

The Rabbit and the Horseshoe Crab

The LAL method, which is today's industry standard for the detection of gram-negative bacterial endotoxin, was largely preceded by the rabbit pyrogen test by about 30 years. In order to better understand the benefits of current testing, as well as, the industry's shift from the rabbit to *Limulus polyphemus* or the Atlantic horseshoe crab, it is important to follow the path through which testing has evolved.



The Rabbit and the HORSESHOE CRAB

When speaking of rabbit pyrogen testing, we would first need to discuss pyrexia. Pyrexia, or fever, is the elevation of the body temperature from the normal. A pyrogen is a substance which induces pyrexia. Endotoxin, also known as lipopolysaccharide (LPS), is our main pyrogen of interest due to its deleterious effects on the human body.

The term "endotoxin" was coined in 1892 by Richard Pfeiffer during his studies of *Vibrio cholerae*, the bacterium responsible for cholera. Pfeiffer observed that the bacteria produced a toxin that was attached to their cell wall. The toxicity of this substance was unchanged by killing the bacteria. Around the same time, Eugenio Centanni successfully isolated endotoxin from bacterial lysates using a complex work-up. He observed that all gram-negative bacterial species could produce this toxin. Additionally, Centanni noted the high heat stability of the toxin and suggested that it was likely not proteinaceous, as had been previously assumed.

Many researchers later improved upon Centanni's workflow to produce highly pure endotoxin isolates. These efforts culminated in the work of Otto Westphal and Otto Luderitz, who developed the hot phenol-water extraction method. This protocol, which is still used to this day, enabled researchers to isolate protein-free LPS isolates, proving to be a boost to the field of endotoxin research for decades to come.

Today, purified LPS is a staple for any immunology laboratory. Scientists continue to unravel the mystery of how this simple

molecule can trigger an intense immune cascade and how we can develop better treatments against septicaemia.

LPS is made from a lipid (fat), which anchors the structure to the cell wall, and an oligosaccharide (sugar), which extends from the bacterial surface. The lipid moiety, known as lipid A, is the main structure that's recognised by the immune system. Specifically, lipid A is recognised by a complex of two immune system proteins: Toll-like receptor 4 (TLR4) and MD-2. When activated, this complex triggers an innate immune cascade to fight the pathogen.

Both the lipid and oligosaccharide components of LPS vary considerably between different species of bacteria. These variations can have a dramatic effect on the host's ability to recognise and respond to the infection. The oligosaccharide tends to have the highest degree of variability in terms of both size and structure. In fact, the LPS oligosaccharide is frequently used to classify different strains of bacteria.

In contrast, variations of lipid A between bacterial species are more subtle. The general structure of lipid A consists of two units of glucosamine (a form of sugar), each of which is attached to one phosphate group and several acyl (fatty acid) chains.

The most common variety of lipid A contains six acyl chains. This form is found in many common gram-negative bacteria, including *E. coli*, and can elicit a strong immune response via the TLR4/MD-4 complex; however, other species of bacteria express forms of lipid A with more or fewer than six acyl groups. These unusual forms of lipid A do not stimulate the TLR4/MD-2 complex as strongly.

The rabbit pyrogen test is basically a test which involves inoculating a rabbit

with a product sample to determine the presence or absence of pyrogens. If a pyrogen is present in the test substance, it will cause an elevation of the animal's body temperature from normal. Rabbits have a similar temperature response to endotoxins as humans. For this reason, they are ideal for use in pyrogen detection.

The U.S. Pharmacopeia gives a description on the administration of pyrogen testing. A test solution is administered intravenously into the animal's ear. The rate of administration is not to exceed 10ml/kg for 10 minutes. All materials, diluents and solutions used should be sterile and pyrogen-free. Healthy, mature rabbits are selected and housed in temperatures of 20 to 30 degrees Celsius. They are also kept free from any disturbance or excitement. This will later be discussed further as there are other situations which may cause increases in body temperature. A rabbit can only be subjected to a test solution once every 48 hours and well outside of two weeks of having a body temperature greater than 0.6 degrees Celsius from its normal. The baseline temperature of the rabbit is measured not more than 30 minutes prior to the testing. It should not exceed 39.8 degrees Celsius. Rabbits used in group testing should have a variance in temperature of only one degree from each other. The rabbits are tested in temperatures similar to their natural environment. Food is withheld during the test. Water may be withheld or be made available to the test animals. Temperature is measured through rectal probes while the rabbit is restrained in its natural resting position. Test solutions are injected into the ear vein after being warmed to between 35 and 39 degrees Celsius. Temperature readings are taken at 30-minute intervals for the next 1-3 hours. Three rabbits will be tested per solution.

A negative test is an elevation in the rabbit's temperature of less than 0.5



degrees Celsius. The test solution may then be considered pyrogen-free. A temperature elevation of 0.5 degrees or more will warrant a repeat of the testing in five more animal subjects. A negative result is an elevation in temperature of 0.5 degrees or greater in only two out of the eight rabbits or if the total temperature rise in all eight rabbits does not exceed 3.3 degrees Celsius.

The endotoxin limit is expressed as K/M, where K is the threshold pyrogen dose for both humans and the rabbit animal subjects. K has a value of 5 EU/kg. M is the maximum human dose to be administered per kilogram in one hour. Intrathecal injections have a K value of 0.2 EU/kg.

There are numerous limitations in using rabbit pyrogen testing for determining endotoxin content. Endotoxins and pyrogens are two entirely different entities. Endotoxin is a molecule contained in the cell wall of gram-negative bacteria. It is a known pyrogen. Pyrogens are any substance which causes febrile responses in both humans and rabbits and they encompass several entities and situations beyond endotoxins. Mycobacteria, fungi and viruses which do not contain endotoxins may still cause febrile reactions. We have endogenous pyrogens, substances inherent to our metabolic system which can induce fever. We have interleukins which are known to raise body temperature during activation

of the inflammatory pathway. Our thyroid and oestrogen hormones are known to regulate body temperature and can elevate or decrease it depending on its level and activity. We have exogenous pyrogens that, when introduced inside of our bodies, can cause a febrile response. Blood and blood products when transfused may cause a rise in body temperature. Medications we take in may cause different bodily reactions which may include elevated temperatures.

Other conditions may lead to elevations in body temperature as well. Exercise causes an increase in metabolic rates. The increase in blood flow and rapid cellular metabolism causes a transient fever. Emotional stress or

excitation may cause a similar response. A tumour or malignancy, with its increased metabolic requirements and release of endogenous pyrogenic substances can also produce fever episodes. Temperature control of the body is regulated by the hypothalamus; therefore, any defects or injuries to the hypothalamus and the central nervous system can alter the regulation of body temperature.

As there are so many factors which may cause pyrexia, there are, in turn, also many contributors to variability in rabbit pyrogen testing. Being an *in vivo* test, various studies have shown that multiple exposures to endotoxins and pyrogens produced tolerance in the test animals. Over time and after numerous tests, the febrile response of the rabbit became diminished. The bacteria *Legionella pneumophila* has also been found to induce little pyrexia in rabbits but is readily detectable through the LAL procedure. In fact, a 1000-fold difference was found between the results of the two procedures in detecting *Legionella* endotoxin. *L. pneumophila* is the bacteria responsible for causing the notorious pneumonia outbreak among the attendees of the American Legion convention at the Bellevue-Stratford Hotel in Philadelphia in July 1976. That outbreak involved 182 cases and resulted in the death of 29.

The rabbit test was determined to be inadequate for detecting pyrogenic substances in radiotherapy products, chemotherapy drugs, steroids, narcotics and other substances which had inherent probabilities to react with the human immune system and produce pyrogenic reactions even in the absence of bacterial pyrogens. This would lead to numerous false positive results. Rabbit pyrogen testing is also obviously more time-consuming, rigorous and expensive. The biggest drawback of this test procedure, however, is the inability to quantify the endotoxin levels.

As the fable goes, the rabbit was fast and started early. In the comparison of rabbit pyrogen testing with the LAL method, the rabbit is compared this time to the horseshoe crab, which, like the turtle, is a slow-going resident of the sea. The horseshoe crab from the Atlantic, commonly known as *Limulus polyphemus*, has existed for more than 200 million years. It has been classified under the category arthropods since the 19th century, and not under crustaceans as it was first thought. This close relative of arachnids

has historically been harvested for its use as a fertiliser or bait, and more recently it is used for the extraction of blood for the *Limulus* ameobocyte lysate (LAL) Test, used for the detection of bacterial endotoxins in pharmaceutical products and other medical applications.

Limulus polyphemus plays a vital role in human medicine. It is a model for the study of the innate immune system. As an invertebrate that does not have immunoglobulins, the horseshoe crab has developed a unique mechanism for detecting and responding to the antigens from the microbial surface, such as lipopolysaccharides (LPS), lipoteichoic acids, lipoproteins, peptidoglycans (PGN) and 1,3- β -glucans. This mechanism is the coagulation of the hemolymph. The blood cells of *Limulus*, called ameobocytes, clot in the presence of antigens from the microbial surfaces mentioned above. This reaction involving the clotting of the hemolymph is precisely the biochemical principle of the *Limulus* ameobocyte lysate (LAL) Test. These days it is accepted that the coagulation phenomenon of the *Limulus* blood is not an isolated reaction, but a cascade of enzymatic activation steps which end with excision of the protein, leaving an insoluble product that combines via an ionic interaction. If enough quantities of this coagulate are found, turbidity appears, followed by the formation of a clot with the consistency of gel. From this the fundamentals appeared which gave rise to the method to detect the presence of these bacterial endotoxins, better known as the *Limulus* ameobocyte lysate test (LAL) or bacterial endotoxin test (BET).

The LAL method was officially accepted by the FDA (U.S. Food and Drug Administration) in the 1970s and has remained until now the official basis for endotoxin testing across the globe. In fact, the U.S. Pharmacopoeia (USP) establishes the quantification of pyrogens or lipopolysaccharides for more than 90% of parenteral-use drugs via this method.

Though the main use of the LAL test is for the detection of LPS in pharmaceutical products, its use extends to the diagnosis of endotoxemia associated with cirrhosis, cancer, meningitis, ocular diseases, infections of the urinary tract, as well as the analysis of water quality. Today, we continue to find new applications for the LAL test, such as its use in the detection of bacterial contamination in food, including frozen products.



Rabbit pyrogen testing was adapted by the U.S. pharmacopeia in the early 1940s, predating the use of LAL endotoxin testing by 30-40 years. Due to the advantages of the LAL procedure in terms of cost, specificity, less variability, convenience and being quantifiable, the LAL test has now replaced pyrogen testing in detecting the presence of bacterial endotoxin. Horseshoe crabs are a vital species whose ancient longevity has not only intricately tied them to the wellbeing of our ecosystem, but also to the safety of modern health and medicine. Their annual spawning activities along the shoreline provide a necessary source of food for migratory birds. Horseshoe crabs are caught by fishermen who rely upon them as a source of bait. They are also a critical resource used by biomedical companies, who collect their blood to produce a reagent for the LAL assay, an FDA required quality test of intravenous pharmaceuticals for bacterial endotoxin contamination.

Thus, in our story, the horseshoe crab has undeniably outraced the rabbit.



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