LAL Update®

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Letter from the President

Because of the unusually mild winter on Cape Cod this year, it seems like our laboratory should already be full of horseshoes crabs. Fortunately, winter allows us some time to squeeze in workshops or attend to writing and research.

Recently I discovered that since our founding, ACC has authored or co-authored over 100 original articles on LAL or endotoxin. And this does not include the numerous articles which have appeared in the LAL UPDATE over the past 15 years! Although ACC does not manufacture therapeutic drugs, devices (other than LAL), or reagents for tissue culture, we have tested these products or used them in our research. We therefore write from experience. In this LAL UPDATE, Dr. Dawson continues to provide practical information with an article that should be of special interest to cell culture and biotechnology specialists.

Remember our technical staff is always ready to help with any endotoxin testing/removal applications you may have. Please contact us by phone, fax, or e-mail and be sure to visit our web site.

Sincerely,

Thomas J. Novitsky, Ph.D.

The Significance of Endotoxin to Cell Culture and Biotechnology

By Michael E. Dawson, Ph.D.

Manufacturers of biopharmaceuticals have two concerns about endotoxin. First, the concentration of endotoxin in finished injectable products must be below the endotoxin limit. This concern applies to all injectable drugs and biologicals and non-pyrogenic medical devices. It is well documented and will not be discussed in this article. The second concern is the influences of endotoxin on the expression systems used to produce biopharmaceuticals and upon the products themselves.

The effects of endotoxin vary greatly for different cell types or cell lines. One should be aware of the effect upon the cells of interest so the appropriate level of control can be instituted. Even parent and daughter cell lines may show very different sensitivities to endotoxin.[1] Awareness of the surface receptors of the cells being used can alert an investigator to the potential for endotoxin sensitivity. The presence of the receptor, CD14, on human cells is strongly associated with endotoxin sensitivity.[2] This is acutely important where the culture medium contains serum. Serum components, such as lipopolysaccharide binding protein (LBP) and septin complex, can potentiate endotoxin activation of CD14-bearing cells.[3,4] Conversely, some mammalian and invertebrate cell systems are tolerant of endotoxin. The presence of endotoxin in the media for bacterial and yeast culture is generally less of an issue. The effects of endotoxin on examples of these biological systems considered below.

In studies involving cell culture, one must guard against spurious results caused by biological responses to contaminants. Also, the properties of the products must be distinguished from those of contaminant endotoxin. This is especially true for cytokines.

Membrane and Morphological Effects

Endotoxin is an amphipathic membrane component of gram negative bacteria. It is not surprising that it might interfere with the membrane structure and function in other cells. Through its phosphate groups it reacts with cationic proteins, and its fatty acid chains interact with lipid membranes and hydrophibic regions of proteins. The interaction of endotoxin with cell membranes may be apparent as morphological changes, such as perturbed Chinese hamster ovary cell membranes,[5] surface ruffles and increased organelles,[6] large vacuoles in the cytoplasm,[7] morphological damage and decreased hexose uptake,[8] and severe membrane damage.[9] Sometimes morphological changes are reversible upon exposure to clean media.[10] Also, the effects upon morphology may vary with different endotoxins.[11]

If severe, the effects of endotoxin upon membrane structure and function may be cytotoxic. The degree of toxicity varies between different endotoxins[12] and between cell lines.[13] With at least some cells, however, it is possible to induce tolerance to endotoxin.[14]

Secretion/Production

Membranes are critical to the synthesis of cell products and endotoxin can influence the processes. This is clearly of critical concern in cell based research and in the development of commercial cell lines. The sensitivities of cells are diverse; in one example, two non-responsive T cell lines were cloned from an endotoxin responsive parent.[15] Also, the effects of endotoxin may be modified by other factors, such as temperature.[16]

The influence of endotoxin upon cytokine production by single cell types *in vitro* has been widely reported. Endotoxin has been cited as the most potent stimulus for tumor necrosis factor (TNF) production.[17] Effects upon transcription have been noted[18,19,20,21] and it has been suggested that the primary regulation of stimulation may occur at this level.[22]

A range of cell products other than cytokines are also influenced by endotoxin. Products for which synthesis by mammalian cells is stimulated or enhanced include prostaglandin,[23] acid phosphatase,[6] fibrinolytic inhibitor,[24] collagenase production,[25] nerve growth factor,[26] secretion of factor B,[27] polypeptides,[28] platelet activating factor,[29] adhesion inhibitor,[30] adhesion molecule-1[31] and procoagulant (dose dependent).[32]

Inhibitory effects have been observed for angiotensin converting enzyme activity,[33] and synthesis of proteoglycan[23] and alpha2 macroglobulin.[34] These responses may be dose dependent and in some cases are quite different for different endotoxins. For example, at some doses *Bacteroides* endotoxin stimulated phosphatase production, while that from *E. coli* was inhibitory.[11]

Mitogenicity

Endotoxins are powerful mitogens to some mammalian cell types and lines. This effect can create problems in the culture of susceptible cells. On the other hand, some long-established cell lines are tolerant to high endotoxin concentrations in the culture medium. A correlation between mitogenicity and both LAL reactivity and pyrogenicity of several different endotoxins has been reported.[35] However, positive correlations are not always evident and endotoxins of equal mitogenicity may differ significantly in their LAL reactivity and pyrogenicity.[36] Therefore, LAL activity is not a good predictor of endotoxin mitogenicity.[37]

Some mitogens are heavily contaminated by endotoxin (Table 1) and the high concentrations raise questions about the contribution of endotoxin to their mitogenicity. Similarities in the effects of endotoxin and mitogens have been reported.[38]

Concanavalin A	**<0.003 – 9.2 x 10^5 ng/mg**
Pokeweed mitogen	**1.1 x 10^6 ng/mg protein *(sic.)***

Table 1. Endotoxin concentrations in two mitogens[39]

To add to the complexity, endotoxin can inhibit cell division[10] and, as with stimulation of mitosis, these effects depend on the origin of the endotoxin.[40] There may be a concentration dependent effect, ranging from promotion of mitosis to inhibition at higher concentrations. It is interesting to note that the concentrations of endotoxin in some commonly used mitogens are as high as those found to inhibit cell division in some cell lines.

Other Effects

Other *in vitro* cellular effects of endotoxins reported in the literature include: influence upon adherence,[41] decreased insulin binding and endocytosis,[42] loss of TNF binding sites,[43] protection from HIV infection;[44] tumoricidal activity,[45] and increases in potassium current and in the number of potassium channels.[46]

Multiple Effects and Synergism

The multiple biological responses elicited by endotoxin may result in confusion about cause and effect. The influence of endotoxin upon secretion of cell products can be indirect. For example, an antibody to interferon was shown to prevent the apparent inhibition of elastase production by endotoxin. This suggests that the interferon produced by the cell caused the inhibition. In the presence of the antibody, endotoxin actually increased elastase secretion. The inhibitory effect was therefore secondary and due to endotoxin stimulated interferon production.[47] Similarly, an 80% inhibition of lipase production by cultured heart cells is mediated by endotoxin induced TNF produced by the cells.[48] These observations are a clear warning that observed effects may be indirect consequences of endotoxin exposure. Similarly, mitogenicity may be a secondary effect. Endotoxin stimulated IL-1 production by macrophages and neutrophils has been shown to be mitogenic in spleen cells.[49] In this case the effect was elicited by *Bacteroides gingivalis* endotoxin but not by that from enteric bacteria.

An interesting synergistic interaction between lymphokines and endotoxin results in the induction of chemiluminescence in macrophages. This response has been suggested as a replacement for a cytotoxicity test.[50] In another study, both synergism and antagonism between endotoxin and cytokines in the same system were reported.[51] Granulocyte-macrophage colony stimulating factor makes cells responsive to endotoxin, which induces TNF production. TNF stimulates IL-6 production which inhibits further TNF production in a negative feedback loop.

In vitro Fertilization

In vitro fertilization is a special application of tissue culture technology. Improvements in the fertilization rate have been achieved in media of low endotoxin concentration.[52] In the absence of detectable endotoxin in the media (at a detection limit of 0.1 ng/ml), a pregnancy rate of 27.2% was achieved, compared with only 6.3% when endotoxin was present. Morphological and ultrastructural changes were evident in the presence of endotoxin. In another study,[53] an increasing degree of fragments in the conceptus was observed at endotoxin concentrations above 1 ng/ml. The oocyte fertilization rate was 66% when endotoxin concentrations were less than 1 ng/ml in the medium, compared with 53% when greater than this. Differences were more marked when pregnancies were compared. The pregnancy rate was 8% if the endotoxin concentration in the medium exceeded 1 ng/ml but 32% when no endotoxin was detected (<0.1 ng/ml).

Conclusion

Because of the great variability in the response of cells to endotoxin, it is not possible to state a critical level at which endotoxin begins to interfere with cell function and growth, or what form that interference might take. The effects of endotoxin, and the sensitivity of the cells in question, can only be established by growing cells in a medium free of detectable endotoxin. This may or may not be practicable or possible. If it is, critical levels of contamination can be established and limits can be set.

The effects of endotoxin upon cellular processes has long been recognized. Particular attention has been paid to endotoxin contamination of sera[54] because of the widespread use of sera in cell culture media. Fumarola and Jirillo[55,56] have called vigorously for recognition of the effect of endotoxins on cell lines and the need for appropriately low endotoxin concentrations. They state that "contamination with endotoxin of some preparations commonly used in biology may give false results." There has been at least one call for the "absolute absence"of endotoxin in tissue culture media.[57] These authors also discuss the use of endotoxin to stimulate cells and enhance their reactivity, obviously in a controlled way.

Endotoxin contamination of products should be minimized, and not only because of concerns about pyrogenicity and endotoxin limits. The properties of some products of biotechnology are shared by endotoxin. These products cannot be properly evaluated if the preparation is contaminated with endotoxins.[58] This was well illustrated by the experience of an investigator using an End-X B15 endotoxin affinity resin (Associates of Cape Cod, Inc.). Endotoxin was successfully removed from a protein solution to well below levels of concern. The specific enzymatic activity of the protein was unaffected by treatment. However, the mitogenicity of the preparation dropped dramatically. The investigator eventually concluded that the mitogenicity of the original preparation was probably due to the substantial endotoxin contamination and was not an inherent property of the protein.

The question of endotoxin contamination should be addressed early in product development and certainly before any scale-up. This will enable both minimization of deleterious effects and proper determination of the product's properties. Early attention to endotoxin related issues is then likely to follow through to full-scale production. This will have benefits in final release testing when the endotoxin specification is as important for biopharmaceuticals as it is for conventionally produced therapeutics.

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