CORMICA®

Animal-Free Bacterial Endotoxin Testing

The Road to Recombinant Reagents

As we look to the future of bacterial endotoxin testing with animal-free reagents at Cormica, we wanted to provide our customers with a brief overview of endotoxins, and the journey the industry has taken to get to this point.

Endotoxins

Any discussion around endotoxins must first begin with an explanation of their relationship with pyrogens. A pyrogen is anything that causes a rise in body temperature (El-Radhi, 2019). While endotoxin does happen to be the most potent pyrogen, it is not the only one. From a pharmaceutical manufacturing perspective, examples of pyrogens would include lipoteichoic acid, yeasts, moulds, and of course, endotoxin.

The definition of endotoxin itself has evolved several times as our understanding has developed, but broadly speaking, endotoxin is Lipopolysaccharide (LPS). LPS are the hair like appendages found on gram-negative bacteria such as E. coli, Pseudomonas sp. and Salmonella sp. They form an integral part of the cell membranes of these bacteria, within which hides one of the most toxic molecules in microbiology; the Lipid A region of LPS.

To briefly entertain the minutia of the endotoxin definition: When endotoxin is released naturally, either through the release of outer membrane vesicles containing LPS, or as a result of gram-negative bacterial cell death, they are made up of cell wall fragments in addition to the LPS molecule. This means that naturally occurring endotoxin is structurally different to a pure LPS molecule. This is an important distinction to make when it comes to their detection, as the structural differences affect the charge of these molecules, which means they behave differently when in solution (McCullough, 2015).

However, the main point from a patient safety perspective is that, whatever your definition of endotoxin is, it will always include LPS, which is extremely toxic when introduced into the bloodstream. As little as 1 to 2 mg of LPS entered intravenously can be lethal (Khan & Farhana, 2023). Because of its toxicity, any parenterally administered product must be tested for bacterial endotoxins, and this is where the road to recombinant reagents comes in.

The Road to Recombinant Reagents

As the understanding of microorganisms was limited in the early 20th century, it was some time before the "Injection Fever" experienced by some patients was linked to anything other than just "pyrogens" (Akers et al, 2022). Therefore, the first test for endotoxins was called the Rabbit Pyrogen Test (RPT). This involves injecting a sample into three rabbits, and monitoring their temperatures at 30 minute intervals over a period of 3 hours. If there is a rise in body temperature above 0.5 ˚C, the sample is deemed to be pyrogenic (Gimenes et al, 2015).

Aside from the obvious ethical implications of using rabbits for testing, there are other limitations of the RPT. Not only is it susceptible to false positives as rabbits can exhibit a temperature increase for a multitude of reasons, it is also not an endotoxin-specific test. This means its use is limited in quantifying a specific amount of endotoxin in a sample, as any true pyrogenic effect could have been caused by other pyrogens within the sample.

The RPT was first recorded as being used in 1912 before being further refined in the 1920s by Seibert, Bourn and Mendel (Vipond et al, 2015). As a result of the dramatic increase in the use of injectables during World War II, the RPT was then added to the United States Pharmacopeia (USP) in 1942 (Roberts, 2007). So, for decades, every batch of injectables was tested for endotoxins using rabbits. This was the case until the immense potential of the Horseshoe Crab (HSC) was realised.

The HSC is an arthropod which has a fossil record dating back to over 400 million years. There are four main species of HSC today: Limulus polyphemus, Carcinoscorpius rotundicauda, Tachypleus gigas, and Tachypleus tridentatus. One thing that all of these species have in common is their unique immune system. As HSCs have a single sinus cavity instead of the miles of circulatory system that we have, these crabs have a dramatic response to infection; their blood will clot when exposed to endotoxin.

This clotting mechanism is driven by a three-factor enzymatic cascade. When it is extracted from the blood cells of Limulus polyphemus, this cascade is referred to as Limulus Amoebocyte Lysate (LAL). As LAL is made up of three different Factors, each one activating several others in the next level of the cascade and therefore significantly amplifying the signal, it is triggered by miniscule amounts of endotoxin. Not only does this give the HSC an immensely sensitive immune response, it also provides us with one of the most useful biological assays in history.

The effects of LAL were initially described by Frederik Bang in 1953 (Bang, 1953). He was then joined by Jack Levin at the Marine Biological Laboratory in Woods Hole, Massachusetts, USA, where they worked together to further our understanding throughout the 1960s. In 1968, Bang and Levin published their findings that this coagulation was both caused by endotoxin and that the mechanism was located in the amoebocyte granules (Bang & Levin, 1968).

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It was not until almost a decade later, however, that Stanley Watson of Woods Hole Oceanographic Institute commercialised the first LAL reagent for the gel clot test. He went on to receive the first FDA license to manufacture these reagents in 1977, and was closely followed by the other LAL manufacturers currently holding a license today.

The gel clot test uses LAL to cause a solid clot to form if a sample is above a specific sensitivity. After years of comparability studies demonstrating the gel clot test was equivalent to the RPT, it was added to the pharmacopoeias in 1980 (Tirumalai & McCullough, 2019). This was then followed by the development of the kinetic LAL assays, which provide a far more sensitive test through the use an absorbance reader – first as the turbidimetric modification and later on followed by the chromogenic assay.

In the chromogenic assays, as the LAL reagent reacts with the sample, it will cause a colour change in that sample over time as the LAL cascade is activated. This colour change will reduce the amount of light that is able to pass through the sample and be read by the absorbance reader. After the amount of light passing through the sample has reduced to a preset threshold called the optical density threshold, the software will assign what is called an onset time for that sample. By comparing the onset time of the sample with those of the standard curve run in parallel, this method is able to provide a quantitative result for endotoxin activity.

An important point to note here is that the LAL test is referred to as the Bacterial Endotoxin Test (BET) in the pharmacopoeias. This is to account for the fact that not all of the reagents used come from the Limulus polyphemus HSC. There are other reagents manufactured using the blood of Tachypleus sp.

Accepting the LAL test as a replacement for the RPT marked a dramatic shift away from using mammals as the test subject, and only relying on an aliquot of the arthropods blood as part of the preparation of LAL. However, at this point in history, a brand-new technology was developed allowing a truly animal-free test for endotoxin testing.

In 1973, the first recombinant DNA molecules were generated which paved the way for the development of various therapeutic agents and diagnostic tools using this technology in the 1980s (Khan et al, 2016). This recombinant technology was first applied to the field of endotoxin detection with the work of Dr. Jeak Ling Ding and her team in the late 1980s and early 1990s. Dr. Ding went on to successfully express the part of the cascade that was sensitive to endotoxins from the Carcinoscorpius species of HSC in 1995 (Ding et al, 1995). This provided the first recombinant reagent for bacterial endotoxin testing, known as recombinant Factor C (rFC).

While rFC first became commercially available in 2003, it did not enjoy the fanfare one may expect for such an accomplishment, as these reagents are still deemed as alternative methods by the pharmacopoeias for finished product testing two decades later.

Nevertheless, one cannot discount the significant efforts of Dr. Ding and her team in developing this reagent, as it not only provided the first animal-free reagent in history, it also paved the way for the latest generation of recombinant reagents for bacterial endotoxin testing – the recombinant Cascade Reagents (rCR).

Stay tuned for our next article which will provide an overview of the currently available recombinant reagents, including rCR. Should you have any questions about animal-free endotoxin testing, please do not hesitate to reach out to us at info@cormica.com.

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