LAL Update®
ASSOCIATES OF CAPE COD, INCORPORATED

June 2005 • Volume 22, No.2

Letter From the Editor

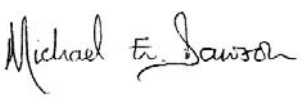
Dear LAL User,

In this issue Dr. Malcolm Finkelman provides an introduction to (1,3)-ß-D-glucans. LAL Update articles in 2001 and 2002 addressed quantitation of (1,3)-ß-D-glucans and their presence in cotton containing medical products. This article reviews their chemistry and occurrence in nature and discusses their significance as contaminants of healthcare products. It reflects the increased understanding of the biology of glucans since the last overview to appear in the LAL Update in 1990. Unchanged is that the majority of positive LAL test results are attributable to endotoxin. One of the frustrations of glucans, and the native reactivity of LAL to them, is the seed of uncertainty that they plant. Is a positive result due to endotoxin contamination? Or is it caused by glucans? We offer all the tools needed to resolve that uncertainty.

The article briefly mentions the Fungitell™ assay, our clinical diagnostic kit. This 510K cleared device measures serum glucan level, which has been shown to be an effective indicator of invasive fungal infection. The test is rapid. Results are available within two hours of sample receipt, as compared with the days required for results of cultures. The kit is available in Associates of Cape Cod's LAL product catalog or samples can be sent for analysis to Beacon Diagnostics™ Laboratory, our CLIA certified reference laboratory (see www.acciusa.com or www.beacondiagnostics.com).

We are also pleased to introduce our reorganized biochemicals division, Northstar BioProducts™. Northstar BioProducts offers a wide range of products. We will continue to maintain our core product line based upon the highest quality reagents from Seikagaku Corporation for carbohydrate analysis and biochemistry. To this we are adding a number of new products. Erik Paus, the Director of Northstar BioProducts, provides details of this exciting development.

Sincerely,



Michael E. Dawson, Ph.D.

(1,3)-ß-D-Glucan:
Biological Properties and Implications for LAL Testing

By: ***Malcolm A. Finkelman, Ph.D.***
Chief Technical Officer
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Introduction

The *Limulus* Amebocyte Lysate (LAL) cascade is triggered by two substances, bacterial endotoxin and (1,3)-β-D-glucan. It is important that users of LAL be aware that positive tests may result from the presence of (1,3)-β-D-glucan, as well as bacterial endotoxin. Measurement of (1,3)-β-D-glucan contamination has been previously addressed in the LAL Update[1,2]. This article provides an introduction to (1,3)-β-D-glucan, including its chemistry, biological effects, detection, and sources of (1,3)-β-D-glucan contamination in pharmaceuticals and medical devices. It reflects advances in our understanding of the topic since it was first introduced in 1990[3].

Both bacterial endotoxins (lipopolysaccharides), and (1,3)-β-D-glucans, are considered "Pathogen-Associated Molecular Patterns", or PAMPS, substances which elicit inflammatory responses in mammalian systems[4]. Their presence in drugs and medical devices may lead to adverse events. In recognition of this concern, compendial limits on endotoxin have been established world-wide. In contrast, the understanding of the biological responses to (1,3)-β-D-glucan is at an earlier stage. Although clearly an elicitor of innate immune responses, and having been associated with pre-clinical and clinical adverse events, its presence in drugs and devices is as yet unregulated.

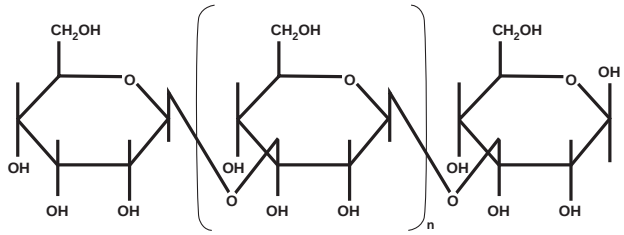
PN001480 Rev#000

LAL Technical Report

Structure and Occurrence

The basic structure consists of a homopolymeric backbone of glucose molecules, linked through (1,3)-β-D-glucosidic linkages (Figure 1). This type of linkage produces a repeating structure that organisms, such as fungi, utilize to produce mechanically stiff structures, including cell walls. In addition to the repeating structure of the backbone, these glucans are typically branched, at multiple points, to other glucans, glycans, and proteins. These molecular matrices form the complex structures of the fungal cell envelope. (1,3)-β-D-glucans are rather insoluble and difficult to characterize. Analysis may involve multi-step preparatory procedures to break down complex materials[5,6].

Figure 1 - (1,3)-β-D-glucan structure



Given their ubiquitous presence in nature, there are many opportunities for pharmaceuticals, and medical devices, to become contaminated with glucans. In contrast with endotoxins, which are unique to Gram negative bacteria, (1,3)-β-D-glucans are produced in nature by a range of organisms from different kingdoms.

Fungi

(1,3)-β-D-glucan is present in the cell walls of most fungi. It comprises up to 50% of the dry matter of the cell wall and it provides mechanical integrity and structural rigidity. A complex of synthetic and degradative enzymes are involved in fungal morphogenesis. During growth and development, (1,3)-β-D-glucans are sloughed into the environment of the hyphae. This is analogous to shedding of endotoxin as Gram negative bacteria grow.

Plants

(1,3)-β-D-glucan is a very minor component of plants. Typically, the (1,3)-β-D-glucan is present as a structural component of specialized tissues such as the apical meristem (growing tip), vasculature (plasmodesmata), and in wound repair tissue. In plants, (1,3)-β-D-glucans are referred to as callose[7]. It is difficult to discern the origin of (1,3)-β-D-glucan in processed plant matter, as it is often highly contaminated with fungal hyphae. Plant material, such as wood chips, which are subject to outside storage under natural conditions, are rapidly colonized by saprophytic fungi, whose hyphae contribute to the (1,3)-β-D-glucan burden of the plant material upon which they are growing. Plant (1,3)-β-D-glucan often consists of the linear (1,3)-β-backbone with (1,4)-β-branch side chains.

Algae

Some genera of brown algae (e.g. *Laminaria*) are excellent sources of (1,3)-β-D-glucan, which is used commercially in a variety of industrial applications including food, coatings, fillers and gums[8]. They are very hydrophilic and also modify final product viscosity properties.

Bacteria

A few bacteria, including certain *Cellulomonas* and *Alcaligenes* species, produce (1,3)-β-D-glucan as extracellular products. Among the latter, *Alcaligenes faecalis* is a source of a linear (1,3)-β-D-glucan that is also used in food products[9]. Although there are no reports of glucan contamination from these sources in pharmaceutical products, one should be aware of the possibility.

Sources of (1,3)-β-D-glucan Contamination

Sources of (1,3)-β-D-glucan contamination in pharmaceutical products include fungal fermentation, cellulosic depth filters and plant hydrolysates. Typically, (1,3)-β-D-glucan contamination is identified during the investigation of an LAL Out Of Specification (OOS) event. Investigation of a LAL positive sample should always include the possibility of (1,3)-β-D-glucan contamination.

Fungi

If fungal fermentation is part of a process, cell wall (1,3)-β-D-glucan may be present and be carried through purification. The unit operations performed during the purification of the product may eliminate most, if not all, of the contamination, however this must be verified. Other processes, such as ultrafiltration, may concentrate (1,3)-β-D-glucan. Careful analysis of the process operations must be conducted to identify steps where (1,3)-β-D-glucan contamination may occur and be affected, positively or negatively.

Depth Filters

Many cell culture, fermentation fluids and blood products are clarified using disposable depth filters. These often contain a mixture of diatomaceous earth and cellulose as the filtration media. The plant tissue that is the source of the cellulose

typically contains (1,3)-β-D-glucan. During pharmaceutical processing, it may be leached from this source. Depth filter manufacturers are aware of this and some have taken steps to mitigate its release. The leaching of pro-inflammatory (1,3)-β-D-glucan, from depth filters has been described[10], including the impact upon therapeutics such as blood fractionation products. Similarly, the use of cotton or gauze-based materials can also generate (1,3)-β-D-glucan-rich leachates[2].

Plant-derived materials

Plant-based raw materials or components, used in either primary production or downstream processing, may be contaminated with (1,3)-β-D-glucan and, thus, contaminate the product. Any plant-derived material such as a sugar or protein hydrolysate should be considered suspect, unless demonstrated to be free of (1,3)-β-D-glucan. Recently, an adverse event was described in which (1,3)-β-D-glucan was introduced to a protein purification process in a sugar component[11]. In this report, volunteers, in a Phase I drug study, developed fever and had C-reactive protein and white cell elevations. Investigation identified the presence of contaminating levels of (1,3)-β-D-glucan, introduced with the sugar, and concentrated during a diafiltration process step. The material in question was free of contaminating endotoxin and also passed the rabbit test for pyrogens. Paper and cardboard fines or dust can also be sources of both glucan and endotoxin, and can result in contaminated products or LAL tests.

Cellulose dialysis membranes

Cellulose dialysis membranes have been associated with the release of (1,3)-β-D-glucan to levels that can be observed in the blood of patients. In the early 1980s, the phenomenon was ascribed to LAL-Reactive Material, or LAL-RM, and was associated with adverse events in dialysis patients[12]. The LAL-RM was once thought to be cellulose, based upon enzymatic analysis that cannot be considered definitive. Pure cellulose, free of (1,3)-β-D-glucan, does not activate LAL[13]. Digestion of activating polysaccharides with (1,3)-β-D-glucanase, eliminates any activating principle.

Significance

Regulatory

(1,3)-β-D-glucan contamination is typically identified by LAL OOS results. Determination that (1,3)-β-D-glucan contamination is the cause opens new issues. The regulatory approach to (1,3)-β-D-glucan contamination is complex. Unlike bacterial endotoxin, (1,3)-β-D-glucan is not specifically regulated. This does not mean that manufacturers may ignore it. The only FDA statement on glucans was issued in 1992, in a memorandum from the Chairman of the LAL Task Force entitled "Statement Concerning Glucans and LAL-Reactive Materials in Pharmaceuticals and Medical Devices"[14]. The onus is placed on the manufacturer to investigate and assign root cause and, unless shown otherwise, positive LAL results must be considered to be endotoxin-related. This document concludes that glucans and LAL-RM do not appear to be a problem in pharmaceuticals. Given that the measurement of (1,3)-β-D-glucan was very difficult and rarely performed at that time, that conclusion was based on little data. Since 1992, there has been enormous growth in the scientific literature describing the biological effects of (1,3)-β-D-glucan. A whole field of study has evolved that is clarifying the cellular and physiological response to (1,3)-β-D-glucan. The developments in (1,3)-β-D-glucan research have included (1,3)-β-D-glucan drugs, specific cellular receptors, signal transduction cascades, gene regulation, and an adjuvant role for (1,3)-β-D-glucan in cancer therapy.

US and European regulations require manufacturers to characterize the levels and sources of contaminants in drugs. Elaborating upon contaminant characterization in the 1991 Office of Regulatory Affairs Biotechnology Inspection Guide[15] (under PROCESSING AND FILLING, A. Processing), the FDA noted: "Most BDP [biologically derived products] cannot be terminally sterilized and must be manufactured by aseptic processing. The presence of process related contaminants in a product or device is chiefly a safety issue. The sources of contaminants are primarily the cell substrate (DNA, host cell proteins, and other cellular constituents, viruses), the media (proteins, sera, and additives) and the purification process (process related chemicals, and product related impurities)." Similarly, the International Committee on Harmonization has addressed the need to characterize contaminants in the ICH Guideline Q6B.

LAL OOS Occurrences and Investigation

LAL OOS events are a major area of concern for Quality Control personnel. These trigger costly and time consuming investigations and can delay or prevent product release. Following an OOS result, a simple initial screen for the presence of (1,3)-β-D-glucan is the performance of the LAL test using a (1,3)-β-D-glucan-blocking buffer such as the Glucashield™ Buffer offered by Associates of Cape Cod, Inc. (ACC). If the reactivity obtained with Glucashield buffer reconstituted LAL is reduced or eliminated, (1,3)-β-D-glucan contamination is the likely source of part or all of the positive LAL result (see below for more information on discriminating between glucan and endotoxin).

When an LAL OOS is attributable to glucan contamination, all manufacturing and testing processes should be evaluated for their likelihood of contributing (1,3)-β-D-glucan contamination. In the manufacturing environment, the investigation should include those factors discussed in the "Sources of (1,3)-β-D-glucan contamination" section, above. In the testing environment, one should evaluate environmental sources, laboratory filters, other materials being tested in the lab (such as powders), use of cotton-plugged pipets, etc.

Differentiating Endotoxin from (1,3)-β-D-glucan

In the event that (1,3)-β-D-glucan contamination is a possible root cause of an OOS result, ACC offers a number of approaches to the analysis of the source of the OOS signal.

Glucashield™ Buffer: ACC's Glucashield buffer contains a glucan blocking agent that renders any of our LAL reagents, endotoxin-specific. If the OOS sample contains no endotoxin, use of the Glucashield buffer will result in the absence of signal, providing evidence of a) the absence of endotoxin in the sample, and b) the LAL signal being caused by (1,3)-β-D-glucan. (Catalog # GB051).

PyroTurb™ ES Reagent*: PyroTurb ES lysate is an endotoxin-specific turbidimetric reagent that is only activated by endotoxin. Lack of signal when testing with this reagent indicates the lack of endotoxin in the sample (but not lack of (1,3)-β-D-glucan). (Catalog # PT032).

Pyrosate® Kit*: Pyrosate lysate is an endotoxin specific gel-clot reagent packaged in single test vials with matched positive product control vials. This reagent does not react with (1,3)-β-D-glucan. (Catalog # PSD10, PSD101, PSD25 and PSD251).

Glucatell® Kit: This chromogenic reagent is specific for (1,3)-β-D-glucan. It does not respond to endotoxin. It has a limit of detection as low as 1.5 pg/mL. (Catalog # GT001, GT002, GT003 and GT004).

Diagnostic Application

In addition to use of Glucatell in the identification and management of (1,3)-β-D-glucan contamination, the reagent has an important role in the identification and management of invasive fungal infection (IFI). Clinical investigations have demonstrated that the detection of the (1,3)-β-D-glucan at levels above 80 pg/mL, with ACC's Fungitell™ kit (a registered diagnostic device), is a sensitive and specific indicator of the presence of fungal pathogens[16]. Also, serum glucan level monitored throughout the course of infection and treatment has been found to be useful in monitoring treatment efficacy.

Conclusion

(1,3)-β-D-glucan is currently an unregulated contaminant, which is found in some pharmaceutical and medical device products. Depending upon the LAL reagent and the concentration of the (1,3)-β-D-glucan, LAL OOS results can occur. (1,3)-β-D-glucan is a biologically active material but its significance as a contaminant is, at this point, unclear. Recent findings have demonstrated that both adverse events and changes to the properties of immunological therapeutics can be triggered. The availability of methods for the quantitation of (1,3)-β-D-glucan, such as the Glucatell kit, facilitates the identification of (1,3)-β-D-glucan contaminants, assists in their control, and permits the assessment of their relative safety. (1,3)-β-D-glucan analysis can also help resolve the question of whether or not a positive LAL test result is caused by endotoxin.

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**May be used for all endotoxin testing applications not requiring CBER-licensed LAL.*

Northstar BioProducts™

Specialists in Glycobiology and Glycoanalysis

Erik J. Paus, Director

Northstar BioProducts™ reagent business is founded on the high quality reagents from Seikagaku Corporation for carbohydrate analysis and biochemistry. We offer a diverse and complete product portfolio in glycoscience, supplying high purity reagents and bulk products for carbohydrate purification, biochemical and structural analysis, labeling, and detection of glycoconjugates. We integrate all the reagents and supplies required for comprehensive profiling of glycoconjugates. Our products enable complete structural analysis of glycans, proteoglycans, oligosaccharides, glycoproteins, and any other carbohydrate containing biological sample or solution.

Carbohydrates are remarkable for their structural diversity. Differences in a proteins' glycosylation profile can profoundly affect its biological functionality. The understanding and characterization of varying glycoforms has become fundamental in elucidating and understanding a specific glycoproteins' function. Consequently, glycoproteins are evaluated for quality control and drug discovery purposes. Regulatory requirements for accurate and comprehensive glycoprofiling data have now been established by both the US Food and Drug Administration (FDA) and the European Medicines Agency (EMEA). Northstar BioProducts reagent business offers the products to meet these stringent requirements in an accurate and cost effective manner.

The typical steps involved in the characterization of a glyco-conjugate are:

1. Capture and Purification of the glycoprotein from solution
2. Enzymatic treatment of protein to free glycans
3. Separation of glycans from reagents, enzymes, detergents, and protein core
4. Chemical labeling of glycans
5. Purification of labeled glycans
6. Analysis and quantitation

To facilitate the analysis and profiling of complex carbohydrate structures, Northstar BioProducts is proud to distribute the LudgerMap Glycoprofiling System which enables rapid labeling and profiling of glycosylated proteins as described above. The LudgerMap Glycoprofiling System includes:

- LudgerTag™ Kits - Fluorescent labeling
- LudgerClean™ Kits - Post labeling clean up
- LudgerPure™ Glycan standards - purified glycans for HPLC and MS analytical use

Northstar BioProducts also distributes defined molecular weight and labeled hyaluronic acid (HA) standards and markers from Hyalose, LLC. These synthetic HA polymers are useful in many applications, for example:

Select-HA™ is HA of uniform and narrow size distribution produced by a new method developed by Hyalose LLC. Data sheets are provided for the absolute average molecular weight determination of each Select-HA as characterized by SEC-MALLS (size exclusion chromatography - multiple angle laser light scattering). All other currently available commercial HA preparations have much greater size heterogeneity and run as a smear, but any given Select-HA runs as a distinct band. (Note: the product number signifies the molecular mass in kiloDaltons [kDa]).

Products available from Hyalose LLC include:

- Select-HA Ladders: Hyalose LLC has assembled different mixtures of linear Select-HA polymers of very defined size ranges for use as size standards on gels or other separation methods. The sizes (masses) of the Select-HA samples have been determined by SEC-MALLS. Two ranges for higher (HiLadder) or lower molecular weight (LoLadder) HA are now available.
- Tagged Select-HA: A fluorescent or biotinylated narrow-size distribution HA polymer with a single fluorescein or biotin group.
- Mega-HA Ladder: A mixture of streptavidin/biotin Select-HA complexes of well defined size ranges for use as size standards on gels or other separation methods. The ladder contains the original size material plus larger complexes that are 2 times, or 3 times, or 4 times the size of the starting Select-HA polymer.
- Nano-HA: A variety of different sizes of HA oligosaccharides are available from HA 4 to HA 24 (Note: the product number signifies the number of monosaccharides, thus HA12 comprises twelve monosaccharide units).

In addition, the product line includes an expanded selection of enzymes to include deglycosylation kits, PNGase F, and many recombinant sialidases that complement and complete our product offering to make Northstar BioProducts the premiere supplier of all the necessary reagents for total analytical carbohydrate biochemistry.

LAL News and Events

JULY

American Association for Clinical Chemistry (AACC) Annual Meeting
July26-28, 2005
Orange County Convention Center
Orlando, FL
Booth: 1262

SEPTEMBER

LAL Training Course
September 13-15, 2005
Minneapolis Airport Marriott Hotel
Bloomington, MN

Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC) Annual Meeting
September 21-24, 2005
Ernst N. Morial Convention Center
New Orleans, LA
Booth: 631

OCTOBER

LAL Training Course
October 4-6, 2005
Falmouth Technology Park
East Falmouth, MA

Infectious Disease Scociety of America (IDSA) Annual Meeting
October 6-9, 2005
Moscone Convention Center
San Francisco, CA
Booth: 1411

LAL Training Course
October 25-27, 2005
Courtyard Philadelphia Downtown Hotel
Philadelphia, PA

For more information or to register for a workshop, visit our website at www.acciusa.com or call (888) 395–2221

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LAL Update®
June 2005 • Volume 22, No.2