Alternatives to Animal Use for the LAL-Assay

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Summary

The Bacterial Endotoxin Test (BET or Limulus Amoebocyte Lysate (LAL) test) is performed to quantify endotoxin (lipopolysaccharide, LPS), the most effective pyrogen known. Due to its persistence against cleaning/inactivation procedures, LPS has an enormous impact on the Pharma, Life Sciences, and Medical Devices sectors. The BET has replaced the Rabbit Pyrogen Test (RPT) for most products and is obligate for many source materials, intermediates, and final products.

However, the "alternative" BET is an indirect animal test. The Limulus lysate is obtained from wildlife populations of horseshoe crabs (mainly Limulus polyphemus). Despite immense efforts by the BET-industry, the mortality of this procedure is approximately 10-15%. The Asian Tachypleus population (especially in China) is threatened severely. The still-growing demand for lysate cannot be satisfied by the wildlife population, which may result in a major drug safety problem.

The 3Rs could be applied immediately, addressing Replacement and Reduction (validated alternatives such as the Monocyte Activation Test (MAT) and recombinant LAL assays), as well as Refinement (reduced lysate volume). Furthermore we describe a more sensitive and accelerated MAT version as a more feasible alternative.

Keywords: BET, LAL, MAT, drug safety, 3Rs

1. Introduction

Testing for the absence of pyrogenic (fever inducing) substances (Ding and Ho, 2001) is of utmost importance for a safe administration of parenteral medicines (or "medicines for parenteral use") such as infusion solutions, plasma derivatives, vaccines, cell therapeutics, gene therapeutics, and tissues. In general, the group of pyrogenic agents able to activate monocytes includes bacteria, viruses, fungi, their components, and a variety of other substances up to chemicals. The most potent and best known pyrogen is endotoxin, the main constituent of the outer cell wall of Gram-negative bacteria. Other pyrogens (or Toll-like receptor (TLR)-agonists) are of a more diverse nature (Lien and Ingalls, 2002) than endotoxins, and therefore the classification into endotoxin and non-endotoxin pyrogens (NEP) was established. Ingression of pyrogens into the bloodstream can lead within minutes to adverse reactions, including life-threatening consequences. Therefore, pharmacopoeias worldwide demand pyrogen testing for the safe administration of medicines for parenteral use.

The Rabbit Pyrogen Test (RPT) has long been the standard animal test for pyrogens. It has been commonly accepted and established for more than 60 years, it is still performed – and it is still associated with extensive use of animals. Great efforts during the past 15 years have led to alternative methods, some of which are animal-free. Alternative methods, including the BET, which indirectly consumes animals, take advantage of the archaic host defense of horseshoe crabs (e.g., *Limulus polyphemus*) against endotoxins (Bang, 1956; Ding and Ho, 2001). A recombinant BET (based on a genetically engineered protein from the *Carcinoscorpius* clotting cascade), that does not consume any animals, has been developed and validated (Ding and Ho, 2001; Loverock et al., 2010). A different approach in pyrogen testing, mimicking the human fever reaction, can be found in the Monocyte Activation Test (MAT) (Dinarello, 2004; Hartung et al., 2001; Hoffmann et al., 2005; Gaines Das et al., 2004; Werner-Feldmayer et al., 1995; Eperon et al., 1997; Moesby et al., 1999; Hansen and Christensen, 1990; Schindler et al., 2009). Using human monocytes from different sources, such as fresh blood, peripheral blood, mononuclear cells (PBMC), and monocytic cell lines (cryopreserved whole blood and PBMC can also be used), this test directly measures the pyrogenic response to endotoxin and NEP. We will discuss in detail the aforementioned tests with their respective advantages and disadvantages, elucidating some of the rationales for choosing the most appropriate variant for one's needs.

2 The Rabbit Pyrogen Test (RPT)

The RPT as an animal test is able to detect endotoxin as well as NEP by monitoring the changes of body temperature in response to intravenous injection of samples (Nakagawa et al., 2002). As the standard test for pyrogens, the RPT has been

used for more than 6 decades and has proven reliable for a great variety of products. Due to substantial similarities in sensitivity and specificity between the rabbit's immune response and the human's, this test can be considered safe. As a true pyrogen test, the RPT is able to detect endotoxin and NEP alike and therefore possesses a broad spectrum of applications (Nakagawa et al., 2002). To perform this test, 3 rabbits per sample are needed. A pre-test has to be performed, and within 2 further days the main test follows. To pass the main test, the sum of temperature rises must not exceed 1.15°C. The whole procedure takes about 3 days.

The drawbacks of the RPT are animal pain and animal consumption, a considerable amount of work, plus the need for facilities and well trained personnel. It should be mentioned that the RPT may remain negative in the case of NEP, which induce pyrogenic adverse reactions in humans (Martis et al., 2005).

3 The Bacterial Endotoxin Test (BET)

The extensively used BET is based on parts of the archaic host-defense of horseshoe crabs (*Limulidae*). As a response to the intrusion of Gram-negative bacteria, the hemolymph-born amoebocytes of these marine arthropods "seal" the site of infection by an enzymatic mediated clotting-reaction. Different variations, utilizing different readouts (gel clot, turbidimetric, chromogenic, etc.) of this highly sensitive and specific test were developed and are widely used for in-process testing and release-testing alike. Modern BET have the ability to detect down to 0.5 pg/ml of endotoxin within 2 hours.

For the production of the respective reagents (except for the recombinant variants), horseshoe crabs are collected by hand on sandy beaches on the east coast of the United States, brought to facilities and partially bled (approximately 30% of the total hemolymph) by puncturing the pericardium. Despite careful treatment, the mortality of this procedure is around 10-15%. Although the fishery for horseshoe crabs has been regulated since 1998 by the Fishery Management Plan, the estimated total mortality for biomedical use exceeded the limit of 57,500 (established 1998) for the last four years in a row (Eyler et al., 2011). The local use as bait or food contributes to the pressure on these populations. In 2007 the first "International Horseshoe Crab Conference" was organized (Tanacredi et al., 2009).

It remains unclear whether the Deepwater Horizon oil spill in 2010 will have a negative impact on the ecosystem, including the population/fertility of *Limulus polyphemus* (Whitehead et al., 2011; Suchanek, 1993). The oil and its metabolites already have entered the marine food chain (Graham et al., 2010). The Delaware Bay (center of the Limulus population on the east coast) is the entrance to the most important harbor for oil-tankers on the east coast, and it has seen several oil-spills before.

Worldwide, all three genera (*Carcinoscorpius*, *Limulus* and *Tachypleus*) of the family *Limulidae* are on the IUCN (International Union for the Conservation of Nature) red list of threatened species, with *Tachypleus* close to extinction, and practically no method for farming established. The biomedical use of horseshoe crabs could be diminished according to the 3Rs by the following approaches:

1. Replace:

Those BET performed as a pyrogen test should be replaced by the MAT (see next chapter), which is able to detect more than endotoxin.

2. Reduce:

The use of recombinant BET-assays (Ding and Ho, 2001; Loverock et al., 2010) could help in protecting the horseshoe crab populations. Additionally the reaction to 1,3β-glucan is missing in this recombinant assay due to the lack of the factor G-pathway.

3. Refine:

Some LAL-techniques using classically derived lysates can be performed with diminished lysate volumes (Jimenez et al., 2010; Gee et al., 2008).

The BET is a fast, sensitive, and highly specific test for the detection and quantification of endotoxins. It is extensively used by industry for in-process testing and batch release of relevant products. Testing is performed on basic material, intermediates, and end products on a large scale. Different test compositions and variations guarantee the suitability for the respective sample needs.

Being specific for endotoxins precludes the BET from being a complete alternative for pyrogen testing (Huang et al., 2009). Because non-endotoxin pyrogen contaminations are not detectable by this test (Kikkert et al., 2007) but may also cause severe inflammatory and pyrogenic complications, the BET cannot be seen as a true pyrogen test (Martis et al., 2005; Huang et al., 2009). The difference in endotoxin detection (BET) and the prediction of pyrogenicity (MAT) is reflected by different test results on modified LPS-specimens (Gutsmann et al., 2010; Brandenburg et al., 2009; Stoddard et al., 2010). The fact that different LPS-species display various BET-activities (not related to their pyrogenicity) makes the use of the BET as a pyrogen test even more questionable (Dehus et al., 2006).

4 The Monocyte Activation Test (MAT)

The MAT mimics the human fever reaction (Dinarello, 2004). Monocytes have a central role in the innate immune response, and the identification and characterization of the different TLR (Poltorak et al., 1998; Lien et al., 1999) explains their reactivity to more pyrogenic stimuli than endotoxin.

The MAT was established as a true alternative for pyrogen testing (Hartung et al., 2001; Hoffmann et al., 2005; Gaines Das et al., 2004; Werner-Feldmayer et al., 1995; Eperon et al., 1997; Moesby et al., 1999; Hansen and Christensen, 1990; Schindler et al., 2009), detecting LPS, NEP (Nakagawa et al., 2002; Hermann et al., 2001), and mixtures (Kikkert et al., 2007). In 2009 it was implemented into the European Pharmacopoeia (Monograph 2.6.30. Monocyte Activation Tests) as an alternative to the RPT. The new European Directive 2010/63/EU on the protection of animals used for scientific purposes enforces the replacement of animal tests when validated alternatives exist.

The different MAT versions employ monocytes by using human fresh blood, cryoblood, PBMC (cryopreservation possible, too), or monocytic cell lines for the detection of pyrogenic substances. The use of cell lines (or a single donor, both representing single genetical backgrounds) bears the risk of choosing a non-responder (Carlin and Viitanen, 2005).

To perform this test in its standard procedure, e.g., fresh blood is diluted with appropriate cell culture medium and incubated overnight at 37°C and 5% CO_2 in 100% humidity with sample and a series dilution of standard-LPS, respectively. The next day, an ELISA is performed for the detection of the cytokines of interest (e.g., IL-1β, IL-6, TNF-α). The MAT delivers information about true pyrogenic events in a species-relevant manner. Depending on the setup of the test, it is possible to perform it with pooled blood leading to mean values of responses compensating for non-responders or creating special cohorts of interest (e.g., age, gender, illnesses, etc.).

In standard procedure the MAT is able to detect at least 50 pg/ml LPS in samples. The MAT provides reliable and reproducible results for many final products (Perdomo-Morales et al., 2011; Spreitzer et al., 2002; Andrade et al., 2003), but it might be too time-consuming in several cases, e.g., for inline-production-testing or cellular therapeutics with an extremely short shelf life (Montag et al., 2007). Confronted with the need for a faster and more sensitive test, a variant of the MAT was developed, measuring intracellular accumulated cytokines in individual cells by flow cytometry. Here, monocytes in fresh human whole blood are chemically impaired regarding the export of cytokines and then incubated with samples. The accumulated cytokines are detected intracellularly by fluorescence-labeled antibodies. This method is capable of detecting 6.25 pg/ml LPS after two hours of incubation with a strong potential for further improvement in both time and sensitivity.

With the advent of bead-supported multiplex-analyte methods, new variations of the MAT capable of detecting multiple cytokines in small volumes in a short time are under development.

5 Conclusion

Regulatory authorities demand safe administration of medicines for parenteral use, such as infusion solutions, vaccines, blood products, cell and gene therapeutics, and tissues, which has to be guaranteed by the manufacturers of such products. Among other tests, the pharmacopoeias require safety testing for pyrogenic-acting substances such as endotoxin and NEP. The well-established RPT has been used for decades for pyrogen testing and has proven to be reliable and safe. The resulting animal pain and animal consumption, the considerably larger amount of work, the need for facilities and well trained personnel, all led to substitution with the BET, which is comparably easier to perform. This test is very sensitive but at the same time highly specific for endotoxins, and thus no full alternative to the Rabbit Pyrogen Test. Furthermore, the BET consumes animals indirectly, since even careful harvesting causes the death of numerous animals. In addition, the new European Directive 2010/63/EU demands the replacement of animal tests when feasible alternatives exist. One of those alternatives could be found in the MAT, a pyrogen test using species specific monocytes for the detection of endotoxin and NEP alike. In our standard procedure the MAT is as sensitive as the RPT. Variations of this test are capable of detecting 6.25 pg/ml LPS with strong potential for improvement.

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