 1β protein. Lyophilized IL-1β were resuspended in phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM NA_2HPO_4, 2 mM KH_2PO_4, pH 7.2) and added with equal volume of Freunds' complete adjuvant and mixed vigorously into white homogenous micellar emulsion, which was administered intramuscularly at the thigh region of the hind limb. Booster doses were administered every 28th day post-primary immunization, with a 1:1 emulsion of Freunds' incomplete adjuvant. The specificity of the antibody thus raised was confirmed by dot blot assay wherein the purified IgG from pre-immunization serum and post-immunization (1st booster) were used to detect 50 ng of purified recombinant