Endotoxin and pyrogen testing

11.1 Introduction

Bacterial endotoxin is the lipopolysaccharide (LPS) component of the cell wall of Gram-negative bacteria. It is pyrogenic, and it is a risk to patients who are administered intravenous and intramuscular preparations. This chapter outlines the pyrogenicity and the structure of endotoxin and moves onto examine *Limulus* amebocyte lysate (LAL) testing and other, alternative, methods of assessing pyrogens and endotoxin.

For these reasons, pharmaceutical products that are injected into the human body are tested for pyrogenic substances. The most common, and arguably most important pyrogen, is bacterial endotoxin. Bacteria endotoxin presents a significant risk to many pharmaceutical products, especially parenteral products. This is because endotoxin is [1]:

- ubiquitous in nature;
- has potent toxicity;
- is stable under extreme conditions;
- is likely to occur in the manufacturing process.

The pathological effects of endotoxin, when injected, are a rapid increase in core body temperature followed by extremely rapid and severe shock, often followed by death before the cause is even diagnosed. However, there needs to be large quantities of endotoxin within the human body the body for this to happen and it the endotoxin needs to be injected into the blood stream.

Of the available endotoxin tests, the LAL method is the most widely used [2]. The pharmacopoeial monographs for the LAL test (USP <85> and Ph. Eur. 2.6.14) are long established and relatively comprehensive and have been applied to the testing of parenteral products and water systems for bacterial endotoxin since the 1980s.

11.2 Pyrogenicity

Bacterial endotoxin can be classed, among other things, as a "pyrogen." Pyrogens are substances that, when injected into the mammalian body, will cause a variety of symptoms, the most recognizable of which is an increase in core body temperature. The association of fevers (pyrexia) has a long history. When injected into mammals (including humans), at a certain threshold, pyrogens will cause a number of adverse physiological responses. These responses include:

- increased body temperature;
- chilly sensation;
- cutaneous vasoconstriction;

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- pupillary dilation;
- decrease in respiration;
- increase in arterial blood pressure;
- muscular pain;
- nausea and malaise.

Of these, a rise in body temperature represents the most common response, and the effect has been known since 1865, where it was reported that distilled water, later reasoned to be contaminated, triggered hyperthermia in dogs [3]. This physiological response is associated with the Greek word "pyrogen" (pyro, meaning "fire"; and gen signifying "beginning"). The term pyrogen was first used in 1876 [4].

In the early days of the pharmacopoeia, drug substances were classed as apyrogenic or pyrogenic, based, from 1942 and until the 1980s, on the "pyrogen test" (whereby a quantity of the drug was injected into three rabbits, and the temperature response of the rabbits was noted). The rabbit pyrogen test was first described by Florence Seibert in 1911, and it became a mainstay test for medicinal products from 1923 [5].

The rabbit test is no longer widely used, and it has largely been replaced, for the testing of parenteral drug products, by the LAL test. The reason for this is because the most common type of pyrogen found in the pharmaceutical industry is bacterial endotoxin, and for which, LAL (with some limitations explored below) is a specific test for. This risk from endotoxin is due, not least, to the large quantities of water used in the manufacture of pharmaceutical products as Chapter 10 describes. The predominance of the LAL assay is not to suggest that rabbit pyrogen testing has been completely eliminated, but that its use is in decline. Moreover, there are alternatives to the LAL test, such as enzyme-linked immunosorbent assay (ELISA) methods and the monocyte activation test (MAT). These are considered at the end of this chapter.

The LAL test is a method, of the bacterial endotoxin test (BET), for detecting the presence, and to go some way to determining the level, of Gram-negative bacterial endotoxins in a given sample or substance. Current editions of the pharmacopoeia carry statements to the effect that where the term apyrogenic or pyrogen-free is used it should be interpreted as meaning that samples of the product will comply with a limit for bacterial endotoxin.

However, it was not until the early twentieth century with the development of a rabbit pyrogen test that an understanding emerged in which bacteria could be classified into pyrogenic and nonpyrogenic types, correlatable to their Gram stain. Gram-negative bacteria were found to be pyrogenic, Gram-positive bacteria were generally not: and killed cultures of Gram-negative bacteria were comparable to live cultures in their ability to induce fevers [6]. Due to the association with living and dead bacteria, by the 1920s, it was apparent that sterility in parenteral pharmaceuticals could be no guarantee of nonpyrogenicity, and that if pyrogenicity was to be avoided it was imperative to avoid bacterial contamination at every stage of manufacture of parenteral pharmaceuticals.

In recognition that the causative agent of pyrogenicity was filterable and heat stable, efforts were applied to identifying its chemical composition. Trichloracetic acid and phenol–water extractions of bacteria were found to be effective in

isolating the pyrogenic element from bacteria. These extracts were chemically identifiable as LPS (or what is commonly described as bacterial endotoxin). With pharmaceuticals, the greatest concern with pyrogens is with large volume parenterals. This is because these are products injected into the vein in relatively large volumes [7].

11.3 Bacterial endotoxin

The structural rigidity of the bacterial cell wall is conferred by a material called peptidoglycan (also known as murein). It is a polymer consisting of sugars and amino acids that forms a mesh-like layer outside the plasma membrane of bacteria forming the cell wall [8].

In Gram-positive bacteria, peptidoglycan is present as a thick layer that is outer-most in the cell wall. In Gram-negative bacteria, the peptidoglcan is only a thin layer, and it is not the outermost layer. Gram-negative bacteria instead have an outer membrane, and they are sometimes described as having a cell envelope rather than a cell wall. The outer membrane functions to maximize the ability of the bacterium to derive nutrients from the external environment. The outer layer also functions as a permeability barrier effective against diffusion of exo-enzymes into the external environment. This is an evolutionary feature that has arisen to allow Gram-negative bacteria (illustrated in Figure 11.1) to survive and increase in numbers in environments such as water in which there are only low concentrations of organic nutrients. Macromolecular organic nutrients are trapped in the cell envelope as the water flows by, and then within the cell envelope they are hydrolyzed to smaller molecules that can be taken into the cell.

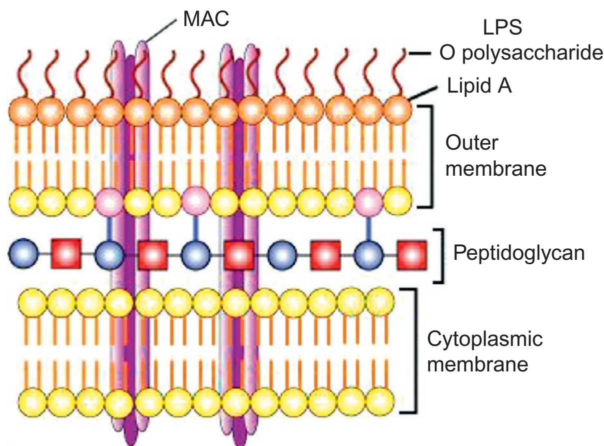

Figure 11.1 Gram's stain light microscope image, showing rod-shaped Gram-negative bacteria.
Image from Creative Commons Library.

The important evolutionary advantages conferred by LPS are:

- it contributes to adhesion of Gram-negative bacteria to surfaces allowing them to form as biofilms in aqueous environments;
- it “attracts” and “entraps” organic macromolecules from aqueous environments;
- it allows entrapped organic macromolecules to be “recognized” by the cell so that specific enzymes can be synthesized to break them down into smaller fragments capable of passing through the peptidoglycan layer into the cell;
- it increases the negative charge of the cell membrane and helps stabilize the overall membrane structure;
- it retains the enzymes synthesized by the cell, so that they are not lost into the external environment [9].

The outer membrane is composed of phospholipid and LPS protein. LPS is an amphiphile molecule—a hydrophilic polysaccharide attachment to a hydrophobic lipid moiety. LPS molecules vary in molecular weight from 1000 to 25,000 Da. LPS is rarely found as a unimolecular entity, it normally aggregates to form vesicles [10].

LPS is pyrogenic, and bacterial endotoxin is a synonym for LPS. LPS is the toxin which is synthesized endogenous to the bacteria cell structure. When Gram-negative bacteria are destroyed, endotoxin is released. In the human body, endotoxin triggers the activation of the body’s defence system, which, in turn, elevates the body temperature and elicits the pyrogenic response [11]. LPS is located outside a thin structural layer of peptidoglycan, as shown in Figure 11.2.

Although intimately associated with the cell envelope of Gram-negative bacteria, LPS is constantly shed by the bacteria into the environment, much like the shedding of the outer layers of human skin. When Gram-negative bacteria die and lyze, all of their LPS is shed into the environment.

Furthermore, when bacterial cells are lysed by the immune system, fragments of membrane containing lipid A are released into the circulation, causing fever, diarrhoea,

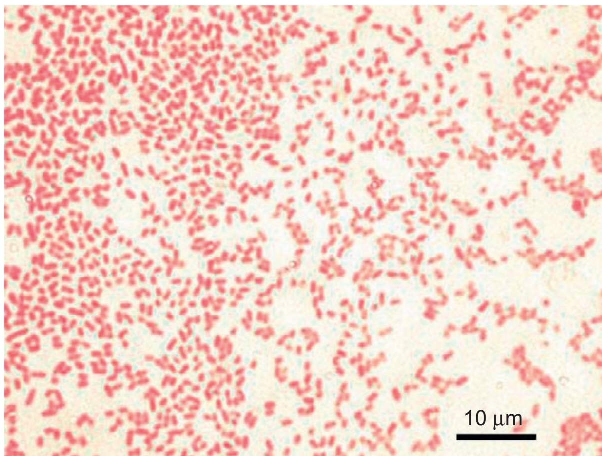

Figure 11.2 Diagram of the outer bacterial cell wall.
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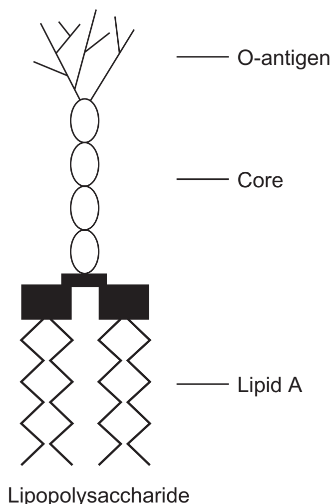

Figure 11.3 Diagram of LPS.
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and possible fatal endotoxic shock (also called septic shock) [12]. There are some other substances that are also pyrogenic. They are unusual and are extremely rarely found associated with pharmaceutical preparations.

LPS has three distinct chemical regions, as illustrated in Figure 11.3:

- an inner core called lipid A;
- an intermediate polysaccharide layer;
- an outer polysaccharide side chain.

Lipid A, embedded in the bacterial outer membrane, is responsible for pyrogenicity (endotoxic).

11.4 Quantifying endotoxin

An important assessment with any application of endotoxin testing is with endotoxin limits. Here, the universal unit of assessment is the “endotoxin unit” (EU). The approximate pyrogenic threshold dose for an adult human is 5 EU/kg/h, as set out in the pharmacopoeias. Any application of a product at a dose in excess of the 5 EU/kg/h will most likely result in a pyrogenic response. It has, therefore, been established that 5 EU/kg/h is the maximum limit for most injectable products. It is also assumed that the mean adult human weight is 70 kg, and so the total amount of endotoxin that can be administered to a human per hour should not exceed 350 EU/h (70 kg × 5 EU = 360 EU/adult).

It is recognized that injectable products that are administered into the cerebrospinal fluid (intrathecal) require a much lower threshold limit of 0.2 EU/kg/h, or 14 EU/adult/h. It is important to consider the route of application when selecting the

endotoxin limit. The term K is commonly used to represent the endotoxin limit. For a standard injectable product $K = 5$ EU/kg/h, where intrathecally administered products K is 0.2 EU/kg/h.

The calculation of the endotoxin limit for a nonintrathecal, injectable drug product is illustrated in the following example:

Product A has a maximum human (whole body) dose of 1 g

$$\text{Dose per kg} = \frac{1\ \text{g}}{70\ \text{kg}} = 0.0143\,\text{g/kg} = 14.3\,\text{mg/kg}$$

$$\text{Endotoxin limit} = \frac{K}{M} = \frac{5\ \text{EU/kg}}{14.3\ \text{mg/kg}} = 0.35\ \text{EU/mg}$$

The concentration of the product (potency in final product) is used to convert the endotoxin limit into EU/mL. If the concentration of product is 100 mg/mL, the following calculation will provide the EU/mL of the finished product. It is useful to use these units, as this is usually the units used within the LAL assay (as described below)

$$\text{Endotoxin limit} = 0.35\ \text{EU/mg} \times 100\ \text{mg/mL} = 35\ \text{EU/mL}$$

It is important to note that this limit is based upon a specific product concentration. If the concentration of the product changes, then the endotoxin limit will change. This provides the maximum limit for this product at this concentration. It is common for manufacturers to select an endotoxin limit that is lower than this maximum limit.

The endotoxin limit of each sample requires determination prior to conducting endotoxin testing.

11.5 The *limulus* amebocyte lysate test

The most widespread endotoxin test is the LAL test. The principle of the LAL test is a reaction between LPS and a substance (clottable protein) contained within amoebocyte cells derived from the blood of the horseshoe crab, as illustrated in Figure 11.4 (of which *Limulus polyphemus* is the most commonly used species, although other species, such as *Carcinoscorpius* and *Tachypleus* demonstrate the same effect). The reaction of the horseshoe crab to endotoxin (the formation of a clot) has been known since the 1950s [13]. LAL is an aqueous extract obtained after lysis of blood cells (amoebocytes).

When endotoxin comes into contact with LAL, it initiates a series of enzymatic reactions that result in the activation of a pathway to the production of at least three serine protease zymogens (factor C, factor B, and pro-clotting enzyme). This pathway alters amoebocyte coagulogen (an invertebrate fibrinogen-like clottable protein) to form coagulin gel.

Serine proteases are enzymes that cleave peptide bonds in proteins, in which serine serves as the nucleophilic amino acid at the active site. They are found in humans as wells as in horseshoe crabs (and indeed in all mammals). In humans, they are responsible for co-ordinating various physiological functions, including digestion, immune

Figure 11.4 Image of the *Limulus* "horseshoe crab."
Image from Creative Commons Library.

response, blood coagulation, and reproduction. It is the blood coagulation reaction that is similar in both humans and horseshoe crabs [29]. It is on this basis that LAL has become the sensitive test reagent made from the soluble protein extract (lysate) of horseshoe crab blood cells (amoebocytes). All commercial lysates can detect picograms (10^{-12} g) of endotoxin.

The reference test in the pharmacopoeias is the gel clot (or gelation) and is conducted on the end-point principle. The description of the test and the necessary validation and accompanying controls is so detailed in both US Pharmacopeia (USP) and European Pharmacopoeia (Ph. Eur.) (harmonized since 1999).

The clotting mechanism of the blood of the crab is designed to prevent the spread of bacterial contamination throughout the horseshoe crab's biochemical system. When the endotoxin of Gram-negative bacteria contacts with the horseshoe crab's amoebocytes, a series of enzymatic reactions begin. The pathway alters amebocyte coagulogen into a fibrinogen-like clottable protein, which forms a coagulin gel. The defence mechanism is also effective against fungi, hence a similar reaction occurs in response to a fungal infection, which triggers the clotting cascade. In the reactions, β-glucans trigger the protease enzyme factor G, whereas endotoxin triggers the factor C enzyme, although the end result—coagulin—is the same [14].

A considerable amount more glucan (1000 times) is required to trigger the clotting cascade than the equivalent amount of endotoxin. The glucan required to trigger factor G can be of varying molecular weights (such weights range from 3 to 100 kDa) [15]. The clotting reaction is illustrated in Figure 11.5.

Glucan as an interfering substance is explored below.

The LAL reagent used for the gel-clot is supplied with an identified sensitivity or label claim (λ), for example, 0.03 EU/mL. This means that when mixed with an equal volume of the material under test, a gel or clot will form if the material contains 0.03 EU/mL or greater. For the kinetic methods, the lysate does not come with a label claim. The test sensitivity is determined by the lowest point of the standard curve used with each assay [16].

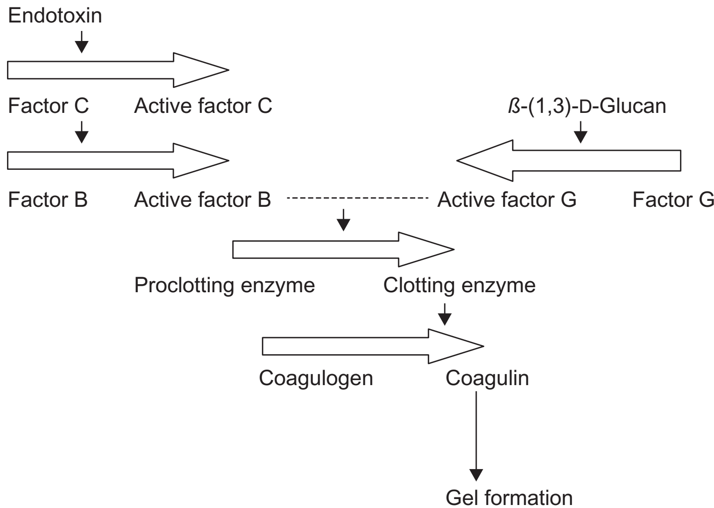

Figure 11.5 LAL-clotting cascade.
Adapted by the author.

When it is necessary to quantify endotoxin concentration in a material, it is usual to test a series of doubling dilutions against the reagent in temperature-controlled conditions. The greatest dilution that gives a positive result (formation of a gel or clot) is the end point, and the concentration of endotoxin in the original material can be calculated by multiplying the dilution factor at the end point by the sensitivity of the LAL reagent.

LAL tests require validation for each technician and each product before being applied routinely and, even then, valid assays require careful internal standardization. LAL tests require the use of standard endotoxin, termed a positive product control (PPC), which is a known amount of endotoxin mixed with a test material to confirm the absence of interference.

In the early days of the LAL test, endotoxin standards were variable. The potency varied with the method of purification, the bacteria of its origin and how it was formulated. Initially LAL test results were reported as units of weight. The problem with this was that results between tests and laboratories were not comparable. This was because different endotoxins, of the same weight and from different species of bacteria, can have different potencies. This led to the need for an endotoxin standard. This standard was based on *Escherichia coli* 0113: H10 K negative. This introduced the EU, which is a measure of the activity (potency) of endotoxin against the LAL test, irrespective of mass [17].

The units of measurement for the LAL test are EU. These are a measure of the activity of the endotoxin. Endotoxins differ in their biological activity or potency; the pyrogenicity or LAL reactivity of one endotoxin preparation may be very different from that of another of the same weight. Conversely, two endotoxin molecules may be different sizes and different weights but may have the same reactivity in an LAL test.

The potency of an endotoxin determined with one LAL reagent lot may differ from that determined with another lot. Expressing endotoxin concentrations in EUs avoids the issues of different potencies of different endotoxins and allows us to compare results of different LAL tests performed in different laboratories. Consequently, it is not the usual practice to convert results of an LAL test in EU/mL to units of weight of endotoxin per milliliter.

The origin of the test standard is from when the USP and US Food and Drug Administration (FDA) commissioned Rudbach to resolve the issue by preparing a reference endotoxin that was stable, could be chemically characterized, could be lyophilized without loss of activity and that was free from biologically active proteins [18]. This is termed reference standard endotoxin (RSE). Since RSE is expensive and potentially exhaustible, control standard endotoxin (CSE), also manufactured from *E. coli* 0113, is normally used for routine work but not necessarily for fundamental research. The potency of a given batch of CSE is determined relative to the current Ph. Eur., USP, or FDA lot of RSE and is specific to particular batches of LAL reagent. Thus, a purified form of LPS is used to manufacture a reference standard for the LAL assay. The endotoxin control is lyophilized with lactose and polyethylene glycol.

11.6 *Limulus* amebocyte lysate test methods

The primary LAL test methods are the gel-clot and two photometric methods: chromogenic and turbidimetric. The former is an end-point method while the latter two are kinetic methods. The kinetic methods are more sensitive than the gel-clot method (as low as 0.001 EU/mL). This is because changes in turbidity and color are discernible by light-scattering devices at lower concentrations of endotoxin than those at which gels form [19].

11.6.1 Gel-clot

Gel-clot is a method that utilizes the endotoxin-mediated clotting cascade, that naturally occurs within horseshoe crabs, to produce a gelatinous clot after incubation at 37 ± 1 °C with endotoxin. The gel-clot mimics the clotting of *Limulus* blood *in vivo*. Here a clotting protein is cleaved by an activated clotting enzyme, at which point the insoluble cleavage products coalesce and form a gel.

The gel-clot method is performed using depyrogenated glass tubes. The assay comprises of an equal volume of test solution and lysate (typically 0.1 mL of test solution and 0.1 mL of lysate) that are gently mixed together and, due to its sensitivity to vibration, incubated in an unstirred water bath or dry block heater at 37 °C normally for 1 h. After which, the end point is read by carefully inverting the tube through 180°. The tube is deemed positive for endotoxin if, after inversion through 180°, a solid clot (gelation) has formed and remains intact. The tube is deemed to be negative for endotoxin if no clot forms, or if the clot breaks through inversion of the tube.

11.6.2 Turbidimetric

The turbidimetric assay is, together with the chromogenic assay, a photometric method. With this test, during the process of clot formation, the lysate-sample reaction mixture becomes increasingly more turbid.

During the LAL reaction, the concentration of insoluble coagulin increases as its precursor, coagulogen, is cleaved. This process causes a corresponding increase in optical density (OD) of the reaction mixture. It is this increase in OD that is detected in a spectrophotometer (typically a microplate reader or a tube reader). The rate of increase of the OD is directly proportional to the endotoxin concentration present in the well. This is the basis for the turbidimetric LAL assay. The assay measures the increase in turbidity as a function of endotoxin concentration measured against a standard curve and, from this, estimates the endotoxin concentration in a sample.

11.6.3 Chromogenic

The chromogenic assay utilizes the initial portion of the endotoxin cascade. Here a synthetic chromogenic peptide is substituted for the clotting protein. The peptide generates a yellow color.

The chromogenic assay utilizes a synthetic chromogenic substrate that contains a specific sequence of amino acids that are designed to mimic the cleavage site in coagulogen. Activated clotting enzymes cleave this site and cause the liberation of the chromophore (para-nitro aniline, pNA), which has a yellow color. The liberated pNA absorbs light at 405 nm. In the chromogenic assay, the measurement of this absorption of light at this 405 nm that is measured. The degree and the rate of increased absorption is proportional to the endotoxin within the sample.

When using kinetic methods, the most important aspect is the standard curve for the endotoxin concentrations. This is because the standard curve selected, and how it performs, determines the test sensitivity. Therefore, the high point and low point in a standard curve determines the lower and upper levels of endotoxin that can be detected.

Although the LAL test today is more robust, it remains open to a degree of variation [20].

11.7 *Limulus* amebocyte lysate test applications

As indicated above, the widest application of the LAL test method is with the testing of samples of water (primarily water-for-injection; WFI) and for assessing final products, especially those administered by injection. A related area is with depyrogenation studies.

WFI and sample testing are important because endotoxicity is not necessarily lost with the loss of viability of micro-organisms. LPS is not destroyed to any significant extent by sterilization treatments such as steam sterilization, gamma radiation, ethylene oxide, and hydrogen peroxide. LPS also passes through 0.22-μm bacteria-retentive filters. It is claimed that endotoxin may be removed from liquids by up to $4 \log_{10}$ reductions using 0.025-μm ultrafilters (which function as a molecular sieving process).

With depyrogenation, there are two commonly used ways of eliminating endotoxin from materials, removing them and inactivating them: by rinsing or by dry heat depyrogenation. The normal method of removal is by rinsing the material with WFI. This is normally applied to rubber stoppers for vials. It is also what is done to vessels and major pieces of equipment used for sterile parenteral manufacture. A question arises as to whether this can be validated and assured. In answer, the sampling statistics is likely to be poor, and the test method is inaccurate and probably there is not much there in the first place.

Inactivation is achieved by dry heat. If there are materials and glass vials that are required for sterile parenteral manufacture that can be depyrogenated by dry heat, then they should be depyrogenated by dry heat in an oven or a tunnel.

The regulatory standard for validation of an endotoxin inactivation (depyrogenation) process is that it should be capable of reducing an endotoxin challenge through $3\log_{10}$ reduction. To ensure that this limit works there is also a requirement to clean materials prior to dry heat depyrogenation with WFI—otherwise at least in theory, an item could be contaminated with 10,000 EU prior to entering a validated endotoxin inactivation process and still emerging with 10 EU intact and ready to contaminate the product.

Dry heat depyrogenation is a complex process that is still poorly understood with contradictory research data. The phenomenon that complicates the picture is that inactivation may approximate to the second-order chemical kinetics with a high initial rate of inactivation, then tail off to nothing. What this means in practice is that, at any particular depyrogenating temperature, it will be subjected to some degree of inactivation in some period of time or other but beyond that point no further inactivation will occur by holding the material at that temperature [21].

11.8 *Limulus* amebocyte lysate test interference

The LAL assay may be interfered with by the sample being tested; these are "LAL reactive materials (LRMs)" or "LAL activators." The phenomenon of substances reacting with LAL, such as thrombin and ribonuclease, has been known for many years (such as Elin and Wolff [28]). By "reacting," these LRMs can cause a positive LAL reaction for something other than endotoxin. This interference effect may be caused by a number of different factors such as pH, protein concentration, and presence of chemicals (such as NaOH from rinsing cycles). Interference may affect the lysate or the endotoxin. Inhibition or enhancement is normally detected through the use of spiked controls. Inhibition is arguably the greatest concern because it can result in a failure to detect the true level of endotoxin in a sample.

When conducting LAL tests, the dilution of samples is important. This is in order to minimize the effects of any component of the material being tested, which may be inhibitory to the LAL reaction. However, it is imperative that the material being tested is not diluted too much because this would ensure negative results, possibly false negatives. The way this is avoided is by having a maximum valid dilution (MVD), which is derived from the ratio of the endotoxin limit to the test sensitivity.

One potentially major source of interference is with β-glucans. These are polysaccharides of D-glucose monomers linked by β-glycosidic bonds. Glucans are important because they can react with certain lysates and cause interference with the LAL test [22]. Furthermore, glucans can, in certain circumstances, cause physiological effects in humans (β-glucans are known as "biological response modifiers" because of their ability to activate the immune system). According to Cooper et al., glucans are the most common LRMs likely to occur within pharmaceutical manufacturing [23]. Like endotoxin, glucans are large polysaccharides (homopolymers of glucose) of a high molecular weight. Therefore, the LAL test reacts with two types of polysaccharides: glucan and LPS (endotoxin). There are several sources of (1,3)-β-glucan. These include fermentation and cell culture media, fungal organisms, plant-derived materials, process unit operations' equipment, and packaging [24].

The extent to which glucans are a problem is a matter dependent upon the level of glucan and the patient population. Glucans are "biologically active" (immunostimulatory), although without the acutely toxic responses observed for similar levels of endotoxin. In some cases, glucans can cause a pro-inflammatory cytokine reaction. The main risk cited is for patients undergoing long-term haemodialysis. They cause a range of physiological reactions (such as the use of swabs in surgery) and are becoming increasingly regarded by regulators as a problem.

In response, most of the major LAL reagent manufacturers now produce lysate that is either glucan specific or endotoxin specific (in addition to the standard LAL reagent that could potentially react to either glucan or endotoxin). It should also be noted that the commercially available LAL reagents all differ in their reactivity to glucans (up to 200 fold in some studies, such as the experiments conducted by Roslansky) [25]. An alternative to modifications of the lysate is the use of a buffer, which can be added to the lysate to make it endotoxin specific. The buffer blocks factor G. Whether a glucan blocker should be used depends upon whether the testing laboratory wishes to know if glucan is present in the material under test or not.

LAL tests are only valid in situations where standard endotoxin can be shown to be detectable with the same efficiency in a test sample as in a control consisting of water (LAL reagent water, LRW) known to be endotoxin free (LRW).

11.9 Alternative test methods

There are alternatives to the LAL test. These are ELISA-based assays including the MAT.

MAT is an *in vitro* test used to detect or quantify substances that activate human monocytes or monocytic cells to release endogenous mediators. MAT works by predicting the human response to pyrogens on the basis of human fever, rather than animal models. MAT is based on human whole blood, and the test is theoretically capable of measuring all pyrogens relevant to the human patient (the MAT is based on the human fever reaction). In brief, the test uses heparinized human whole blood drawn from a healthy donor. The blood is diluted and introduced to the test sample. In response to any pyrogens present, monocytes contained within the blood sample will produce

pro-inflammatory cytokines interleukin (IL)-β1 or IL-6, as measured by ELISA. These cytokines are pyrogens; in essence, all endogenous pyrogens are cytokines, molecules that are a part of the immune system. They are produced by activated immune cells and cause the increase in the thermoregulatory set point in the hypothalamus [26].

MAT is suitable for the testing of medical devices, blood products, toxic or immunomodulatory drugs, dialysis liquids, lipidic parenterals, and air quality [27]. In 2010, the MAT was accepted within Europe as an alternative endotoxin test method (Ph. Eur. 2.10).

An alternative to MAT is the endpoint fluorescent microplate assay called EndoLISA®. This revolutionary rapid method is based on an LPS-specific phage recombinant protein, which specifically binds to the entire substance group of LPS (endotoxin). The phage protein is precoated to the wells in the EndoLISA® microtitre plate, and as the sample is added to the well, the endotoxin (LPS) in the sample is bound to the phage protein. Any sample matrix with potentially interfering components is then completely removed by a washing step. Therefore, the subsequent detection by recombinant factor C (rFC) and a fluorescence substrate is left unaffected by inhibitors, facilitating a reliable quantification of endotoxin in the sample.

With the traditional LAL test, the industry appears to be moving towards the use of recombinant lysate, and the reliance upon the horseshoe crab may one day decrease. With recombinant, factor C in the clotting cascade has been replaced by a recombinant factor. Detection is by a fluorogenic substrate.

11.10 Conclusion

Pyrogens are a concern for pharmaceutical drug products and for many of the ingredients used to formulate them. This is especially so for products that come into contact with human blood. Here, by far the most concerning pyrogen is bacterial endotoxin. In relation to this, the chapter has considered the risks of endotoxin to pharmaceutical processing and some of the control measures in place to reduce the risk of endotoxin contamination.

Furthermore, the chapter has provided an introduction to endotoxin as well as to the primary method for detecting endotoxin: the LAL test. Here the chapter has examined the three main types of LAL test: gel-clot, chromogenic, and turbidimetric, as well as considering alternative test methods. Such tests are an essential feature of most pharmaceutical microbiology laboratories.

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