INTERNATIONAL RESEARCH JOURNAL OF PHARMACY
Available online http://www.irjponline.com
Review Article

AN OVERVIEW ON PYROGEN REDUCTION METHODS

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Article Received on: 17/11/10 Revised on: 30/11/10 Approved for publication: 09/12/10

ABSTRACT
Pyrogenic contamination is a recurrent and troublesome problem in the preparation of solutions for parenteral use. Pyrogens, a chemically heterogeneous group of fever inducing compounds which are derived from bacteria, viruses, fungi or even from the host. Consequently, to assure the quality and safety of any pharmaceutical product for parenteral application in humans, pyrogen testing is mandatory. Endotoxins are the major contaminants which contribute to the pyrogenic response and complicate the interpretation of experiments using in vivo or in vitro biological responses. This review explains the different methodologies which are use to reduce pyrogens.

KEYWORDS: Endotoxin, Pyrogen, Parenteral application, In vivo, In vitro.

INTRODUCTION
Pyrogenic substances are endotoxins (lipopolysaccharides, LPS). Endotoxins are highly pyrogenic cell wall components of all gram negative bacteria and they are very resistant to degradation. Endotoxins are not inactivated efficiently by the terminal heat sterilisation procedures. Even though the pyrogenicity of gram positive microorganisms and the cell wall components from gram positive bacteria are less potent compared to endotoxins, they can cause serious clinical problems if present in pharmaceutical preparations.[1] Monocytes/ macrophages react to microbial products during an immune response by producing endogenous pyrogens such as prostaglandins and the proinflammatory cytokines interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor-a (TNFa). Depending on the type and amount of pyrogen challenge and the sensitivity of an individual, lifethreatening shock-like conditions can be provoked.[2]

MATERIALS AND METHODS
Rabbit test
This test is official in Indian Pharmacopoeia, British Pharmacopoeia and United State Pharmacopoeia. The test involves measurement of the rise in body temperature of rabbits following the intravenous injection of a sterile solution of the substance under examination. It is designed for products that can be tolerated by the test rabbit in a dose not exceeding 10 ml per kg injected intravenously within a period of not more than 10 minutes.[3]
Limulus amebocyte lysate test (LAL test)
This test is official in United State Pharmacopoeia. It is 5-10 times more sensitive than the rabbit test. It is vitro test method for pyrogens has been developed utilizing the gelling property of the lysate of the amebocyte of limulus polyphemus. In the presence of pyrogeneic endotoxins from gram negative bacteria, a firm gel is formed within 60 minutes when incubated at 37°C.[4]
Helox therapy
The effectiveness of a gas environment consisting of 80% helium and 20% oxygen (Helox) in reducing a rabbit's fever due to an i.v. injection of endotoxin is dependent on the amount of pyrogen injected. When

a relatively large dose of pyrogen is injected, the helox environment used in these experiments reduced the mean maximum temperature reached during the fever from 41.5 to 40.5°C, but the helox does not significantly alter the change in temperature from baseline levels prior to the injection (a 1.4°C increase in air and a 1.1°C increase in helox). When a relatively low dose of pyrogen is injected, the helox environment increased the change in temperature from baseline at peak fever, but does not produce a significant change in the actual temperatures attained during the fever. [5]

Electrical stimulation therapy

Electrical stimulation of sites located in the medial paraventricular nucleus of the rabbit suppresses fever normally induced by systemic administration of a bacterial pyrogen. But these results indicate this stimulation does not affect normal thermoregulatory processes in the afebrile animal. Electrical stimulation of sites outside this region of the brain has no effect on either the absolute magnitude or the duration of the fever.[6]

Interferon-g-primed monocytoid cell lines method

Reducing or even replacing animal tests by in vitro methods is generally desired for ethical reasons. In vitro alternatives are often economic, easy to perform and can be adjusted for automation. As a possible alternative to animal testing for pyrogenic contamination of pharmaceuticals for parenteral application, the use of monocytoid cell lines and the detection of various pro-inflammatory cytokines including IL-1, TNF-a or IL-6. Using monocytoid cell clones selected for high endotoxin sensitivity and TNF-a as a read-out, this cellular system correlated well with the rabbit pyrogen test and appeared to overcome some of the limitations of the LAL test.[7]

A new cell-based innate immune receptor assay

Medical equipment and products (implants and medication applied i.v.) are test for pyrogenic residues in order to prevent sepsis by the transmission of pyrogenic residues into the human bloodstream. Similar to the LAL test this assay can detect specific pyrogens like lipopolysaccharide (isolated from E. coli) in medical supplies or devices. Samples are collect from the surface in endotoxine-free water with the help of cellscrapers and analyze with the TLR4/CD14 test system. Other pyrogen test systems like in vitro pyrogen test or rabbit test detect all possible pyrogens, whereas this assay is focus on synthetic ligands (Pam2CysSK4, Pam3CysSK4, MALP-2, FSL-1 and lipid A).[8]

Cryopreservation of differentiated HL-60 cells

All-trans retinoic-acid (ATRA) differentiated HL-60 cells can be used to detect pyrogens such as bacteria, bacterial components, yeasts and fungi. Differentiated HL-60 cells obtain neutrophil like characteristics and if it is stimulated it produce reactive oxygen species in a dose dependent manner. Culturing and differentiation of cell lines are time consuming activities and require suitable facilities, cryopreservation of pre-differentiate cells can provide the basis for an easily distributable an in-vitro in-house pyrogen testing kit. Cryopreservation of granulocytes has proven to be very complicated and neutrophils are especially difficult to cryopreserve, most likely due to their large degree of granulation. HL-60 cells can be differentiate with ATRA and subsequently cryopreserve. Upon thawing the cells retain their reactive oxygen species producing capabilities and reactivity towards pyrogens. The cells retain their ability to react dose dependently towards lipopolysaccharide (LPS), lipoteichoic acid (LTA) and zymosan. At pathophysiologically relevant concentrations of LPS, LTA and zymosan the cells retain full reactivity for at least two months when stored in liquid nitrogen.[9]

Anisomycin protein inhibition method

The synthesis of protein within tissue of the hypothalamus is a functional requisite for the development of a pyrogen induced fever. Anisomycin possess a pharmacological property of its own which interferes with thermogenesis, the long latency in the onset of the pyrexic response is due to inhibition of protein synthesis. The fever generated by endogenous pyrogen derived either from cat or rabbit exudate is similarly modified by anisomycin provides a possible delineation of the metabolic step at which protein synthesis inhibition affects heat production.[10]

Lipoxygenase inhibitors

Leukocytic pyrogen (LP), the endogenous mediator of fever, is synthesized and released from mononuclear phagocytes following activation by several microbial and immunologically derived substances. Purified fractions of LP also stimulate thymocyte proliferation and LP seems to be

indistinguishable from lymphocyte activating factor. Inhibition of lipoxygenase-mediated transformations of arachidonic acid significantly decreases the production of LP from human monocytes. The decreased LP production is due to inhibition of processes taking place at the time of cell activation rather than during the period of LP synthesis. The increased Prostaglandin production during the activation of phagocytic cells, the subsequent synthesis and release of LP are not conditioned by Prostaglandin E_2 levels or by cyclooxygenase activity in general. Products of arachidonate lipoxygenase have an important role in the initiation of signal which ultimately leads to de-repression of the LP-genome.[11]

Noradrenaline and adrenergic receptor antagonist

Pretreatment of the hypothalamus of rabbits with 6 hydroxydopamine causes degeneration of noradrenergic nerve terminals significantly reduced the febrile response to intravenous pyrogen injection. 6-Hydroxydopamine also cause a decrease in the febrile response to prostaglandin E_1 (PGE_1) injected into the hypothalamus. An intrahypothalamic injection of 50 µg of the alpha-adrenergic antagonist phenoxybenzamine significantly reduced pyrogen fever. Also, addition of 50 µg phenoxybenzamine to the PGE_1 injection resulted in a significant reduction of the febrile response. Propranolol, a beta-adrenergic blocking agent when injected in the same dose and in the same manner as phenoxybenzamine, has no significant effect on either the pyrogen- or the PGE_1 induced fevers.[12]

Synthetic cannabinoid HU-210

(-) l I-OH-A8- A9-Tetrahydrocannabinol dimethylheptyl (HU-210) has a profound and long lasting hypothermic effect. HU-210 is considerably more active than other known natural or synthetic cannabinoid drugs. Also there is a strong correlation between the biological effect of HU-210 and its high affinity binding to the cannabinoid receptor.[13]

Prazosin, dihydrobenzperidol and nifedipine

The thermoregulatory effector processes are investigate after treatment with prazosin, dihydrobenzperidol and nifedipine apply to the thermosensitive zone of the anterior hypothalamus (PO/AH) on normothermic and feverish rabbits (LPS, lipopolysaccharide E. coil;l mcg/kg, i.v.). The alpha-noradrenergic receptor antagonists prazosin and dihydrobenzperidol apply to the PO/AH produce an abolishment of fever elicite by pyrogen i.v. injection mainly because of vasodilation of ear skin vessels and attenuation of metabolic rate. Calcium channel blocker nifedipine also induce a decline in the rabbit's core temperature in the same manner. All these drugs given to the PO/AH do not change the body temperature in normothermic rabbits.[14]

Moist heat sterilization method

Pyrogenic activity of S. aureus peptidoglycan and lipoteichoic acid is reduced when exposed to moist heat sterilisation. The efficacy of standard moist heat inactivation of peptidoglycan at 121.C for 15 min and 134.C for 3min are in both processes less than 1-log reduction. The efficacy of the standard moist heat inactivation of lipoteichoic acid is approximately a 1-log reduction at 121.C for 15 min and 134.C for 3min. This reduction far from fulfills the demand of a 3-log reduction.[1]

Polymyxin B-Sepharose 4B (PB-Seph 4B) affinity binding and endotoxin-protein dissociation with the dialyzable surfactant, octyl-fl-o-glucopyranoside (OBDG) method

A method of reducing endotoxin contamination in protein-containing solutions is using a combination of PB-Seph 4B affinity binding and endotoxin-protein dissociation with the dialyzable surfactant, OBDG. Using the LAL assay to detect endotoxin, greater than 1000-fold reduction of endotoxin reactivity can be accomplish from a contaminated commercial preparation of bovine catalase. This occurred with only a 24% protein loss and an 11% loss of catalase enzymatic activity after treatment: The treated catalase appeared to be largely endotoxin-free since it no longer elicited a pyrogenic response in rabbits or primed for intravascular coagulation of the generalized Shwartzman reaction. OBDG treatment of Salmonella minnesota Re595 lipopolysaccharide enhanced its ability to bind to serum high density lipoproteins which might contribute to decreased in vivo toxicity. In quantitative studies using radiolabeled endotoxin, the OBDG is show to be capable of dissociating protein-bound endotoxin thereby facilitating its binding to the PB-Seph 4B adduct. The technique is also useful in removing radiolabeled endotoxin added to human IgG. The methodology has general usefulness in reducing endotoxin contamination of macromolecular solutions that can bind and retain endotoxin.[15]

By Ether

Pyrogen and hemagglutinin of influenza and newcastle disease viruses are not distinguishable by their thermal sensitivity, ultraviolet irradiation or formaldehyde treatment will completely destroy the pyrogenic activity without impairing the hemagglutinin. Since the virus particles lose their pyrogenic activity and their capacity to induce tolerance pari passu, it is postulated that the pyrogen itself is responsible for inducing tolerance. The separation of G antigen, hemagglutinin, and lipid substances by treatment of the virus particles with ether, completely abolishes their ability to induce fever, even when the components are mixed in the original proportion. Treatment with ether releases the building blocks of the elementary particle by dissolving its outer lipid shell. The liberated nucleoprotein and the enzymatically active hemagglutinin retain their specific properties, indicating that the ether treatment does not go beyond its action on the lipids.[16]

BE 2254 (2-[β-(4-hydroxylphenylethyl)aminomethyl]- tetralone)

BE 2254 (2-[β-(4-hydroxylphenylethyl)aminomethyl]- tetralone), a new more selectively acting alpha l-adrenoceptor antagonist is investigate on LPS induced fever. It is administered in feverish rabbits reduced the metabolic as well as pyretic activity produced by both doses of pyrogen. It is suggest that stimulation of the thermoregulatory heat production which contributes to a febrile rise in body temperature is dependent on alpha adrenoceptor mechanisms.[17]

Salicylate therapy

When given in sufficient doses, peripherally administered Pseudomonas~polysaccharide can cause fever. Injections of salicylate in the anterior but not the posterior hypothalamus prevent the development of hyperthermia associated with peripheral injections of pyrogen.[18]

High-performance affinity capture-removal of bacterial pyrogen from solutions

Synthetic peptide S3Δ has high affinity for bacterial endotoxin or LPS. Under tested conditions of pH 5–9 and 0–0.4 M NaCl, the affinity constant, K_D ranged from $2 \cdot 10^{-6}$ to $2 \cdot 10^{-9}$ M^{-1}. A novel affinity matrix based on peptide S3Δ was developed for removal of LPS from solutions such as water, buffers with a wide range of ionic strength and pH, medium for cell culture, and protein solutions under optimized conditions.[19]

CONCLUSION

This review compiles various pyrogen reducing methods which are very useful to assure the quality and safety of any pharmaceutical product for parenteral application. While to date there is no efficient and practical way of removing pyrogen, so in this area further research is required.

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