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Bacterial endotoxin testing of drugs and biologics in the US: Ensuring patient safety

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Bacterial endotoxins can trigger severe inflammation and death if they enter the bloodstream, cerebrospinal fluid, or intraocular fluid. Accurate detection of endotoxin levels in medical products is therefore an important part of the sterility testing process. This article provides an overview of how endotoxins can contaminate injectable drugs and biologics and examines the common methods used in the detection and removal of endotoxins from materials, manufacturing equipment, and final medical products. The authors provide guidance from the US Food and Drug Administration (FDA) for setting acceptable limits of endotoxins and give examples of FDA warning letters issued to sponsors of medical products for endotoxin-related concerns.

Keywords – endotoxins, gram-negative bacteria inflammation, standards, sterility, United States Pharmacopeia

Introduction

Endotoxins are molecules produced by gram-negative bacteria that can contaminate medical products and have lethal consequences. They are shed by bacteria such as *Escherichia coli* (*E. coli*) and *Salmonella* during cell growth, cell division, and particularly in cell death, and are a type of pyrogen – a fever-causing substance. These molecules can have severe consequences if they enter the bloodstream; overexposure to endotoxins has been associated with severe inflammatory responses, septic shock, and death. It is vital that injectable medicines and implantable medical devices undergo endotoxin testing before use to ensure patient safety. This article will discuss the pathogenicity and common sources of endotoxins in drugs and biologics, as well as the testing methods for these endotoxins, their safety limits, and the relevant, related FDA regulations and guidance. Other pyrogens, such as molecules from viruses and fungi and from metals and plastics, can also contaminate medical products and cause harm, although this article does not focus on those types of pyrogens. Medical devices also require endotoxin testing but fall outside the scope of this article.

Function and pathogenicity

Endotoxins contribute to the structure and permeability of the bacterial outer membrane and to bacterial-host interactions. They are lipopolysaccharide molecules containing a lipid domain, a core polysaccharide, and a polysaccharide side chain of repeating units, known as the O-antigen, which varies depending on the bacteria. The lipid portion of the endotoxin molecule anchors the molecule in the bacterial cell membrane and is the part that leads to inflammation during bacterial infection. When endotoxins are shed, the lipid binds to toll-like receptor 4 on animal cell surfaces and triggers the inflammatory response.

Sources of endotoxins

Endotoxins may be introduced into medical products through many sources, including raw materials, excipients, bacterial production systems, equipment in the manufacturing process, and packaging. Bacteria can be used to produce drug substances, and endotoxins may be inadvertently copurified with the desired molecule. For example, brolucizumab is an approved antibody fragment drug produced in *E. coli* for treating wet age-related macular degeneration, and in an early preclinical study of the drug in cynomolgus monkeys, the administration of non–good manufacturing practice (GMP) batches of the drug led to ocular inflammation, believed to be due to endotoxin levels rather than the drug itself.[1]

Reagents such as recombinant growth factors and hormones may be derived from bacterial systems and therefore also contain endotoxins. Previously, fetal bovine serum was often a source of endotoxins because it is derived from animals that can harbor bacteria, but these products are now thoroughly screened for endotoxin presence.[2]

Due to the presence of bacteria in water systems, all water used in production of medical products, including in manufacturing and to wash equipment, should be tested to ensure it is free of endotoxins. Endotoxins are hydrophobic and have high affinity to other hydrophobic materials, such as plastics and glassware, making it particularly difficult to remove them completely. Bacteria are commonly found on human skin and hair, so user contamination of materials can also arise.

FDA requirements and current guidance

The FDA requires that endotoxin levels are assessed in injectable drug products. Generally, the FDA has cited the approach used by the United States Pharmacopeia (USP) for endotoxin testing and limits.[3,4] Endotoxin levels are measured in endotoxin units (EU) – equal to the activity of 0.1 ng of endotoxin from *E. coli.* Reported patient cases of inflammation because of endotoxin presence include reports of ocular inflammation in 80 patients in China resulting from counterfeit bevacizumab that contained 32 EU/ml of endotoxin[5] (more

than 100 times the FDA-recommended endotoxin levels for intraocular use).[6] Even in early research stages, endotoxin contamination of a product and of laboratory equipment can cause immune responses in cell culture and animal models, making it difficult to determine true biological effects of the product.

Acceptable levels of endotoxins depend on the product type, disease indication, type of administration, and quantity of dose to be administered. USP gives the following guidance:[4]

- **Parenteral drugs –** Based on the threshold pyrogenic dose of endotoxins per kilogram of body weight or meter-squared of body surface area per hour, divided by the maximum dose to be administered to the patient within a 60-minute period.
- **Nonintrathecal injections –** USP defines K as equal to 5 EU [endotoxin units] per kilogram of body weight or 100 EU per meter-squared of body surface area per hour for nonintrathecal injections.

Stricter endotoxin limits than those suggested by USP should be considered for certain products, such as unique patient populations (e.g., neonates), concomitant administration of other products, or atypically large volumes or doses.[7] The FDA has expressed the expectation that "sponsors of investigational new drug [IND] applications will justify the proposed endotoxin acceptance criteria for each investigational therapeutic biologic, drug, or combination therapy based on manufacturing experience and established control strategies, such as careful selection of ancillary materials, aseptic processing, and the use of closed systems."[8]

USP notes that endotoxin limits should be monitored throughout product development and that adjustments after the investigational phase may occur: "It is important that the endotoxin limit specification, as a critical quality attribute for a new product, be calculated early in development and monitored throughout development and early-stage clinical trials."[9] The FDA's guidance for drugs and biologics suggests an expectation that sponsors will develop an endotoxin testing procedure as part of an IND submission. For instance:

- The FDA's guidance for current GMPs for Phase 1 drugs describes negative endotoxin tests as an example of a test that would show sterility.[10] The guidance states that a requirement for sterile products includes "[e]nsuring that final Phase 1 investigational drugs are not released until acceptable results of sterility testing are known," suggesting "a negative endotoxin test on the final product" as an example.
- Draft FDA guidance for drugs and biologics for oncological indications notes that "[c]ontrols on pyrogen and endotoxins are part of the chemistry, manufacturing, and control information to be included in an

investigational new drug, a new drug, or a biologics license application for most drug products."[8]

- The FDA's guidance on the content and format of INDs for Phase 1 studies states that sterility and nonpyrogenicity tests should be submitted as part of the chemistry, manufacturing, and controls information section.[11]

Endotoxin levels can be discussed with the FDA during a pre-IND meeting with the agency, as part of the discussion relating to chemistry, manufacturing, and controls issues.[12] The accompanying **Table** provides an overview of FDA and USP guidance and information relating to endotoxins.

Testing methods

Endotoxin testing is typically conducted using the Limulus amebocyte lysate (LAL) test. There are several possible methods, which are detailed in the US Pharmacopeia.[4] These tests require a blood cell lysate that is typically taken from the wild Atlantic horseshoe crab, *Limulus polyphemus*. These ancient crabs have existed for around 450 million years, and their blood is unusual for two reasons. First, it appears blue in color as their systems use copper for oxygen transport, rather than iron, as in humans. Second – and the reason the crabs are useful for the biomedical industry – amebocyte cells in the crabs' blood cause clotting in the presence of bacterial endotoxin.[15] This is part of the crab immune response; amebocytes secrete coagulogen in response to injury when they detect endotoxins to prevent bacterial infection. Specifically, endotoxin

Table. FDA guidance documents and USP chapters relating to endotoxins

FDA guidance
Inspection technical guide: Bacterial endotoxins/pyrogens[13]
cGMP for Phase 1 investigational drugs[10]
Content and format of investigational new drug applications (INDs) for Phase 1 studies of drugs, including well-characterized, therapeutic, biotechnology-derived products[11]
Inspection technical guide: Bacterial endotoxins/pyrogens[13]
Questions and answers on quality-related controlled correspondence[14]
Pyrogen and endotoxins testing: Questions and answers[3]
Setting endotoxin limits during development of investigational oncology drugs and biological products[8]
Sterile drug products produced by aseptic processing — Current good manufacturing practice[7]
US Pharmacopoeia
General chapter <85> Bacterial endotoxins test[4]
General chapter <1085> Guidelines on the endotoxins test[9]

cGMP, current good manufacturing practice; **FDA,** [US] Food and Drug Administration; **USP,** United States Pharmacopeia.

molecules bind to clotting factor C molecules in the amebocyte plasma membrane, activating them and triggering the clotting cascade.[16]

Due to these properties, endotoxin testing has relied on horseshoe crabs since the 1970s. Blood is harvested when the crabs come ashore for annual spawning,[17] with an estimated half a million animals bled per year.[18] The bloodletting process is nonlethal, although the mortality rate of the overall harvesting process is estimated to be around 15%.[19] Blood cells are extracted from the crabs and lysed, and the lysate is purified for use. Lysate can be used in different methods, namely gel clot, chromogenic, and turbidimetric. The gel clot method is the simplest and indicates the presence of endotoxins by clotting when a test sample is incubated with lysate.[20] The latter two methods involve measuring optical changes due to endotoxin interaction with lysate, either as a color change resulting from the cleavage of a chromophore from a chromogenic peptide (chromogenic method), or a change in cloudiness of the sample (turbidimetric method).

Clotting factor C molecules can be synthetically produced as recombinant factor C (rFC), which has been available for two decades. This technology is used by some, such as Eli Lilly,[21] for its improved consistency and sustainability. USP initially announced it would include rFC in its endotoxins chapter. However, it announced in 2020 that rFC would instead be included in a separate chapter in which its use in endotoxin testing would require additional validation to demonstrate that the results with rFC are similar or identical to those with animal-derived LAL.[22]

Before the use of LAL testing, the rabbit pyrogen test was the standard endotoxin assessment.[8] This method is also still sometimes used and involves injecting a product into rabbits and measuring their body temperature to note any fever that might arise. This is based on the principle that humans and rabbits react similarly to endotoxins. However, its use largely ceased when LAL testing was developed, because of the convenience of not having to house and monitor live animals and the quicker testing, given that the rabbits took time to exhibit symptoms.

Endotoxin removal

Endotoxins are difficult to destroy by standard sterilization processes. The molecules are small and can bind to therapeutic molecules, making separation challenging.[20] It is generally better to maintain minimal endotoxin levels in raw materials and monitor in-process levels in intermediates than to rely on endotoxin removal processes for final-stage drug productions. All endotoxin detection and removal methods must be verified and validated to demonstrate their efficiency (see next section for examples of FDA warning letters issued when this does not take place).

Endotoxin removal processes, also referred to as depyrogenation, depend on the product characteristics or the type of equipment. Methods include ultrafiltration, solvent extraction, isothermal extraction, and affinity chromatography.[23] Rinsing with endotoxin-free water may be sufficient to remove endotoxins from equipment. Glassware can also be heated to temperatures of 250 °C for a minimum of 30 minutes.[24]

Failures to manage endotoxins

Failures to adequately determine and control endotoxin levels have been noted in Form 483s and warning letters sent to sponsors by the FDA. A study of endotoxin-related current GMP noncompliance issues identified in FDA inspections (and noted in FDA 483s) showed about 70% arose in inspections of laboratories, with approximately 30% in manufacturers and very rarely (<1%) in suppliers.[25] Most issues in warning letters about endotoxins have tended to relate to insufficient testing, insufficient controls, insufficient records of verification and validation, and insufficient investigation of out-of-specification results.

- **Insufficient testing.** In 2002, the FDA issued a warning to the Cleveland Clinic Foundation after an inspection of a clinical study site for a clinical trial of an investigational biological found that study investigators failed to "perform the protocol-required tests for endotoxin and mycoplasma prior to the infusion of the investigational [redacted] for all study subjects." The Center for Biologics Evaluation and Research also noted that "[f]ailure to perform the quality control tests places subjects at increased risk."[26]

- **Inadequate testing methods.** An FDA inspection of Panacea Biotec in 2020 led to a warning letter about their analysis methods regarding endotoxins after the company was found to have failed to perform critical steps and had not used calibrated timing equipment. The company's response acknowledging the inadequacies was itself deemed inadequate by the FDA, with the agency noting that the company had "failed to perform a retrospective review of [the] laboratory practices including but not limited to the adequacy of [the] test methods" and that "an evaluation of analyst competencies and training sufficiency" was not provided.[27]

- **Insufficient in-process testing.** Ben Venue Laboratories Inc. was found to have failed to reject materials that contained endotoxin levels above the in-process limit but later passed finished product testing. In addition, the FDA believed the company did not provide sufficient evidence to show its sterilization procedure was able to reduce endotoxins in the final product and that it failed "to establish written control procedures to monitor the output and validate the performance

of those manufacturing processes that may be responsible for causing variability in the characteristics of in-process material and the drug product," as is required by 21CFR §211.110.[28]

- **Insufficient controls.** GSK, formerly known as GlaxoSmithKline, was warned about a lack of adequate controls for the purified water system and manufacturing processes at its facility for manufacturing its influenza vaccine, which were viewed as not being sufficient to prevent endotoxins.[29]

- **Insufficient validation.** Following inspections of Catalent Belgium's sterile filling facility in 2021, the FDA concluded that the "laboratory analytical method for endotoxin ha[d] not been adequately validated." Specifically, the sample storage time for the drug product in-process control test had not been validated.[30]

- **Insufficient oversight of testing.** If tests for endotoxins are conducted by a contracted testing facility, it is still the responsibility of the quality unit of a sponsor to maintain oversight and to ensure the product is being manufactured in accordance with current GMP (21 CFR 211.22).[31] A warning letter to Vitae Enim Vitae Scientific Inc. in 2022 highlighted this failing when the company "failed to evaluate chemical and microbiological method validation and verification as part of the supplier quality audit report of the contract laboratory" and failed to possess method verification and validation records.[32] The FDA advised: **"**your [quality unit] are responsible for ensuring the test methods used and data obtained by your contract testing laboratories are accurate and reliable. The release of drug products without assurance they have the identity, strength, quality, and purity they purport or are represented to possess could result in undetected hazards to patients."

- **Insufficient investigation into out-of-specification results.** In 2013, Pfizer subsidiary Wyeth Lederle received warnings for GMP violations regarding a lack of sufficient investigation into out-of-specification endotoxin results for testing of the diluting agent for the company's anticancer drug Torisel (temsirolimus).[33] The company claimed the results were due to cardboard packaging, but the FDA was not satisfied with the level of evidence provided.

Conclusions and outlook

Endotoxin testing of raw materials, intermediates, and final drug products is essential for patient safety. This article highlights the importance of endotoxin identification and current advances in potential low-cost and high-sensitivity (in-process) detection assays using crab blood. The FDA has identified noncompliance with regulations during inspections of laboratories and

manufacturers, leading to the issuance of warning letters to sponsors. These warning letters are essential for protecting patients from the potential health risks posed by inappropriate endotoxin levels. Research is ongoing to improve safety – for instance, with work on endotoxin-free *E. coli* strains for the manufacture of reagents and certain drug products, technologies for nonanimal-based endotoxin detection, and for endotoxin removal from equipment and drug products.

Abbreviations

EU, endotoxin units; **FDA,** Food and Drug Administration [US]; **GMP,** good manufacturing practice; **IND,** investigational new drug; **LAL,** limulus amebocyte lysate; **USP,** United States Pharmacopeia.

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