Biomedical Applications of Limulus Amebocyte Lysate

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Abstract This year celebrates the 30th anniversary of the licensing of *Limulus* amebocyte lysate (LAL) by the US Food and Drug Administration (FDA) as a test for the presence of endotoxin in biologicals, pharmaceutical drugs, and medical devices. LAL is currently recognized by several major pharmacopoeias and is used worldwide. That a suitable alternative for the detection of endotoxin has not supplanted LAL is indicative of its superior reliability. Since its discovery, LAL has proven its usefulness not only to detect harmful levels of endotoxin (as pyrogens) in pharmaceutical products, but has become an indispensable tool in controlling endotoxin in processes and equipment used to produce pharmaceuticals. Indeed, the exquisite sensitivity of LAL compared to other assays for endotoxin/pyrogen has proven extremely useful in monitoring high-purity water used as a prime ingredient or processing agent for all biologicals, drugs, and devices. LAL has also become the assay of choice for researchers studying both the clinical and the environmental effects of endotoxin. To highlight its usefulness, various specific applications of LAL including modifications of the assay to allow testing of complex substances will be described. Finally, although horseshoe crab mortality associated with LAL production is low, the LAL industry has taken steps to find a synthetic substitute and to produce reagents and methods that use much less LAL than traditional assays. That the horseshoe crab has uniquely contributed a test that profoundly affects the safety of pharmaceuticals should be celebrated and rewarded by continuing to protect this valuable resource.

1 Introduction

This year, 2007, marks the 30th anniversary of the licensing of *Limulus* amebocyte lysate (LAL) by the US Food and Drug Administration (FDA) as an alternative to the United States Pharmacopoeia's (USP) Pyrogen Test (PT)

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for endotoxin in human and veterinary drugs (US Department of Health and Human Services 1987). Since this approval, LAL has become accepted worldwide. Besides inclusion in the USP as the Bacterial Endotoxins Test (BET) (US Pharmacopoeia 1995), LAL test methods are also included in the European Pharmacopoeia (European Pharmacopoeia Commission 1987) and the Japanese Pharmacopoeia (The Pharmacopoeia of Japan 1992). Although the PT remains as an official test, in most cases the BET has completely replaced the PT, i.e., is the required test for certain drugs or is an acceptable alternative.

While LAL has been criticized as not being a true pyrogen test, i.e., there are other pyrogens (fever-causing agents), endotoxin has been shown to be the most common and most potent pyrogen and the one that is most likely (if not the only) pyrogen to contaminate injectable drugs and devices (Twohy et al. 1984). Thus, LAL and the horseshoe crab that is its source of manufacture have become invaluable to insure the safety of the world's pharmaceutical drug supply.

Currently LAL is commercially manufactured from amebocytes carefully harvested from the blood of the North American horseshoe crab, *Limulus polyphemus*, and the Asian horseshoe crab, *Tachypleus tridentatus* (Levin et al. 2003). LAL can also be obtained from the two other species, *Tachypleus gigas* and *Carcinoscorpius rotundicauda*. A synthetic version of LAL based on a recombinant form of the major endotoxin-reactive component of *C. rotundicauda* has been developed (Ding et al. 1999) and is commercially available (Lonza 2007).

This paper focuses mainly on the non-pharmaceutical applications of LAL. With the exception of a few specific examples to illustrate pharmaceutical use, general pharmaceutical applications and related regulations, LAL manufacture and commercialization, and clinical applications have been thoroughly reviewed elsewhere (Levin et al. 2003, Novitsky 1982a, Hochstein and Novitsky 2003, Novitsky 1994, 1996, 1999, Jorgensen 1986)

2 LAL Biochemistry

The biochemical basis of LAL is thought to play a key role in the horseshoe crab's (HSC) ability to ward off infection (Armstrong 2003). As the HSC does not have an immune system, as do the vertebrates, microbial defense is left to a humoral system. The mechanisms of action of the biochemical components that make up LAL originate in the HSC's amebocytes. These components not only recognize bacteria (those of the gram-negative type) but also fungi (those containing β-D-1,3-glucan) (Morita et al. 1981). One can only speculate that early HSCs, known to us through the fossil record, had a similar system and that the protection from infection it provided contributed to the HSC's survival through the millennia. Figure 1 shows *Mesolimulus walchi* (most likely a juvenile) and its tracks at the end of a journey some 150 million years ago when met its end, not to infection, but to stranding and burial in a tropical lagoon (Barthel 1990).

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Fig. 1 Fossil of *Mesolimulus walchi* with tracks from Solnhofen Plattenkalk, Eichstätt, Bavaria, approximately 150 mya (prosomal width = 5.8 cm) *Photo by author*

To adequately understand the advantages and limitations of LAL, it is important to have at least a fundamental understanding of the biochemistry underlying the LAL test and to a certain extent, the chemistry of the substance detected by LAL – endotoxin. It should be noted that commercial LAL differs according to brand/manufacturer. Basically, all LAL is made from blood collected by "bleeding" adult HSCs of both sexes and separating the amebocytes from the plasma or hemolymph. The amebocytes are then broken or lysed to release the biochemical components that form the active ingredients of LAL. Differences in manufacturing occur in all steps. For example, some manufacturers use glass and stainless steel implements for blood collection, while others use plastic. Amebocytes can be lysed by suspending in distilled water, by alternately freezing and thawing, or by mechanical rupture. Various chemicals can also used to prevent the amebocytes from coagulating or from premature rupture during blood collection. Variation in manufacturing results in qualitative and quantitative differences between brands of LAL. This is especially evident when certain samples, usually of complex chemical composition, are tested with multiple brands. The consensus biochemistry of the LAL reaction is shown in Fig. 2 as are the various modifications to allow the assay to be read. The assay can be read in a variety of manners and once again the method of manufacture will differ for the type of assay chosen. The originally described LAL assay utilized the physiological "clotting" reaction. This assay is commonly referred to as the gel-clot test (Novitsky 1988). It basically is an end point test based on the highest dilution of sample that causes the LAL reagent to form a solid clot in a small test tube in a certain period of time at a fixed incubation temperature. A variation of

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this assay utilizes the turbidity or cloudiness that is formed leading up to clot formation as an end point. This type of assay is generally more sensitive and/or more rapid to perform than the gel-clot assay. In order to accurately read this test, however, a spectrophotometer is required. Finally, the component of the LAL that causes turbidity and subsequently a gel-clot to form, coagulogen, can be replaced by a synthetic peptide that contains the amino acid sequence that is cleaved by the clotting enzyme. This peptide also contains a chromogen (or fluorogen), i.e., a chemical that changes color (or fluorescence) when an adjacent chemical is modified. The LAL reagent that utilizes this synthetic peptide is referred to as the chromogenic assay. The chromogen most commonly used is para-nitroaniline which is colorless until the peptide is acted on and then turns yellow. A spectrophotometer using a wavelength appropriate to the chromogen's spectra is required. In one variation of this assay, the yellow dye is converted to its diazo derivative that is a deep purple. This later variation is useful for samples that have an inherent yellow color (Novitsky 1999).

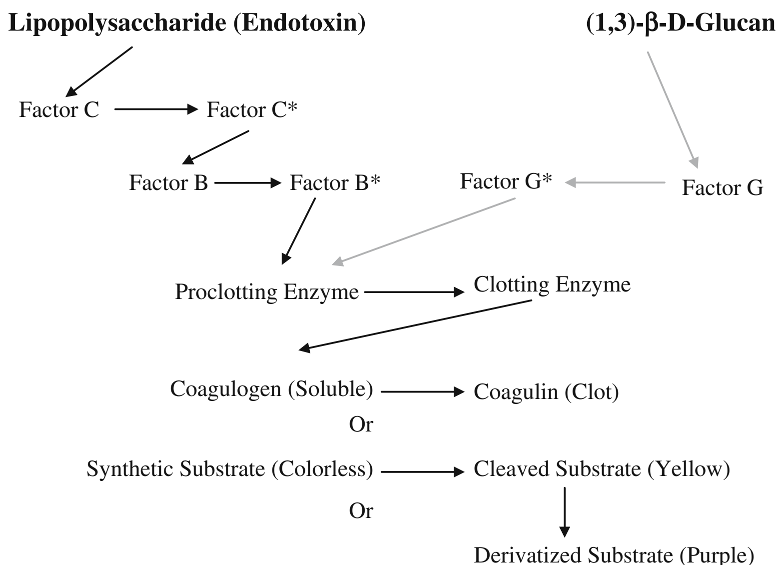

Fig. 2 Consensus biochemistry of the LAL reaction. Gray arrows denote alternate glucan-mediated pathway

3 Source and Chemistry of Endotoxin

Endotoxin is a component of the outer cell envelope of gram-negative bacteria. A more descriptive term for endotoxin is lipopolysaccharide (LPS). A generalized structure of LPS is shown in Fig. 3. Endotoxin or LPS occurs wherever bacterial contamination has occurred. Since LPS is refractory to degradation by

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most physical and chemical methods that would kill or remove bacteria, it can and does remain in solutions that are sterile. As water is an excellent growth environment for bacteria, especially gram-negative bacteria, water is typically the major source of endotoxin (Dawson et al. 1988). Since water serves as the major ingredient or processing component for injectable drugs, vaccines, and medical devices, it is not surprising that insuring water is as free of endotoxin as possible is a major concern of pharmaceutical manufacturers and testing of purified water with LAL constitutes its single largest use (Novitsky 1984, 1987).

O-antigen---Outer Core---Inner Core---Lipid A

O-antigen =	**Polysaccharide composed of repeating sugar sequences**
Outer Core =	**Oligosaccharide**
Inner Core =	**Heptoses + 2–keto-3-deoxy-octonic acid (KDO)**
Lipid A =	**Phosphate + fatty acids**

Fig. 3 Schematic of the general structure of lipopolysaccharide (endotoxin)

The toxic component of LPS and the component that causes LAL to react is the lipid A portion of the molecule (Fig. 3). Lipid A is highly conserved between different species of LPS, i.e., LPS from different species of bacteria, and because of this, equal weights of LPS from widely diverse species of bacteria react similarly. While this makes LAL a perfect general test for endotoxin, LAL cannot be used to differentiate between species.

LPS manifests its toxicity by causing a febrile (pyrogenic) response in animals when a sufficient amount is injected into the blood stream or cerebrospinal fluid. Hence LPS/endotoxin is also referred to as a pyrogen. At a high enough dose, LPS can be fatal. Fortunately, LPS can be tolerated in massive doses if ingested by healthy individuals. In fact, the normal bacterial flora of mammalian digestive systems is mainly gram-negative and is present in extremely great quantity. There is ample evidence, however, that inhaled endotoxin can cause pulmonary problems in susceptible individuals (Milton et al. 1987).

4 Pharmaceutical Applications

Soon after the discovery of LAL, the utility of this test to pharmaceuticals became apparent. LAL was demonstrated to be both a rapid and an accurate test for short-lived pharmaceuticals, i.e., radio drugs (Cooper et al. 1970). One

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of the authors, J. Cooper, then joined the Bureau of Biologics at the FDA and together with HD Hochstein helped start the FDA's program to investigate the feasibility of using LAL as an alternative to the PT for all pharmaceuticals and medical devices (Levin et al. 2003). The LAL test also quickly earned the distinction as a referee for disputed PT results. In one notable instance, the LAL test was used to prove that high levels of endotoxin in the swine flu vaccine introduced in 1976 was responsible for the adverse effects reported to the Centers for Disease Control (CDC) attributed to the vaccine (HD Hochstein, personal communication).

In the years following developments at the FDA and while clinical LAL studies continued to dominate most scientific interest LAL, a wide variety of LAL applications began to appear in the literature. The most notable of these are summarized in the following sections.

4.1 Purified Water

Since water is common to all drugs and devices, either as an integral component or as a processing agent (wash water), and since water is easily contaminated by gram-negative bacteria and hence endotoxin, it is not surprising that water is the substance that accounts for the largest number of LAL tests in the pharmaceutical industry. It is reasoned that if water contains endotoxin below a certain level, it will be unlikely to increase endotoxin in the final product that would render that product pyrogenic. The level as accepted by the USP and FDA is 0.25 Endotoxin Units (EU) ml^{-1} (a EU is equivalent to about 1 ng of purified endotoxin obtained from a strain of *Escherichia coli*). For comparison, ordinary bottled drinking water can easily contain several EU ml^{-1}. A special subset of water testing relates to renal dialysis. Although artificial kidneys (actual reverse osmosis filters) are tested with LAL as medical devices, they become contaminated during use and especially with reuse. In addition, the water used to prime the dialysis machine can add endotoxin to the unit if not closely monitored with LAL. Adverse patient reactions due to endotoxin have been widely documented and although monitoring with endotoxin is required, the dialysis industry has been slow to adopt this critical test (Novitsky 1982b).

4.2 Intravenous Drugs

Since endotoxin toxicity is manifested most commonly when injected into the blood stream, it is recognized that intravenous (IV) solutions should contain levels of endotoxin well below the pyrogenic dose. Although the water used to formulate IV drugs is required to already have sub-pyrogenic levels of endotoxin as determined by LAL testing, the final formulation and sometimes even the individual components are tested with LAL to insure the final IV solution

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(and its container) is within the allowed endotoxin limit. The endotoxin limit for IV drugs is slightly higher than water and based on the anticipated dose that will be used for the particular drug.

4.3 Biologicals

Biologicals include pharmaceuticals made from substances obtained from humans and animals, e.g., clotting factors and insulin. Biologicals also include vaccines which can contain bacterial, or animal components, e.g., from chicken eggs. It is recognized that gram-negative bacteria can easily contaminate biologicals and batches can contain large amounts of endotoxin. Fortunately, biologicals are usually given in relatively low doses and often administered by intramuscular injection. Even so, in 1976 following adverse reactions to a new batch of swine flu vaccine, in one of the earliest uses of the LAL test, it was determined that the batch of vaccine had a particularly high level of endotoxin that was responsible for the adverse effects. Antibiotics, while not strictly characterized as biologicals, also contain large and variable amounts of endotoxin since they have a microbial source (Case et al. 1983)

4.4 Medical Devices

Medical devices such as syringes, catheters, and needles are usually quite clean as a result of their method of manufacture. However, implanted devices, e.g., porcine heart valves, or orthopedic devices of rather complicated manufacture, can contain levels of endotoxin that cause localized inflammation and ultimate rejection of the device. In these cases, it is particularly important that the devices be checked by LAL. Generally the devices are rinsed with LAL-negative water and then the rinse tested. In some cases, the device itself can be immersed in the LAL reagent and the reaction of endotoxin that tightly adheres to the device can be measured. In this later case, the chromogenic version of the LAL test is used so that the reaction solution can be transferred to a reading device to score the test.

4.5 Recombinant Drugs

Relatively recently, i.e., sometime after the acceptance of LAL as an alternative to the PT, recombinant drugs were introduced. These are drugs produced through genetic engineering and are produced by bacteria, fungi, or mammalian cell culture. One can appreciate that recombinant drugs produced by the gram-negative bacterium *E. coli* would have a high potential of being contaminated with endotoxin from its producer organism. LAL is especially critical in

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drugs produced in this manner as it is used as a tool to follow the purification of the drug. Although the fungi (mainly yeast) and cultured mammalian cells used to produce certain drugs do not contain endotoxin per se, the media used for their growth can contain significant amounts of endotoxin that can subsequently contaminate the final product.

4.6 Stored Blood

Between 1987 and 1991, nine cases of red cell-associated *Yersinia enterocolitica* sepsis were reported to the CDC (Arduino et al. 1989). In some of these cases, it was determined through LAL analysis that most of the severe adverse effects (including seven deaths) noticed with patients who received transfusions of the contaminated red cells were not related to the infection per se but rather to the large amount of endotoxin associated with the contaminating bacteria. In one case, over 20,000 ng ml^{-1} of endotoxin was detected. Although it was hoped that LAL could be used routinely to screen stored red cells immediately prior to transfusion, sampling (i.e. removing a sample from the blood bag immediately prior to transfusion) without compromising the sterility of the unit(s), and sending a sample to the laboratory (versus bedside testing), made the LAL test impractical for this application. In addition, the incidence of infection was extremely low (43 million units collected between April 1987 and October of 1990 administered to 13 million patients resulted in nine reported cases and seven deaths). The problem was eventually solved by shortening the storage time for collected red blood cells. The shorter storage time did not allow potential contaminating bacteria to grow to levels (and produce sufficient endotoxin) to cause the problem.

5 Environmental Applications

5.1 Seawater and Marine Sediments

The oceans are teeming with bacteria. It was originally thought that in the marine environment, bacteria could only grow near the surface where there was abundant food, reasonable temperature, and low pressure. With the advent of the LAL test, it was shown that bacterial-associated endotoxin was present throughout the water column down to the deepest parts of the ocean, i.e., >4,000 m (Watson et al. 1977). Furthermore, endotoxin as determined by LAL was shown to highly correlate with bacterial number and even more so with bacterial biomass (carbon content). Thus, LAL provided a rapid, indirect measure of bacteria. The test was extended to study bacteria in sediments as well as in shore waters and was included in a benchmark survey of the Georges Bank to assess the potential impact of offshore drilling (Hobbie et al. 1987). Due to

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relatively high concentrations of nutrients in coastal waters where horseshoe crabs live and breed, large numbers, e.g., $>10^8$ bacteria ml^{-1} can occur. The HSC therefore lives in a veritable bacterial soup (Watson and Novitsky 1991). The same biochemistry that enabled scientists to measure the number of bacteria and amount of endotoxin in the water, also acts to protect the HSC in this environment.

5.2 Fresh Water

Gram-negative bacteria also occur in abundance in fresh water. Several studies have examined the utility of the LAL test to predict the portability of water assuming the total number of bacteria, indirectly determined from endotoxin content, would correlate with the presence of harmful bacteria or fecal contamination (Jorgensen et al. 1979). In one study, a high degree of correlation between the LAL test and the fecal coliform counts was demonstrated (Evans et al. 1978). This study, however, was a serendipitous occurrence and the LAL test has never gained acceptance as a test for potable water.

5.3 Air Quality

Several studies have shown that inhaled endotoxin can cause respiratory problems (Hasday et al. 1999, Milton et al. 1987, Sloyer et al. 2002). Environments most likely to manifest this type of problem are those where large amounts of aerosols containing gram-negative bacteria and endotoxin occur, e.g., metal working shops and sewage treatment plants (Sloyer et al. 2002). Another type of respiratory problem can be encountered in dusty environments. In cotton milling plants it has been shown that endotoxin associated with cotton dust is responsible for much of the respiratory problems encountered (Milton et al. 1987). The cotton dust/endotoxin association is interesting from an ecological standpoint. Prior to the cotton bole rupturing during the ripening process, the bole is essentially sterile. Once open, however, the raw cotton fibers are quickly colonized by gram-negative bacteria. Additional colonization occurs during processing and depending on the amount of moisture, nutrients, and temperature, large numbers of bacteria can be present along with their accompanying endotoxin. Even if the bacteria are destroyed during processing, the endotoxin usually remains tightly associated with the cotton fiber and can be released along with fine particles of cotton fiber during processing. Tobacco can also contain large amounts of adsorbed endotoxin, and one study employing LAL has shown that endotoxin released during cigarette smoking can cause respiratory problems (Hasday et al. 1999).

5.4 Endotoxin in Space

Recently a specially modified version of the LAL test was tested in space (Charles River Laboratories 2007). While providing on-the-spot results for

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the astronauts for their air and water systems, this was also good news for those of us back on earth in that the test design uses much less LAL reagent than other commercially available assays (Charles River Laboratories 2007).

6 Food Quality

It is well known that many types of food can be and are contaminated by gram-negative bacteria. As with water, studies have shown that presence of large amounts of endotoxin can be indicative bacterial contamination, either current or historical. This is especially true of meat. Thus, the presence of endotoxin was shown to correlate well with meat quality (Jay 1997). Likewise, the endotoxin content of fish has been shown to correlate with quality (Sullivan et al. 1983). While gram-negative bacteria are not the major source of milk spoilage, they are good indicators proper sanitization of the processing plant and of keeping quality of milk, especially for milk intended for ultrapasteurization (Mikolajcik and Brucker 1983). A modification of the LAL assay that uses a blue dye to more easily observe a gel-clot endpoint in microtiter plates was developed especially for analyzing milk (Mottar 1987).

7 Medical Research

7.1 Biological Effect(s) of Endotoxin

While one of the most obvious manifestations of endotoxin is a febrile response, reactions at the humoral and cellular level are manifold and complex. LAL has therefore become an indispensable tool for researchers studying the effects of endotoxin in mammalian models and to investigate the role of endotoxin in existing diseases (Romero et al. 1988, Warren et al. 1985, Novitsky et al. 1985).

7.2 Search for an Antiendotoxin Drug Therapy

In the study of gram-negative infection and sepsis, endotoxin plays a key role in the pathophysiological response of the host (Riveau et al. 1987). It has been postulated that if the adverse effect(s) of endotoxin could be abrogated, sepsis survival could be improved. Since the toxic portion of endotoxin (LPS), i.e., lipid A, is the same part of the LPS molecule that causes the LAL reaction, anti-endotoxin compounds could be screened with LAL prior to testing in animals (Novitsky et al. 1985, Warren et al. 1987a, b, c). Several studies have shown this to be an effective strategy and in fact an endotoxin-neutralizing compound with therapeutic potential was isolated from *Limulus* hemolymph and was studied extensively (Alpert et al. 1992, Garcia et al. 1994, Novitsky et al. 1996, Stack

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et al. 1997, Warren et al. 1992). A recombinant form of this protein was also constructed and found to be active (Andrä et al. 2004, Kuppermann et al. 1994, Nelson et al. 1995, Saladino et al. 1994, 1996a, b, Siber et al. 1993, Wainwright et al. 1990, Weiner et al. 1996).

8 LAL Reactivity to Fungal Glucan

8.1 Results of the Academic Medical Center Consortium (AMCC) Sepsis Study

While LAL is used in Japan as a clinical test for the presence of endotoxin in blood as one tool to diagnose sepsis, this is not allowed in the United States unless approved by the FDA. Part of the approval requires a clinical study to demonstrate utility. In 1988, LAL was included as part of the AMCC Sepsis study to see if there would be sufficient utility to license LAL as an aid in diagnosing sepsis. Prior to this trial numerous independent studies indicated the likelihood that LAL was a predictor of gram-negative sepsis. Unfortunately, under the conditions of this trial, no clinical utility could be demonstrated (Bates et al. 1998, Ketchum et al. 1997). An interesting result of the trial, however, was that a strong correlation was found when a patient was shown to have both a bacterial and a fungal infection (Ketchum et al. 1997). This finding, along with a growing literature indicating that the LAL reagent under certain conditions could also react with a fungal cell wall component, β-D-glucan, lead to more research into the feasibility of making an LAL test that was sensitive specifically to glucan.

8.2 Development of a Test for Fungal Infection

In 1988, Japanese investigators described an alternate pathway that resulted in the LAL reaction (Morita et al. 1981). This is shown in Fig. 2 (gray arrows). Numerous studies in Japan using the two different LAL formulations: one sensitive only to endotoxin and the other sensitive to both endotoxin and glucan proved the value of LAL in detecting fungal infections and eventually resulted in a fungal diagnostic (Miyazaki et al. 1992, Obayashi et al. 1995). Subsequent research resulted in an LAL reagent sensitive to only glucan, simplifying the assay. Although the glucan assay was already widely used in Japan, further development in the United States was necessary before the approval of Fungitell™ by the FDA as an aid in the diagnosis of invasive fungal infection (Associates of Cape Cod, Inc. 2007). While not a universal test for fungal infection, the glucan assay has been reported to be especially useful for the early detection of *Aspergillus* and *Candida* infections (Associates of Cape Cod, Inc. 2007).

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9 Conclusions

From the numerous examples presented, it is clear that LAL has a proven track record as an endotoxin test. As such, it is now considered the gold standard. Although the importance of LAL should justify its continued use for biomedical applications, especially as the major pyrogen test for pharmaceutical products, all of us concerned with the preservation of the HSC need to be vigilant and encourage the LAL industry to be a leader in conservation efforts (Novitsky 2001). To this end, the LAL industry has already made great strides in commercializing a recombinant-based LAL reagent and has introduced tests that use much less LAL than the original assay. While it is exciting to see a new health-related assay from the HSC – the fungal diagnostic based on the glucan pathway – this assay will undoubtedly put new pressures on the harvesting of HSCs. With increased awareness of its biomedical importance and attention to conservation to help ensure its survival, the HSC will continue to disclose life-saving secrets and help our understanding of healthy as well as disease-causing biochemical mechanisms.

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