LAL Update®
ASSOCIATES OF CAPE COD, INCORPORATED

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Letter From the Editor

Dear LAL User:

In this issue of the LAL Update, we look at the significance of endotoxin to cellular and tissue based therapies. This important topic shares some concepts with the article on biotechnology in LAL Update vol.16, no. 1. We also include a brief but important article on CSE Potency, Test Variability and LAL Reagent Label Confirmation.

Last year the United States Pharmacopeia (USP) published in Pharmacopeial Forum (PF) (volume 33, number 3) a revision of the Bacterial Endotoxins Test (BET) chapter. This is a stage 4 draft. When the revision was published, we expected it to be a major topic for an LAL Update article. However, despite the appearance of a complete rewrite, there are no fundamental changes to the chapter. The principles that were established in the BET revision in the second supplement to USP 24, when the harmonized chapter was published, remain unchanged. However, I urge you to look at the draft in PF so that you are aware of the subtle differences from the BET in USP 30.

All of us at Associates of Cape Cod thank you for your business. We wish you the very best for 2008.

Sincerely,

Michael Dawson

Michael E. Dawson, Ph.D., RAC

Endotoxin Testing of Cellular and Tissue Based Therapies

By: James L. Powers and Michael E. Dawson

Introduction

Spurred by scientific advances and by new investment hoping to capitalize on these advances, researchers and clinicians are developing innovative cellular therapies. Control of endotoxin contamination in such therapeutic preparations is important for two reasons. Firstly, preparations to be administered to patients must not be significantly contaminated because of the effect of endotoxin on the recipient. Secondly, endotoxins are potent elicitors of a wide range of cellular responses that may alter the therapeutic value of the cells.

Explanation of cell therapies

Cellular and tissue based therapies (referred to in this article as cell therapies) are a special class of parenteral product in which live cells are administered to the patient. The cells may be derived from the patient (autologous), which prevents immune response to cells. Alternatively, the cells are collected from another individual (an allogeneic source), in which case the donor tissue may be matched to the recipient to reduce the immune response to the therapy, but such a therapy typically requires the use of immunosuppressant drugs. A third approach is the use of xenogeneic cells (cells obtained from another species), which may also necessitate immunosuppression.

The cells are transferred to culture media for subsequent storage, growth, manipulation, transformation, or harvesting (and any combination of thereof). In the simplest form of treatment, the cells are returned to the patient without any manipulation, of their own cells that have been previously harvested. This is typically performed after therapy that reduces lymphocyte count. For other therapies, the cells or tissue administered may be manipulated or stimulated prior to administration.

Cell therapies may be administered by infusion; injection; or surgical implantation, either in aggregated form or with supporting or encapsulating material, and may be considered a medical device. The cells in the donor (who is often the patient) are generally sterile and free of detectable endotoxin. The concern is that they become contaminated during collection, storage, manipulation (if applicable) and return to the patient.

Significance of endotoxin

Endotoxin is a potent biological response modifier. If present as a contaminant in a cell therapy, could affect the patient and/or the cells being administered. The first of these concerns, potential effect upon the patient, is common with other injectable products and medical

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devices that contact the blood, lymphatic system or cerebrospinal fluid. These products penetrate or bypass the protective barriers of the skin and intestinal wall and have the potential to introduce endotoxin directly into the blood. Endotoxin taken in through the mouth generally does not cause deleterious health effects because it does not cross the intestine wall and enter the blood. Endotoxin is produced by bacteria in the mouth and in the intestine; it is in food and tap water, sometimes in concentrations in excess of 1000 EU/mL. In contrast, endotoxin is dangerous if it enters the blood, causing fever (a pyrogenic response) and a wide range of other possible effects, including septic shock and death. Consequently, treatments that introduce the therapeutic agent directly into the patient's blood, such injections, infusions or as medical devices have to be controlled for endotoxin contamination.

The second consideration is the effects of endotoxin upon the cells to be administered. This is unique to cell therapies. The influence of endotoxin upon cells was discussed in LAL Update vol.16, no. 1 in the context of cell culture for biotechnology. The same principles apply to cell therapy. Effects of concern include induction of mitosis, stimulation of production of cytokines (which may be pyrogenic themselves), morphological changes, and cytotoxicity. Consequently, in the presence of endotoxin the properties of the cells being administered may be quite different from those expected. Such contamination may cause the failure of the therapy, as is suggested by Vargas *et al.*[1] in the case of pancreatic islet transplantation.

There is not a great deal in regulations and guidance documents that specifically addresses endotoxin contamination of cell therapies. A few points are raised in the US Food and Drug Administration (FDA) document: Guidance for Human Somatic Cell Therapy and Gene Therapy.[2] The endotoxin testing requirements are consistent with those for other parenteral products. In section III: Characterization and Release Testing of Cellular Gene Therapy Products" it is stated that the final product to be administered and production process and materials used should be subject to QC testing. Under the heading "Purity" (subsection B), the document recommends that a suitable LAL test method for detection of endotoxin be validated. That is, it must be demonstrated that the cell preparation being tested does not interfere with the ability of the LAL test to detect endotoxin. The guidance references the FDA "Guideline on Validation of the Limulus Amebocyte Lysate Test…" of 1987[3]. Section VIII: "Preclinical Evaluation…" does not mention endotoxin (LAL) testing or pyrogen (rabbit) testing. A pyrogen test might be considered is at this stage, but this is not stated in the Guidance. As many cell therapies produce pyrogenic cytokines, a positive pyrogen test may not indicate endotoxin contamination.

The European Agency for the Evaluation of Medicinal Products (EMEA) issued "Points to Consider on the Manufacture and Quality Control of Human Somatic Cell Therapy Medicinal Products"[4] in 2001. Under the heading "Source and characterization of other materials and reagents" it is stated that the "low endotoxin level of ancillary products should be assured." There are no specific statements about release testing of cell therapy products with LAL prior to administration.

Practical considerations

Undiluted cell suspensions may interfere with photometric methods (turbidimetric and chromogenic). For this reason, du Moulin and coworkers[5] used the gel-clot method for testing peripheral blood mononuclear cells before being returned the patient in autolymphocyte therapy. These authors were interested in the potential of the endotoxin serving as an indicator of bacterial contamination. In a subsequent paper[6], this group described validation of a photometric (chromogenic) assay.

Different types of cell preparation are likely to have different interference characteristics. In addition, cell preparations of the same type derived from different donors may also have different patterns of inhibition (or enhancement). To address these possible differences, method development should result in a rugged test so that validation of the test can be conducted on three different batches of the therapeutic preparation (preferably using cells from three different sources) by conducting the test for interfering factors. Despite best efforts to develop a rugged test, it may still be necessary to have build flexibility into the test procedures to overcome interference from troublesome samples. If overcoming the interference requires a change in the treatment of the sample, the revised treatment should be subject to a test for interfering factors when the parameters of the original validation are exceeded.

Another practical consideration is the possibility of false positive (i.e. non-endotoxin) LAL test results caused by (1→3)-β-D-glucan contamination. Anderson *et al.*[7] report on positive LAL gel-clot test in autologous dendritic cells to be used in clinical trial of HIV immunization. In this case, the source of the contamination was shown to be a cellulose nitrate filter. Like endotoxins, glucans are biological response modifiers but there is no limit specified for glucans in any pharmacopeia or regulatory document. There is therefore the potential for glucans to influence the properties of a cellular therapy and or to affect a recipient of that therapy. FDA has written that a positive (failing) LAL test result must be considered as a failure until it has been proven otherwise. Glucan specific LAL reagent (Glucatell, ACC catalog number GT002) and a glucan specific LAL test (use Glucashield glucan blocking buffer, ACC catalog number GB0051) can help discriminate between endotoxin and glucan contamination. It is recommended that cellulosic filters be tested for glucans to avoid positive in LAL tests and possible effects of glucans on cells.

Testing for endotoxin in cell therapies is challenging because the materials under test may vary with the blood chemistry of the donor. There is often acute time pressure on the technician performing the LAL to provide a result before a cell therapy can be administered to a patient. It is therefore important that the test method be rugged and not overly susceptible to interferences, which will result in invalid tests and the need for repeat tests. The ability to dilute the sample is critical to achieving accurate endotoxin results. Photometric methods are significantly more sensitive than the gel-clot method, resulting in a substantially greater maximum valid dilution (MVD). Thus, these methods enable greater dilution of the sample to overcome interferences and reduce turbidity caused by cells in suspension. Instrumentation and reagents for photometric techniques utilizing a 96-well microplate reader or the Pyros Kinetix tube-reader (ACC catalog number PKX02) are available from Associates of Cape Cod, Inc. The Pyros Kinetix is the most sensitive endotoxin detection system available. Consequently, it gives the greatest MVD and scope for dilution for overcoming interference.

Conclusion

The principles that apply to endotoxin testing of parenteral products or medical devices apply equally to cell therapies. There is a similar concern about the effect of endotoxin upon recipients and this drives the compliance requirement. However, living cell therapies carry the additional risk that the endotoxin may affect the properties of the cells and their therapeutic value. For both of these reasons, endotoxin testing of cell

therapies is very important. Because of the potential for endotoxin to alter the properties of the cells, it is strongly recommended that monitoring for endotoxin contamination be initiated from the earliest stages of research into a potential therapy. In addition, an early awareness of the importance of endotoxin control will assure that upon transfer to a cGMP manufacturing process, appropriate considerations about control of endotoxin are also transferred.

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CSE Potency, Test Variability and LAL Reagent Label Confirmation

By: Michael E. Dawson

Control standard endotoxin (CSE) is a secondary standard and its use depends upon determination of its potency relative to the United States reference standard endotoxin (RSE) (or the United States Pharmacopeia endotoxin reference standard, which is the same material as the RSE).

CSE distributed by Associates of Cape Cod, Inc. (ACC) is labeled in units of weight. For the most commonly used of ACC's CSEs (product code E0005) that is 0.5 µg/vial, or 500 ng/vial. When reconstituted with 5 mL this gives a concentration of 100 ng/mL. The endotoxin concentration of reconstituted RSE is expressed in endotoxin units (EU) per milliliter.

In order to determine the potency of a given lot of CSE, dilutions series are prepared for both RSE and CSE. The concentrations of CSE used are those expected to give similar reactivity to the RSE dilutions. It is common to use more dilutions of CSE than of RSE to assure that the full range of RSE potency is covered. A useful guide is to assume a CSE potency of 10 EU/ng in order to determine the dilutions of CSE to prepare and test. For the gel-clot method, the two standard endotoxins are tested in parallel (four replicates of each endotoxin concentration) with a specific lot of LAL reagent. The endpoint (the lowest endotoxin concentration to form a solid clot) is determined for each replicate series of concentrations for the two standards. The geometric mean (GM) of the replicate endpoints is calculated for RSE and for CSE. The potency of the CSE is the result of dividing the GM endpoint of the RSE by the GM endpoint of the CSE.

Associates of Cape Cod provides certificates of analysis which state the potency of CSE. Each certificate is specific for a potency determination made for a particular lot of CSE and stated lot of LAL reagent. If the lot of either CSE or LAL reagent changes then a new certificate is required. When CSE and LAL reagent are ordered together, certificates of analysis are provided automatically. If ordering CSE or LAL reagent separately and you need a certificate, please request one and specify the lot for the half of the CSE/LAL pairing that is not being ordered. Certificates may be obtained from our website (http://www.acciusa.com/lal/cert_analysis.html) or from Customer Service (800-LAL-TEST (800-525-8378); custservice@acciusa.com).

Gel clot tests are performed with twofold dilutions of standard endotoxin and this limits the resolution of the method. Consequently, an error of +/- a twofold dilution is assigned to get-clot results. In some tests, the result falls clearly at one endpoint and may be consistently reproducible. In others, the endpoint may be at, or close to, the breakpoint between two dilutions, such that in some tests one result is obtained and in repeat tests another is obtained. Occasionally, some replicates in a given test may give an endpoint at one dilution and other replicates at the next. This error or potential variability applies to all LAL tests, including the determination of label claim sensitivity and to CSE potency determinations.

In an assay in which a CSE is used, there is potential combination of variability. Sources of variability are:

- Labeled sensitivity of the LAL reagent
- Potency determination (involves the results of two assays: one with RSE and one with CSE)
- Test being performed

The stack up of all of the potential errors can occasionally result in a failure to confirm label claim sensitivity with the CSE, resulting in an invalid test.

If label claim cannot be confirmed using CSE, it is possible that there is test interference. This could be caused by plastic tubes, pipette tips, glass, (including the wrong type of glass reaction tube) or other causes. Associates of Cape Cod's Technical Service should be contacted for assistance in resolving the issue. It is also possible that the potency of CSE used is not applicable. This can be tested by performing the test to confirm label claim using USP endotoxin reference standard (endotoxin RS, commonly referred to as RSE). This eliminates the CSE potency as a variable in the test. If label claim cannot be confirmed with RSE, contact Technical Service. Once label claim has been confirmed, the RSE can be used to check the potency on the certificate of analysis. Alternatively, a new lot of CSE with a new certificate of analysis can be obtained. The test to confirm label claim should be performed to verify label claim with the new CSE.

LAL News and Events 2008

2008 ACC TRADESHOW SCHEDULE

PEP Talk	January 7th - 11th	*San Diego, CA*
Aspergillosis (AAAC)	January 16th - 19th	*Miami, FL*
WCBP	January 28th - 30th	*Washington, DC*
Focus on Fungal Inf.	March 7th - 9th	*San Antonio, TX*
PDA Annual Meeting	April 14th - 18th	*Colorado Springs, CO*
PEGS	April 27th - May 2nd	*Boston, MA*
ASCO	May 30th - June 3rd	*Chicago, IL*
IACP	May 31st - June 3rd	*Washington, DC*
Bio2008	June 17th - 20th	*San Diego, CA*
AAPS Biotech	June 22nd - 25th	*Toronto Canada*
AACC Annual Meeting	July 27th - 31st	*Washington, DC*
PDA/FDA	September 8th - 12th	*Washington, DC*
IDSA / ICAAC	October 25th - 28th	*Washington, DC*
Soc. Glycobiology	November 12th - 15th	*Dallas-Fort Worth, TX*
AAPS	November 16th - 18th	*Atlanta, GA*
ASH	December 6th - 9th	*San Francisco, CA*

2008 ACC LAL WORKSHOPS

LAL Workshop - Mid-Atlantic
April 29th - May 1st
Durham, NC

LAL Workshop - Northwest
June 10th - 12th
San Francisco, CA

LAL Workshop - Northeast
June 24th - 26th
East Falmouth, Cape Cod, MA

LAL Workshop - Southern
September 23rd - 25th
San Antonio, TX

LAL Workshop - Northeast
October 21st - 23rd
East Falmouth, Cape Cod, MA

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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