General Internal Medicine and Clinical Innovations

Review Article ISSN: 2397-5237

Endotoxin detection asays

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Abstract

The detection of endotoxin and the measurement of its relative level *in situ* is of paramount importance to medical science. Even small quantities of endotoxin can cause septic shock in humans. It is also crucial to detect pyrogens in the pharmaceutical manufacturing environment as well. In both cases speed is of relative importance in the detection of endotoxin associated with a manufactured product, or the by-product of the disruption of Gram negative bacterial cells in the body. This paper discusses various types of pyrogen detection assays including *in vitro* limulus amoebocyte lysate (LAL) assays and *in vivo* assays.

Introduction

Endotoxin is a complex biological molecule which comprises an integral part of the cell walls of Gram negative bacteria. It is a part of the lipopolysaccharide (LPS) component. LPS is composed of the O-side chain and the polysaccharide side chain, both of which are exposed to the environment. The third component is Lipid A which is the anchor imbedded in the cell wall, and as such it is not exposed to the extra-cellular environment [1]. However, when bacterial cells are disrupted, Lipid A is exposed, and thus free to interact with eukaryotic cells with the result that inflammatory molecules are produced. Very little Lipid A is required. As little as 1 µg/kg can cause shock in humans [2]. The tests described below were developed to detect Lipid A, however they will detect other types of pyrogens as well. Other types of pyrogens include plastics, certain organic chemicals and air pollutants. This is significant as other products can elicit non-specific immune responses. Endotoxin results are expressed in LAL assays in EU/mL or endotoxin units per milliliter. One EU represents 100 pg *E. coli* LPS, which would be the amount purified from just over 10^5 *E. coli* bacteria.

Endotoxin Detection Assays

The Rabbit Pyrogen Test

The rabbit pyrogen test is one of the older assays used to evaluate compounds for pyrogenic activity [3]. It is a compendia method and uses temperature increases in outbred New Zealand White rabbits as the indicator. The basis of this test is that potential pyrogens will cause the temperature in rabbits to elevate over a three [3] hour period of time [3]. The rabbits receive intravenous injections (often via the marginal ear vein) of the compound in question, with physiological saline as the diluent on a weight per volume basis. Temperature is monitored by an anal probe for three hours. Should the temperature in any single rabbit elevate above 0.5^0 of the rabbits' original baseline temperature reading, the test is a failure. Further testing is allowed if a failure occurs.

The advantage of the rabbit pyrogen test is that it is not so sensitive as to give false positive readings. It allows for a reasonably accurate evaluation of how a compound will react in the human body. It is also not necessarily subject to outside environmental influences such as air pollutants. The disadvantage to the test is that it is costly and requires animals and a special facility to conduct the test. It will also not give actual endotoxin levels associated with the compound, it will simply say if the levels are of physiological significance.

Limulus Amoebocyte Lysate (*LAL) Testing*

The basis of all LAL testing is that the blood of a horse shoe crab will turn into a semi-solid clump when placed in the presence of Gram Negative bacteria [3]. The amoebocytes in the horse shoe crab contain clotting factors called coagulogen. Blood is withdrawn from the crab via the pericardial route, and the crabs are returned to their natural environment. Blood cells are separated from the rest of the blood by-products via centrifugation, and then placed into pyrogen free distilled water where they lyse. The resulting supernatant fluid in water is lyophilized. This "lysate" is the basis of all testing and is included as part of a "kit". There are three basic LAL techniques, gel-clot, turbidimetric and chromogenic. LAL testing uses purified *E. coli* LPS as the reaction substance in the standard curve.

Gel-Clot Technique

The gel-clot technique is a simple semi-quantitative test to evaluate for the presence of endotoxin in a given sample [3]. It is a US compendia method that is sensitive up to 0.3 EU/mL. Samples are placed in an endotoxin free glass tube along with an equal volume of lysate. The tubes are incubated undisturbed for 60 min at 37 °C. There is a negative control which has lysate and water as a substitute for product sample. There is also a standard curve which is run at the same time. Once the time period has been completed, the tubes are inverted 180^0. If endotoxin is present in the sample, a clot has formed which will not dislodge when the tube is inverted. This is a positive test. If no clot has formed, there will be liquid in the tube which will be apparent when inversion is attempted. A semi-solid clot is also negative. This means that no endotoxin is present or more likely a non-detectable amount is present, in either case, a negative test. Many samples can be processed at a time, and the test is inexpensive and easy to conduct. However,

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Key words: *Lipid A; lipopolysaccharide; limulus; pyrogen; endotoxin*

Received: May 04, 2018; **Accepted:** May 14, 2018; **Published:** May 17, 2018

it is less sensitive than other types of LAL testing and is not truly quantitative.

Kinetic Turbidimetric

This is a reliable and quantitative assay that will detect endotoxin at the level of 0.001 EU/Ml [3]. This is the most sensitive of all the LAL assays. In this assay, a pro-enzyme in the lysate is activated by the direct presence of endotoxin. The level of activation can be quantified spectrophotometrically as the lysate/sample combination becomes more turbid. The activation rate can be accurately quantified and is proportional in a direct sense to the amount of endotoxin present. The biggest drawback to this method is that it is the most susceptible to interference.

Chromogenic

The Chromogenic technique has a sensitivity to 0.005 EU/Ml [3]. As with the above assays, a sample is mixed with lysate and in this case is placed into a plate reader. A yellow color will appear over time which is indicative of the amount of endotoxin present. This is compared and analyzed by a computer program to which has been integrated with a standard curve. The nature of the chromogenic assay makes it a better choice for the analysis of parenteral and vaccines. It is less affected by inhibitory substances as compared to the turbidimetric and gel clot assays. It is also the most expensive of the LAL assays to conduct which can prove to be its biggest drawback.

The MAT Assay

The monocyte activation assay (MAT) is a relatively new technique which uses the release of interleukin-6 from human blood monocytes as the basis of the technique [4,5]. It is an *in vitro* test with a cellular component. The greater the endotoxin level present in a given sample, the greater the amount of IL-6 measured in the cell culture supernatant fluid. The assay is based on human blood monocyte ability to react to pyrogens in general and endotoxin specifically. The sample compound is placed in culture with the monocytes, and incubated. Monocytes and other cell types produce IL-6 in response to pyrogenic or allergic stimulus. IL-6 is one of the first cytokines produced upon cellular stimulation, and as such is an excellent detector molecule. The levels of IL-6 present in the supernatant fluid are indicative of the amount of pyrogen present in the sample. A standard curve is generated during the test as a positive control. Sample results are compared to the standard curve and the thus the amount of endotoxin in a given sample is quantified. The advantage of this technique is that measurement of cytokine release from a human cell is possible. Therefore, there can be made more direct collations with the human *in vivo* experience. The disadvantage is the need for human monocytes to perform the assay. This issue may be overcome by the development of a continuous human cell line which has the ability to produce cytokines upon endotoxin stimulation. The cell line is currently in development.

Conclusion

The detection of endotoxin will remain of paramount importance in both medical science and bio-pharmaceutical manufacturing. The timely and accurate measurement of endotoxin levels is especially important in the medical science setting where the correct diagnosis of septic inflammatory reactions is critical. The detection of pyrogens in the biopharmaceutical industry is also important due to the very reasonable restrictions that are placed drugs, vaccines and medical devices. The presence of reliable pyrogen detection assays that are easy to use and inexpensive will help to patient safety as new products are developed.

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