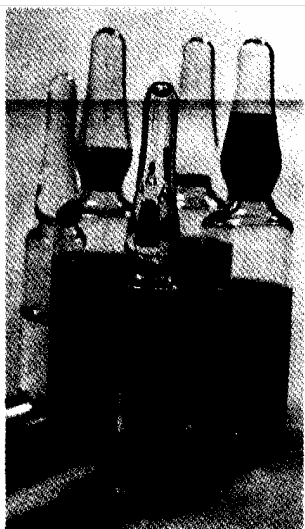


Pyrogens in Parenteral Pharmaceuticals

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Endotoxin, or lipopolysaccharide (LPS), is highly toxic to mammalian cells and is one of the most potent modulators of the immune system. However, quantitative measurement of total bacterial pyrogenicity has not been established. A cell-based method, the monocyte activation-cytokine assay, clarified both false-negative and

false-positive Limulus amoebocyte lysate and rabbit test results in released lots of parenteral products that caused adverse reactions in patients. New evidence points to still-unrecognized consequences of intravenous LPS administration. These findings indicate a need for further studies to better understand the risks associated with intravenous administration of even mildly pyrogenic substances. This may lead to the reevaluation of acceptable pyrogen levels in parenteral products.

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Endotoxin produced by Gram-negative bacteria is of increasing concern in biotechnology. Highly toxic to mammalian cells, endotoxin is one of the most potent modulators of the immune system. Endotoxin, also referred as lipopolysaccharide (LPS), is composed of hydrophobic fatty acid and hydrophilic carbohydrate domains (1). The primary hydrophobic domain, known as lipid A, carries many of the biological activities associated with LPS. Lipid A contains fatty acid chains attached to a phosphorylated disaccharide. The lipid composition of lipid A exhibits strain-specific variations. The core is made of carbohydrates, some of which are phosphorylated and ethanolaminylated. The repeat units are trisaccharides, the number of which varies among the different strains.

The chemical nature of LPS makes pyrogen removal problematic (2). LPS is unusually thermostable and fairly insensitive to pH changes. High concentrations of acids or bases are necessary to destroy LPS within a reasonably short time. The size of LPS varies depending on the environment; reported molecular weights range from 2 kDa to several million daltons. Also, naturally occurring LPS has a Stokes radius smaller than the purified endotoxin typically used to qualify filters (3). This adds to the uncertainty of developing effective LPS removal methods. The heterogeneity of LPS is substantial, and its implications are not fully appreciated in the area of endotoxin removal.

The mechanism of endotoxin action

The mechanism of endotoxin action has been studied extensively, both in vitro and in vivo. Released both by live and dead bacteria, LPS is bound by the LPS-binding protein (LPB). Both LPB (4-6) and bactericidal-permeability increasing protein (7,8) play an important role in the host's response to endotoxin.

The LPB-LPS complex binds to the CD 14 receptor on the cell surface, leading to activation of the cell (9). The main target cells are the circulating mononuclear cells, which produce proinflammatory cytokines such as interleukin-1 (IL-1) and tumor necrosis factor- α (TNF- α) (10, 11). Proinflammatory cytokines are involved in acute and chronic inflammation and modulate the host's response to bacterial infection (12). Even in the absence of fever, IL-1 and TNF- α induce IL-6 and acute phase proteins such as C-reactive protein. This has recently been suggested as a marker for the increased mortality and morbidity of patients with end-stage renal disease (13). Therefore, even in the absence of fever, increased cytokine production caused by LPS contamination can reflect an inflammatory reaction with potentially detrimental consequences.

Besides LPS, Gram-negative bacteria release peptides such as exotoxin A (from *Pseudomonas*), peptidoglycans, muramyl peptides, and other still unidentified substances. These bacterial products act similarly to LPS in terms of inducing the secretion of cytokines. The molecular size of these products also varies greatly. Peptidoglycans and LPS are large molecules, whereas LPS subunits and muramyl peptides may be as small as 2000 Da. In the supernatant of *Pseudomonas*, a major group of waterborne bacteria, a pyrogen with a very low molecular weight (<1000 Da) recently was identified (14,15). In actual final-product samples, a mixture of pyrogens always is present, suggesting that no single modality of pyrogen removal may be adequate.

Pyrogen detection

This heterogeneity of pyrogenic bacterial products raises a question about the effectiveness of pyrogen detection in parenteral pharmaceuticals. Both the United States Pharmacopeia and the European Pharmacopoeia specify the rabbit pyrogen test and the *Limulus* amoebocyte lysate (LAL) test (16,17). However, the LAL test detects only LPS and results in false negatives with certain products. Technically, the rabbit test is an acute toxicity test with an arbitrarily selected end point. There are reports that some parenteral products (e.g., human serum albumin) have caused pyrogenic reactions in patients even after passing the rabbit and LAL tests (18). Therefore, it is possible that even the rabbit test cannot detect all pyrogenic bacterial products.

An alternative pyrogen test is the monocyte activation-cytokine assay (19,20). In this assay, monocytes isolated from human peripheral blood are incubated with test

samples and their supernatant assayed for pyrogenic cytokines such as IL-1 or TNF- α (21). A variation of this test is the whole blood-IL-6 assay (22). A monocytic cell-based assay also has been developed (18).

The disadvantage of the monocyte activation-cytokine assay is that the sensitivity of freshly isolated monocytes can vary depending on the immunological responsiveness of the donors (20). To make this assay suitable for quality control purposes, a well-defined monocytic cell line, which retains its ability to synthesize and secrete cytokines, is needed. This could lead to the development of a quantitative pyrogen test with a broader range of applicability. The Mono-Mac-6 (23) and THP-1 (24) cell lines have been characterized for their suitability in a cell-based pyrogen assay and were found to have the ability to detect bacterial pyrogens (18).

The Mono-Mac-6 cell line has been used successfully to detect pyrogenicity in human serum albumin lots that passed both the LAL and rabbit pyrogenicity tests but gave adverse (i.e., pyrogenic) reactions in humans (18). This suggests that a monocytic cell-based assay is more effective in detecting pyrogens than the standard methods and could successfully complement existing methods.

Physiological effects of pyrogens

The physiological effects of pyrogens in humans are diverse and dose-dependent. First, pyrogens elevate the circulating levels of inflammatory cytokines (e.g., IL-1, IL-6, TNF- α and IL-8) (10) followed by the clinically relevant events of fever, hypotension, lymphopenia, neutrophilia, and elevated levels of plasma cortisol and acute-phase proteins (e.g., C-reactive protein) (13,25). Low doses of pyrogens induce inflammatory reactions without any clinically significant symptoms. Moderate doses of pyrogens induce fever and significant changes in plasma composition (26,27). High doses of pyrogens can lead to septic shock characterized by cardiovascular dysfunction, including myocardial depression and dilatation, vasodilation, vasoconstriction, endothelium dysfunction, and organ dysfunction (e.g., kidney, liver, lung, or brain) followed by multiple organ failures and death (28,29). Endotoxin-mediated endothelial cell injury is also implicated in the pathogenesis of septic shock (28).

Two new lines of evidence point to previously unrecognized consequences of intravenous endotoxin administration. The first came from a clinical study. Introducing LPS intravenously into healthy humans suppressed the cytokine response by peripheral blood mononuclear cells (PBMCs) when rechallenged in vitro with LPS, IL-1, L-1 β , or toxic-shock syndrome toxin-1

(TSST-1) (30). This confirmed that the reduced cytokine synthesis was not caused by endotoxin tolerance but rather by a true immune-suppression reaction. The cytokine synthesis in CD 14+ monocytes did not return to the normal control levels even after 24 h, the duration of the experiment.

This observation is quite interesting in light of the low dose of LPS used (3 ng/kg or 30 EU/kg of body weight). Current endotoxin standards for lot release of parenteral pharmaceuticals is 350 EU/dose. The endotoxin range was defined by introducing the US standard reference endotoxin (31) into healthy male volunteers. Actual product samples always contain a mixture of LPS and non-LPS pyrogens because of the bacterial material present. Characterization of *Pseudomonas* culture filtrate has demonstrated that only ~40-50% of the cytokine-producing activity is caused by LPS-like material (32,33). Because *Pseudomonas* is a major waterborne microorganism, the total LPS-equivalent pyrogenicity in products is more accurately expressed as 700-875 EU/dose. This is in the same order of magnitude as the amount of endotoxin administered by Granowitz et al. (i.e., 1950 EU for a 65-kg average-weight male) (30).

Therefore, the possibility exists that some injectable pharmaceuticals meeting current pyrogenicity standards may in fact be immunosuppressive. Current testing methods are insufficient to answer this question (16,17,31). To accurately determine sample pyrogenicity, both LPS and non-LPS pyrogenicity must be quantified. One must also consider that inflammatory reactions occur in the absence of fever. This makes it even more important to monitor these reactions at the cellular level.

The second line of evidence came from studies investigating the effects of LPS on endothelial cells. Vascular endothelial cells play a major role in the regulation of hemostasis by maintaining an antithrombotic barrier. In response to pyrogenic stimulus, endothelial cells develop prothrombic properties. This involves the production of tissue factor (33,34) the downregulation of thrombomodulin, inhibition of factor C activation (34), leukocyte adhesion, and increased platelet adherence to endothelial cells (35). The denudation of endothelium alters vessel permeability and flow and exposes subadjacent tissue to inflammatory cells and mediators.

The molecular mechanism of endothelial damage by LPS has recently been explained (36). The damage process involves at least three parallel mechanisms. LPS-activated PBMCs cause apoptosis of endothelial cells through membrane bound TNF- α (36-38). The cell-free supernatant of activated PBMCs also trigger programmed

cell death in the absence of TNF- α . In addition, activated PBMCs can induce endothelial cell cycle arrest in G0/G1 (36). This is caused by the production of TGF- β , which inhibits endothelial cell proliferation and repair (39). The data strongly suggest that LPS causes irreversible damage to the vascular endothelium. The implications of this are far-reaching because the industry may have to completely revise current views about allowable endotoxin levels in parenteral products.

The patient population most exposed to this risk is that receiving replacement therapies (e.g., hemophiliacs and diabetics). Another at-risk group includes people in severe trauma (e.g., septic shock). Studying the cytokine-producing capability of the CD 14+ monocytes of hemophiliacs or diabetics could shed light on potential inflammatory reactions induced by the therapy. In septic shock patients, the infusion of the plasma extender serum albumin, although it does meet current pyrogenicity standards, could potentially diminish the already depleted resources of their immune systems. This could lead to a prolonged recovery period and, in some cases, could contribute to the high rate of mortality among this patient group. It seems unreasonable to administer additional pyrogens to patients who already are under severe pyrogenic stress.

A discovery program based on the studies of Granowitz et al. (30) and Lindner et al. (36) would, at the cellular level, clarify the responses of patients to the administration of pyrogens in parenteral pharmaceuticals. Such a discovery program could use the following general outline:

First, the cytokine-inducing ability of the pharmaceutical in question on isolated PBMC and/or a monocytic cell line would be studied in vitro. The secondary cytokine response of product-treated monocytes to endotoxin, IL-1 β , and TSST-1 would reveal any potential immunosuppressive effect of the product.

Activated monocytes, when co-cultured with endothelial cells, would indicate whether apoptosis-inducing effects were present. Experiments using pyrogen-depleted products as well as uninduced monocytes would serve as controls. Readily available technologies can deplete pyrogens from these products. This in vitro portion of the research could be carried out with monocytes from healthy donors and/or a monocytic cell line.

The second phase of research could be performed in a clinical trial by treating patients with standard and pyrogen-depleted products. Monocytes would be isolated before and after administration of the product. The ability of these monocytes to secrete inflammatory cytokines before and after an in vitro LPS challenge would reveal

any immunosuppressive effect caused solely by administration of the product. Co-culturing endothelial cells with the activated monocytes would indicate whether any apoptosis of endothelial cells occurred.

Conclusion

Experimental data suggest that the pyrogenicity limits for parenteral pharmaceuticals might be higher than desired for maximum patient benefit. A discovery study of patients at risk of pyrogen administration should be conducted to obtain additional data. Conclusive evidence of the immunosuppressive effects of currently acceptable pyrogen levels in parenteral pharmaceuticals would require the introduction of new testing procedures and a reevaluation of pyrogenicity standards. If the results of Lindner et al. (36) and Eissner et al. (37) are further confirmed, zero tolerance for pyrogens in parenteral products would be warranted.

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