Assessment of Bacterial Endotoxin by Fluorescent Labeling

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Introduction

New research in the field of bacterial endotoxin allows for the rapid detection, and quantitative estimation of, different endotoxin species. This has long been thought theoretically possible due to similarities and differences with the chemical structure of the pyrogenicity and immunogenicity of lipopolysaccharide,[1] but the technological means to achieve this could not be realized commercially, or reliably, until recently. A new technique, based on microchip electrophoretic methodology, can examine lipopolysaccharides for variations in the length and composition of the O-antigen and core oligosaccharide of the molecule; this is in addition to assessing the number and length of fatty acid chains in the Lipid A portion of lipopolysaccharide. When this information is computed and compared the new method allows for the determination of the bacterial strain of the endotoxin. The technology requires bacterial cells to be grown and a cellular lysate obtained; the probable species is then determined through molecular size comparison.

Initially the technology will be of use for medical research; in time, however, it could conceivably be of use for addressing out-of-limits investigations within the pharmaceutical facility. This type of novel method will not replace the methods described in the compendia for the quantitative determination of the endotoxin concentration in a sample (or even novel methods for endotoxin that are in development, such as the application of quartz crystal microbalance sensors);[2] instead the method has the potential to support the activities of the microbiology laboratory. In the pharmaceutical context understanding the bacterial origin of the endotoxin in materials or a water system, for example, would assist with root cause analysis and the formulation of corrective and preventative actions.

Structure of Bacterial Endotoxin

Bacterial endotoxin refers to lipopolysaccharides and lipooligosaccharides (the term lipooligosaccharide is used to refer to a low-molecular-weight form of bacterial lipopolysaccharides), which are the components of the cell envelope of Gram-negative bacteria, a large group of bacteria comprising important human pathogens as well as commensals. The function of lipopolysaccharide, as the major component of the Gram-negative bacterium cell membrane is to contribute to the structural integrity of the bacteria and to protect the membrane from chemical attack. These molecules are highly immunogenic and directly contribute to bacterial diseases in humans, such as pyrogenic reaction and sepsis.[3] Of interest for this article is lipopolysaccharides, which describe a group of amphiphilic macromolecules which are made up of three defined regions distinguished by their genetics, structures, and function. These are: the lipid A, the core oligosaccharide and a polysaccharide portion called the O-chain.[4] The polysaccharide portion is diverse in length and composition amongst the different Gram-negative bacteria species (this is an important differentiator for the subsequent discussion in this article).

With this Gram-negative bacterial cell wall material, the polysaccharide and lipid portion of lipopolysaccharide contribute to the pathogenic potential of the bacteria; however, it is the lipid component (lipid A) which determines the endotoxic properties of lipopolysaccharide.[5] Given the potential for lipopolysaccharide to cause a pyrogenic response and stemming from the risks that Gram-negative bacteria pose to pharmaceutical production processes it is important that many pharmaceutical products, especially sterile parenteral products, have a final product endotoxin test as well as in-process controls to minimize the risk of endotoxin contamination occuring during the process.[6]

The characterization of endotoxins is of medical and pharmacological importance, since the physiological and pathophysiological (the physiological processes through which an abnormal condition develops and progresses) effects of endotoxin relate to the chemical structure of the molecule. The differences between lipopolysaccharides from different bacterial serotypes and their mutants are primarily variations within the composition and length of their O-polysaccharide chains.

New Technique

The new technique is based upon microchip electrophoretic technology and the method enables the structural characterization of lipopolysaccharide molecules from several different Gram-negative bacteria and for the quantitative evaluation of components of endotoxin extracts.

Central to the new method is electrophoresis, which is long-established in the life sciences for separating out macromolecules based on size. Electrophoresis involves inducing detectable differences in migration behavior between charged species when subjected to the influence of an applied electric field which exerts an electrostatic Coulomb force (this describes the force interacting between static electrically charged particles).[7] This method can be applied to a range of analytes, such as biological macromolecules including DNA and proteins.[8] During the past ten years electrophoresis technology has been adapted into miniaturized microfluidic formats. Microfluidics is concerned with the behavior, control and manipulation of fluids contained within geometrically constrained spaces on the sub-millimeter scale.[9] Technologies within this field are created to achieve multiplexing, automation, and high-throughput screening.

Hence this trajectory towards miniaturized testing has been partly driven by economies of scale (to reduce costs in terms of the numbers of tests run) and portability. These reasons are consistent with the self-contained 'lab-on-a-chip' paradigm, which allows multiple chemical, biological, and biomedical assays to be run.[10] A second reason is to expand the types of analyses that can be conducted, and this is where the application to endotoxin structural analysis comes in.

The application of microchip electrophoretic method for endotoxin analysis has been published, in 2017, in the journal *Methods of Molecular Biology*[11] and it originates from research laboratory of Professor Bela Kocsis at the University of Pécs, Pécs, Hungary. The method describes the direct labeling of endotoxins through the covalent binding of a fluorescent dye or fluorophore (which can re-emit light upon light excitation). Earlier work looked at interaction with dodecyl sulfate and a fluorescent dye, or by a covalently bound fluorescent dye.[12] The latter variant proved to be more effective.

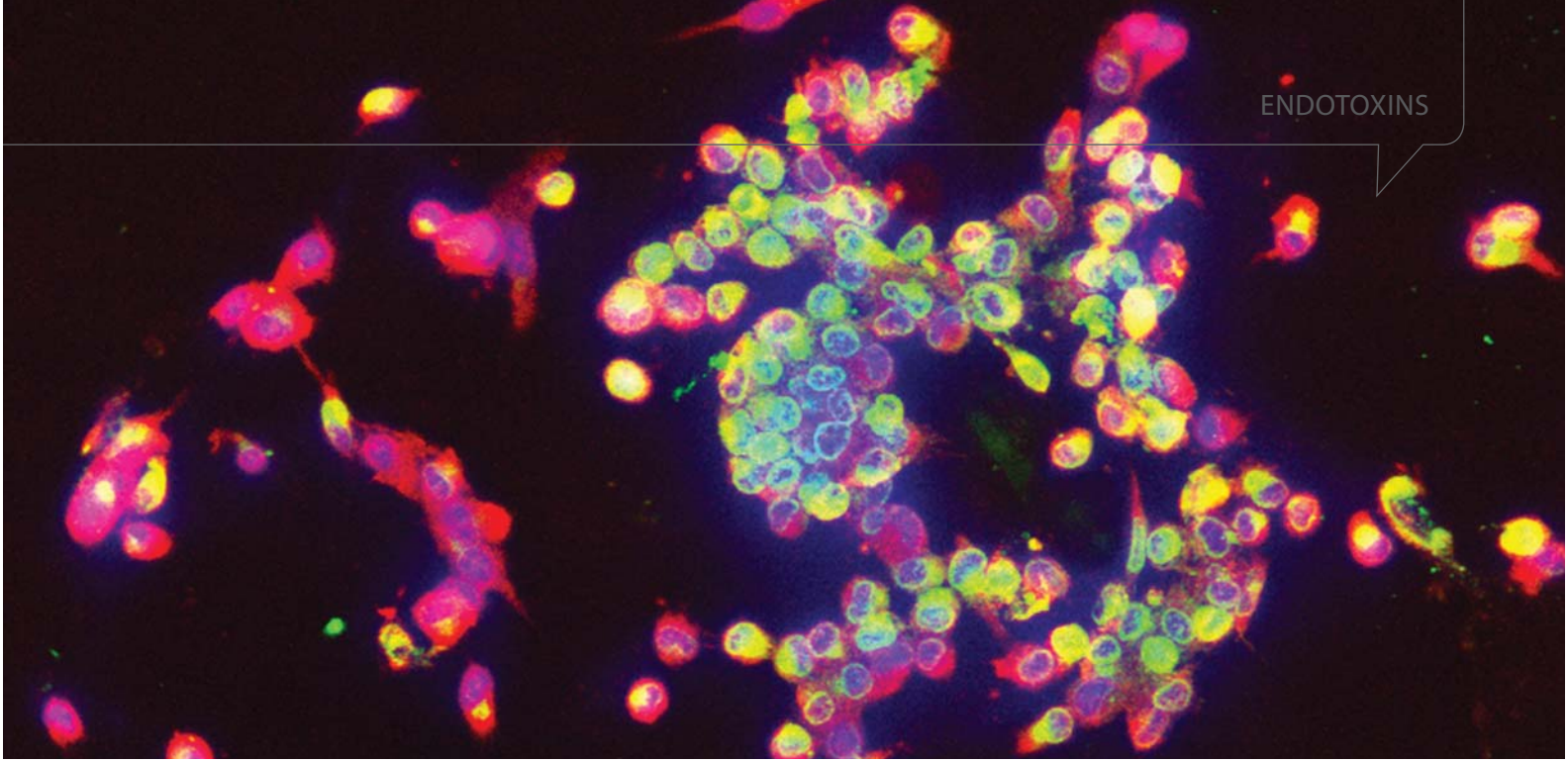
Before labeling the a whole-cell lysate of the Gram-negative bacterium is required (the lysate is a fluid containing the contents of lysed cells). Cell lysis allows the bacterial cells to be broken open so the contents can be further studied. The lysate for the Kocsis work is obtained after the destruction of the proteinaceous components, which is achieved by proteinase K digestion and precipitation of the lipopolysaccharide components. The proteinase K enzyme digests proteins following its activation by calcium. Proteinase K is produced by the fungus *Tritirachium album Limber*. When grown by submerged fermentation the fungus secretes the enzyme.

The use of the dye allows the electrophoretic profile of lipopolysaccharide to be determined. In turn, this allows a differentiation of the 'S-type lipopolysaccharide' be performed since the electrophoretic profiles are characteristic of the different bacterial strains. Previous research has shown how the chain length of the carbohydrate unit of lipopolysaccharide leads to either a long (or 'smooth' S-type) or a short (or 'rough' R-type) O- chains, or both. The array of S- and R-types affects the host-pathogen interaction.[13] The two forms of lipopolysaccharide are not mutually exclusive; most wild-type bacteria, especially Gram-negative organisms found in water, contain a highly heterogeneous mixture of S-form lipopolysaccharide molecules and a varying proportion of R-form molecules.[14] In terms of the level of differentiation, trials have shown, for example, structural differences between the "smooth" and "rough" lipopolysaccharides from *Salmonella minnesota* (both wild-types and laboratory strains) and other organism *Shigella sonnei*.

Kocsis' research, expanded upon in a subsequent book chapter,[15] has determined that, based on the number, distribution, and the variations with the amounts of components from an endotoxin extract, the S-type endotoxins from different Gram-negative bacterial strains can be classified based on differences visualized through differing electrophoretic profiles. The necessary separation of components (importantly S- and R- type lipopolysaccharides) is achieved through the use of the microchip electrophoresis. Further work has led to the microchip technique having the potential to be standardized, as well as offering a relatively fast and sensitive method (in terms of processing speed this is typically less than one minute per sample).

This method represents an advancement upon earlier attempts to characterize endotoxin through purification, such as by using mass spectrometry for profiling lipid A structures extracted from whole-cell lysates. With mass spectrometry (such as using matrix-assisted laser desorption/ionization mass spectrometry analysis) a variation arises with both the purity of the end product and downstream phenotypic variations stemming from the type of bacterial growth media used to culture different Gram-negative species. In contrast, microchip electrophoresis provides high-resolution separation of pure and partially purified (that is obtained from whole-cell lysate) S and R endotoxins. The use of a fluorescent dye also proved superior to attempting methods like sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). For the visualization of the separated proteins a silver stain is commonly deployed. Microchip electrophoresis was superior to SDS-PAGE by virtue of higher speed and better sensitivity, plus negating the need for laborious gel-preparation and silver staining.[16]

A limitation with the method is that bacteria of concern must be culturable and the correct culture medium and incubation conditions selected. In addition, growth must be sufficient for the method, as with any analytical method, has an inherent limit of detection (the lowest quantity of a substance that can be distinguished from the absence of that substance within a stated confidence limit). The limit of detection can be improved through the use of different buffers during the creation of the lysate. So far the limit of detection of endotoxins from S- and R-type



Gram-negative bacteria stands at 2.6 nanograms and 6.9 nanograms, respectively. This is not particularly restrictive provided the organism of interest can be cultured.

Summary

This article has assessed a new technique involving the solubilization and complexation of the lipopolysaccharide, followed by fluorescent labeling of endotoxin, and as analyzed by electrophoresis on a microchip. It is a novel example of lab-on-a-chip technology and through the technique it appears possible to establish an electrophoretic profile of several Gram-negative bacterial strains. While currently confined to the research laboratory the technique could, in time, become a useful diagnostic aid for assisting with out-of-limits investigations in the pharmaceutical and healthcare setting.

Understanding the type of Gram-negative organism that caused an endotoxin contaminating event could help match the organism profiles to bioburden results and this may help with ascertaining whether contamination arose from particular type of pharmaceutical (or non-pharmaceutical) grade water; a process issue; or even an ecological niche pertaining to the human microbiome (*Acinetobacter*, for example, is an example of a rare Gram-negative bacterial genus associated with the outermost moist areas of the human skin).[17] The limitation with the method is that a bacterial cell needs to grown up and the endotoxin extracted; lipopolysaccharide itself cannot, as yet, be extracted from a sample and profiled.

It may come to pass that a microchip electrophoretic method becomes a supporting diagnostic tool for the pharmaceutical microbiology laboratory.

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