

Endotoxins and Cell Culture

Technical Bulletin



John Ryan, Ph.D.
Corning Incorporated
Life Sciences
900 Chelmsford St.
Lowell, MA 01851

Table of Contents

Introduction	1
What is Endotoxin?	2
Detection and Measurement	2
Sources of Endotoxins in Cell Culture	3
Endotoxin Effects on <i>In Vitro</i> Cell Growth and Function	4
Possible Mechanisms for These Effects	6
Conclusions	6
References	7

Introduction

Contamination of cell cultures has long been a serious problem for researchers as well as for manufacturers producing cell-based parenteral (for injection) drug products. In the past, most efforts for avoiding contamination-induced culture losses have focused on biological contaminants: bacteria, mycoplasmas, yeasts, fungi, and even other cell lines (19). However, for companies producing cell culture-based products, such as vaccines and injectable drugs, endotoxin, a chemical contaminant produced by some bacteria, has also been a major concern. The presence of endotoxin in products for injection can result in pyrogenic responses ranging from fever and chills, to irreversible and fatal septic shock. There is increasing evidence that endotoxins can also create a variety of problems for researchers using cell cultures. Our goals are to review some of the current information on endotoxin: its characteristics, potential sources, and especially its effects on cultured cells; and to offer some suggestions on avoiding endotoxin-induced culture problems.

CORNING

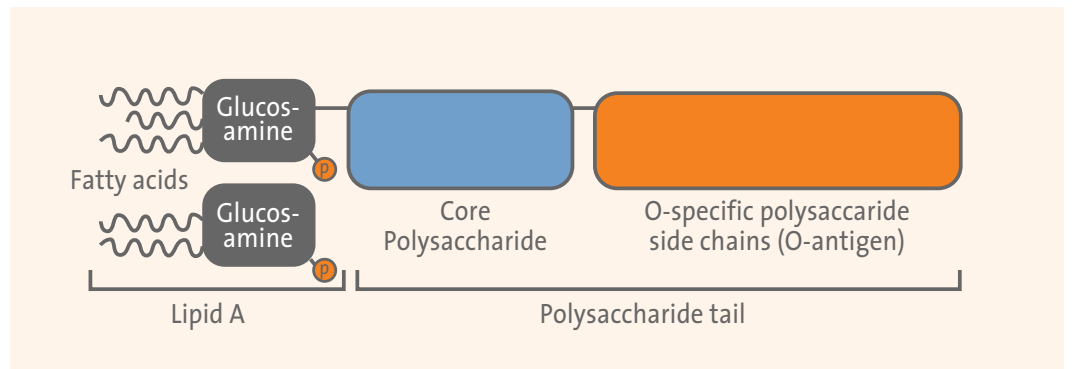


Figure 1. Basic structure of endotoxins produced by gram negative bacteria. Their molecular weight can range from 3,000 to 40,000 Daltons depending on the size of the O-specific polysaccharide side chains. Lipid A is responsible for most of the effects of endotoxin. (Reviewed in 12, 13, 16 and 24)

What is Endotoxin?

Endotoxin is a complex lipopolysaccharide (LPS) which is a major component of the outer membrane of most gram-negative bacteria. A single *Escherichia coli* contains about 2 million LPS molecules per cell. Bacteria shed endotoxin into their environment in small amounts when they are actively growing, and in large amounts when they die. LPS consists of a very hydrophobic lipid group (lipid A) covalently bound to a long complex polysaccharide tail (See Figure 1). Lipid A usually consists of two phosphorylated sugars (glucosamines) which are each coupled with several fatty acids. Lipid A anchors LPS to the bacterial membrane and is responsible for most of its biological effects.

The long hydrophilic polysaccharide tail consists of two domains: a conserved core consisting of a small group of sugars, and a much larger and more variable O-specific polysaccharide region (O-antigen). The core contains heptoses (7 carbon sugars) plus two units of an eight carbon sugar (Kdo: 3-deoxy-D-manno-octulosonic acid) unique to gram negative bacteria that links lipid A to the O-antigen. This region varies both within species and between species, consisting of 20 to 40 repeating units of three to eight sugars each. The O-antigen is responsible for the specific antigenic response in humans that provides immunity to gram-negative infections. (Reviewed in 12, 13, 16 and 24).

Endotoxins are amphipathic molecules characterized by a net negative charge in solution, high heat stability, and a tendency to form very large aggregates (1,000,000 Daltons or more depending on pH, salt concentration, surfactants, etc.) in aqueous solution. Because of their hydrophobicity, they tend to have strong affinities for other hydrophobic materials, especially some plastic products used in the laboratory (18,24).

Detection and Measurement

The first method for detecting endotoxin contamination was the rabbit pyrogen test developed in the 1940's for screening water and solutions used when injecting humans. This test is based on the ability of endotoxin to cause fever (an endotoxin-induced pyrogenic response) when injected into rabbits. Although very successful in reducing pyrogenic episodes, the rabbit test is expensive, time consuming and not very quantitative. In the 1970's, an *in vitro* assay was developed based on observations that the lysate from horseshoe crab (*Limulus polyphemus*) amoebocytes would clot in the presence of very low levels of endotoxin. This *Limulus* amoebocyte lysate (LAL) gel clot assay is very sensitive, detecting down to 0.03 Endotoxin Units (EU)/mL. One EU equals approximately 0.1 to 0.2 ng endotoxin/mL of solution depending on the reference standard used. Endotoxin Units, rather than units of weight, were developed by the U.S. Food and Drug Administration (FDA) for testing comparisons because the potency of an endotoxin for causing pyrogenic effects depends on a

variety of factors: polysaccharide chain length, aggregation, solubility in biological fluids, bacterial source, associated substances, etc. (4). In addition to the gel clot assay, more sensitive LAL kinetic turbidimetric or chromogenic assays have been now developed that can detect down to 0.001 EU/mL.

Sources of Endotoxins in Cell Culture

Water

High purity water is essential in any cell culture laboratory, not only for preparing media and solutions, but also for glassware washing. Traditional glass distillation is very effective in removing endotoxin if the equipment is maintained and used properly. Because of the endotoxin's size, reverse osmosis is also very effective. Both methods are recognized by the U.S. Pharmacopeia (USP) for preparing sterile water for human injection (24,26). Water prepared by passing it through ion exchange resin and activated carbon columns coupled with a final ultrafiltration treatment may also be satisfactory. Poorly maintained water systems, especially systems using ion exchange resins, can harbor significant levels of endotoxin-producing bacteria and should be tested if endotoxin problems are suspected or discovered in cultures. Equally important are the storage conditions used for the water after it has been purified. Bacteria are often found growing in glass or plastic water storage containers and associated tubing, and can quickly raise endotoxin levels in the stored water to unacceptable levels. When in doubt about the quality of the water from a purification system, a simple LAL assay should be done to check endotoxin levels. If the water is found to be a source of endotoxin and the problem cannot be fixed, nonpyrogenic water for injection (WFI) can be purchased and used for preparing media and other critical solutions.

Sera

In the past, sera, especially fetal bovine sera (FBS), have been a major source of endotoxin in cell culture. But as improved endotoxin tests (LAL assay) led to an increased awareness of the endotoxin levels in sera, manufacturers were able to significantly reduce these levels by handling the raw products under more aseptic conditions. In the early 1980's, Case Gould (2) found endotoxin levels as low as 0.006 ng/mL and as high as 800 ng/mL when screening FBS from ten sera manufacturers. Out of a total of 111 lots screened, 86 (77%) had less than 1 ng/mL of endotoxin. Most sera manufacturers currently offer premium or high quality, low endotoxin FBS (less than 1 ng/mL); this is usually more expensive than their standard quality FBS and may not be necessary for many cell lines.

Media and Additives

Currently, most commercially prepared media are tested for endotoxin and certified to contain less than 0.1 ng/mL of endotoxin. For laboratory prepared media, the endotoxin level will be determined primarily by the endotoxin level of the water used to dissolve the other components. However, reagents added to the medium after filtration, even though sterile, can also be a source of endotoxin. In a test of five different batches of commercially prepared bovine serum albumin (5 mg/mL), Dumoulin et al. (5) found endotoxin levels ranged from a low of 0.1 ng/mL to a high of 12 ng/mL. Case Gould reported in her survey of media additives (2) that erythropoietin had endotoxin levels ranging from 80 to 2,000 ng/mL; some 1 Molar amino acid solutions had endotoxin levels as high as 50 ng/mL. These amino acids may have been produced by bacterial fermentation. Whenever possible, ask manufacturers to certify the endotoxin levels of their products; when in doubt test the media endotoxin levels both before and after adding any new components.

Table 1. Potential Sources of Endotoxin in Cell Culture

Water used for glassware washing or making media and solutions
Commercially obtained media and sera
Media components and additives
Laboratory glassware and plasticware

Glassware

Endotoxins can adhere strongly to laboratory glassware and may be difficult to remove completely during washing. While standard laboratory autoclaving procedures will sterilize glassware, they have little if any effect on endotoxin levels due to endotoxin's high heat stability. Subjecting glassware to 250°C for more than 30 minutes or 180°C for three hours is recommended to destroy any contaminating endotoxin (24). This has the added benefit of sterilizing the glassware.

Plasticware

The high temperatures used to melt plastic resins during the molding of plastic laboratory products will usually destroy any contaminating endotoxins. However, significant levels of both biological and chemical contamination can occur after molding during the routine handling associated with assembly and packaging. Unfortunately, while the sterilization process (electron beam or gamma irradiation) will destroy microbial contaminants, endotoxin will be left largely intact. Roslansky et al. (18) showed that endotoxin levels in 50 mL sterile polypropylene centrifuge tubes varied widely among four manufacturers, ranging from a low of 0.007 EU/tube to a high of 15.0 EU/tube. To eliminate this potential problem Corning Incorporated, Life Sciences, certifies that all of their cell culture flasks, dishes, and plates; centrifuge tubes; filters; storage bottles and serological pipets are nonpyrogenic, having less than 0.1 EU/mL as shown using an LAL kinetic chromogenic assay (See Table 2).

Table 2.

Corning cell culture products are certified to be non-pyrogenic with an endotoxin level less than 0.5 EU/mL following USP XXIII Bacterial Endotoxin Test <85>. Corning follows the "Guidelines on Validation of the Limulus Amebocyte Lysate Test as an End Product Endotoxin Test for Human and Animal Parenteral Drugs, Biological Products, and Medical Devices" (FDA 12/87); and the "Interim Guideline for Human and Veterinary Drug Products and Biologics on Kinetic LAL Techniques" (FDA July 15, 1991).

Endotoxin Effects on *In Vitro* Cell Growth and Function

In 1984, Case Gould published a review on the effects of endotoxins on cell cultures (2). Among the effects documented were the stimulation of leukocyte cultures to produce tissue factors, the activation of murine macrophages, and the inhibition of murine erythroid colony formation by very low levels (less than 1 ng/mL) of endotoxin. Since then, many more papers have been published that have reported on the effects of endotoxins on *in vitro* cell functions and growth (See Figure 2). While a comprehensive literature survey is beyond the scope of this bulletin, the following examples will give an overview of some of the varied and significant effects endotoxins can have on cells in culture (See also Table 3).

Macrophages and mononuclear phagocytes are known to produce and release a variety of cytokines, including tumor necrosis factor (TNF) and interleukins, in response to endotoxin stimulation both *in vivo* and *in vitro*. These cytokines mediate the harmful effects of the endotoxins *in vivo* leading to endotoxemia which can result in septic shock and multiple system organ failure (Reviewed in 12). Morris et al. (11) found that endotoxin levels as low as 0.5 ng/mL could significantly increase the production of interleukin-6 in equine peritoneal macrophages after only a six hour exposure. Mattern et al. (9) showed that 100 ng/mL endotoxin could stimulate the proliferation of human T cells and their production of lymphokines, but this stimulation required direct physical contact with previously endotoxin exposed monocytes. Sibley et al. (20) reported that 10 to 200 ng/mL endotoxin stimulated a murine B cell tumor cell line (70Z/3) to complete the production of IgM which was then expressed on the cell surface.

Endotoxin has been shown *in vivo* to impair blood vessel vasoconstrictor responses that can eventually lead to circulatory collapse. It has also been shown to have a variety of *in vitro* effects on blood vessel derived cultures. Organ cultures of rat aortic rings increased their release of TNF and IL-1 and exhibited impaired contractility after exposure to as little as 1 ng/mL endotoxin (10). This showed endotoxin affected both the outer endothelial cell layer (cytokine release) as well as the inner smooth muscle layer (impaired contractility) in these aortic rings. Colburn et al. (3) reported that endotoxin (10 ng/mL) increased the intracellular production of heparan sulfate proteoglycan in a rabbit aortic endothelial cell line,

Table 3. Some of the Documented *In Vitro* Effects of Endotoxin

Cell Type	Concentration (ng/mL)*	Endotoxin Effects
Equine macrophages	0.5	Induced production of IL-6 (11)
Human IVF embryos	1	Reduced pregnancy success rates in in vitro fertilization programs 3-4 fold (7,21)
Aortic rings	1	Reduced contractility and induced production of IL-1 and TNF (10)
Aortic endothelium	10	Dose dependent alterations in heparin sulfate proteoglycan production (3)
Murine B lymphomas	10	Increased production of immunoglobulin light chains 30-40 fold (20)
Recombinant CHO	10	Altered protein production (6)
Cardiac myocytes	10	Induced contractile dysfunction (23)
Human T Cells	100	Induced proliferation and lymphokine production in presence of monocytes (9)
Uretal epithelium	5000	Altered clonal efficiency (25)

*Lowest concentration of endotoxin that showed the effect.

while its extracellular level in the matrix was depleted. Tao and McKenna (23) demonstrated that 10 ng/mL endotoxin could induce contractile dysfunction in cultured rat heart myocytes by increasing nitric oxide synthase activity. Porcine aortic endothelial cells were shown by Buchman et al. (1) to undergo apoptosis (programmed cell death) as the result of exposure to 25 ng/mL endotoxin coupled with exposure to a nonlethal heat shock or sodium arsenite treatment. Sugiura et al. (22) showed that higher levels of endotoxin (100 ng/mL) stimulated the release of endothelin, a very powerful vasoconstrictor, from transformed bovine aortic endothelial cultures. Clearly these papers demonstrate that blood vessel derived cells are very sensitive to endotoxins *in vitro*.

What about endotoxin effects on nonblood-derived cells? Wille et al. (25) showed that very high levels of endotoxin (5,000 to 25,000 ng/mL — endotoxin levels far higher than would be expected under worse case conditions in culture) could alter the cloning efficiency of human uretal epithelial cells in serum-free medium. These endotoxins were from a variety of bacteria: some raised the cloning efficiency, some lowered it, and others had no observed effect. This suggests that the highly diverse polysaccharide component of endotoxin may contribute in some way to its effects. Epstein et al. (6) reported that endotoxin levels as high as 20 ng/mL had no detectable effects on the growth of seven cell lines, including the widely used WI-38, 3T3, and CHO cell lines. They did, however, find that 10 to 20 ng/mL endotoxin altered the production of a recombinant protein by CHO cells.

Other interesting endotoxin effects on cell growth are described in papers (7,21) from two different *in vitro* fertilization clinics that reported endotoxin levels of 0.1 to 1 ng/mL reduced the rate of successful pregnancies. In one case (21), the source of the endotoxin was traced to ultrapure water that had been stored for a short time in a plastic container and then polished in a carbon cartridge, both of which contained endotoxin. However, another laboratory (5) reported that endotoxin levels as high as 500 µg/mL had no effect on human sperm viability and it took 50 µg/mL endotoxin to affect fertilization and subsequent *in vitro* development of mouse embryos.

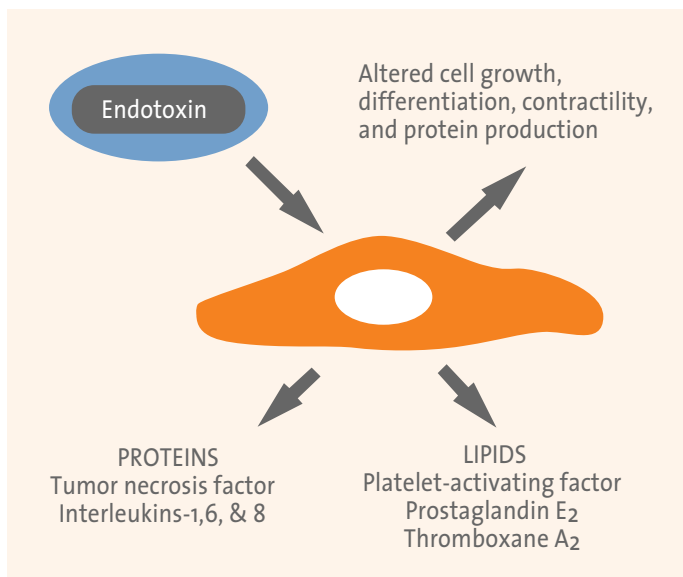


Figure 2. Depending on cell type and culture conditions, endotoxin can have a variety of effects on cell growth and function (16).

Based on the above evidence, it is clear that endotoxins do not affect all cultured cells equally. Some cell cultures, perhaps lacking appropriate endotoxin receptors (See below), may only be sensitive to very high levels of endotoxin — levels not likely to be found in cell culture systems by accident. Cell lines that have been grown in culture for many years (CHO, 3T3, WI-38, HeLa, etc.) may have been naturally selected for resistance to endotoxin by long term, inadvertent exposure to high levels of endotoxins that could often be found in media, sera, and culture additives before endotoxin testing became widely used.

Possible Mechanisms for These Effects

Much is still unknown about the mechanisms through which endotoxins interact with cell cultures (Reviewed in 12, 13, 14, and 15). A 60 kDa glycoprotein, LPS Binding Protein (LBP), has been found in serum that binds LPS through its lipid A domain and greatly enhances its ability to interact with cells of the immune system. This enhancement appears

to require a specific 55 kDa glycoprotein receptor, CD14, on the cell surface. This membrane anchored receptor is found on a variety of immune cells (See Figure 3) and, when bound with the LPS-LBP, starts a chain of events resulting in the cytokine mRNA production and the subsequent release of a variety of cytokines by the cell. Exactly how the intracellular signaling occurs is not fully understood. A paper by Han et al. (8) showed that binding of LPS to CD14 triggered a tyrosine kinase phosphorylation cascade involving a protein, p38, that has been shown to be a MAP kinase. Perhaps CD14 transfers the LPS-LBP complex to a yet undiscovered transmembrane receptor to effect the internal cell signaling.

LPS may also interact with cells by other means, since some cells lacking CD14 receptors have been shown to be affected by LPS. LPS may enter the cell by unknown receptors, endocytosis, or by directly entering the cell membrane (15). Risco et al. (17) demonstrated that LPS could bind to cytoskeleton microtubules, and at high concentrations, inhibit microtubule polymerization. This binding could explain one possible mechanism for some of the cytotoxic effects of LPS that do not appear to involve cytokine activation.

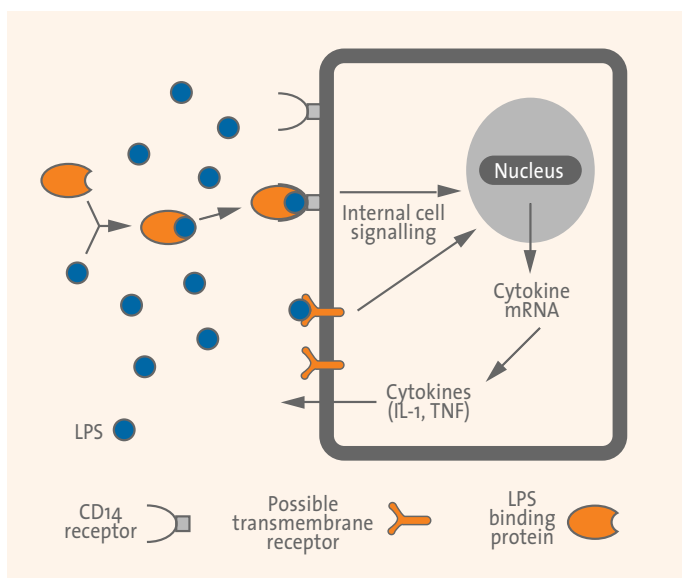


Figure 3. One of the proposed models for endotoxin activating cells (Adapted from 14). The LPS binds to a LPS binding protein (LBP) forming a complex which interacts with a membrane anchored CD14 receptor. This results in the generation of an intracellular signal that turns on cytokine production (TNF = Tumor necrosis factor; IL-1 = Interleukin-1).

Conclusions

While endotoxin may not be a problem for all cell culture users, it clearly is for some. Endotoxin contamination of cell-based products is a major concern to the pharmaceutical industry, since endotoxin in vaccines and other cell-based parenteral drug products render them unfit for use. Endotoxin has also been shown in many cell culture experiments to affect cell growth and function as well as being a source of significant variability (2). This is especially true when using cells known to be sensitive to the low endotoxin levels that are commonly found in cell culture systems.

Therefore, unless you are sure endotoxin has no effect on your cultures and is not a potential source of variability in your experiments, you should reduce the possibility of endotoxin-related problems by taking some basic precautionary steps. The first step is to use cell culture media, sera, and plasticware that are certified either by their manufacturers or through in-house testing to be nonpyrogenic. The second step is to test the source of the water used for making solutions and for washing glassware and plasticware to ensure the water is not a source of endotoxin (Reviewed in 4). As an extra precaution, the culture medium can be tested for endotoxin after any additional components have been added.

For additional assistance in these areas, please contact Corning Life Sciences, Technical Service at 1.800.492.1110 or 1.978.442.2200, or visit our website at www.corning.com/lifesciences.

Acknowledgements

The author would like to thank Marilyn Case Gould, of the Associates of Cape Cod, and Sau Mei Leung of Applied Biosystems, Inc. for their kind assistance in preparing this manuscript.

References

1. Buckman, T.G., Abello, P.A., Smith, E.H. and Bulkley, G.B. Induction of Heat Shock Response Leads to Apoptosis in Endothelial Cells Previously Exposed to Endotoxin. *Am. J. Physiol.* 265:H165-H170 (1993).
2. Case Gould, M.J. Endotoxin in Vertebrate Cell Culture: Its Measurement and Significance. *In Uses and Standardization of Vertebrate Cell Lines*, (Tissue Culture Association, Gaithersburg, MD, 1984), 125-136.
3. Coburn, P., Kobayashi, E. and Buonassisi, V. Depleted Level of Heparan Sulfate Proteoglycan in the Extracellular Matrix of Endothelial Cell Cultures Exposed to Endotoxin. *J. Cell. Physiol.* 159:121-130 (1994).
4. Dawson, M.E., Novitsky T.J. and Gould, M.J. Microbes, Endotoxins and Water. *Pharm. Engineering* 8(2) (1988).
5. Dumoulin, J.C.M., Mehheere, P.P.C.A., Evers, J.L.H., Kleukers, A.P.G., Pieters, M.H.E.C., Bras, M. and Geraedts, J.P.M. The Effects of Endotoxins on Gametes and Preimplantation Embryos Cultured *In Vitro*. *Hum. Reprod.* 6:730-734 (1991).
6. Epstein, J., Lee, M.M., Kelly, C.E., and Donahue, P.K. Effect of *E. Coli* Endotoxin on Mammalian Cell Growth and Recombinant Protein Production. *In Vitro Cell. Dev. Biol.* 26:1121-1122 (1990).
7. Fishel, S., Jackson, P., Webster, J. and Faratian, B. Endotoxins in Culture Medium for Human *In Vitro* Fertilization. *Fertil. Steril.* 49:108-111(1988).
8. Han, J., Lee, J.D., Bibbs, L. Ulevitch, R.J. A MAP Kinase Targeted by Endotoxin and Hyperosmolarity in Mammalian Cells. *Science* 265:808-811 (1994).
9. Mattern, T., Thanhauser, A., Reiling, N., Toellner, K., Duchrow, M., Kusumoto, S., Rietschel, E.T., Ernst, M., Brade, H., Flad, H.D. and Ulmer, A.J. Endotoxin and Lipid A Stimulate Proliferation of Human T Cells In the Presence of Autologous Monocytes. *J. Immunol.* 153:2996-3004 (1994).
10. McKenna, T.M. Prolonged Exposure of Rat Aorta to Low Levels of Endotoxin *In Vitro* Results in Impaired Contractility. *J. Clin. Invest.* 86:160-168 (1990).
11. Morris, D.D., Crowe, N., Moore, J.N. and Moldawer, L.L. Endotoxin-Induced Production of Interleukin 6 by Equine Peritoneal Macrophages *In Vitro*. *Am. J. Vet. Res.* 53:1298-1301 (1992).
12. Morrison, D.C., Dinarello, C.A., Munford, R.S., Natanson, C., Danner, R., Pollack, M., Spitzer, J.J., Ulevitch, R.J., Vogel, S.N., and McSweeney, E. Current Status of Bacterial Endotoxins. *ASM News*, 60:479-484 (1994).
13. Raetz, C.R.H. Biochemistry of Endotoxins. *Annu. Rev. Biochem.* 59:129-170 (1990).
14. Raetz, C.R.H. Bacterial Endotoxins: Extraordinary Lipids that Activate Eucaryotic Signal Transduction. *J. Bact.* 175:5745-5753 (1993).
15. Raetz, C.R., Ulevitch, R.J., Wright, S.D., Sibley, C.H., Ding, A., and Nathan, C.F. Gram-negative Endotoxin: An Extraordinary Lipid with Profound Effects on Eukaryotic Signal Transduction. *FASEB J.* 5:2652-2660 (1991).
16. Rietschel, E.T. and Brade, H. Bacterial Endotoxins. *Sci. Amer.* 267:54-61 (Aug. 1992).
17. Risco, C., Dominguez, J.E., Bosch, M.A., and Carrascosa, J.L. Biochemical and Electron Microscopy Analysis of the Endotoxin Binding to Microtubules *in vitro*. *Mol. Cell. Biochem.* 121:67-74 (1993).
18. Roslansky, P.F., Dawson, M.E. and Novitsky, T.J. Plastics, Endotoxins and the Limulus Amebocyte Lysate Test. *J. Parental Sci. Tech.* 45:83-87 (1991).
19. Ryan J.A. Understanding and Managing Cell Culture Contamination. Corning Incorporated. Technical Publication CLS-AN-020 (2002).
20. Sibley, C.H., Terry, A. and Raetz, C.R.H. Induction of K Light Chain Synthesis in 70Z/3 B Lymphoma Cells by Chemically Defined Lipid A Precursors. *J. Biol. Chem.* 263:5098-5103 (1988).

21. Snyman, E. and Van der Merwe, J.V. Endotoxin-Polluted Medium in a Human *In Vitro* Fertilization Program. *Fertil. Steril.* 46:273-276 (1986).
22. Sugiura, M., Inagami, T. and Kon, V. Endotoxin Stimulates Endothelin-Release *In Vivo* and *In Vitro* as Determined by Radioimmunoassay. *Biochem. Biophys. Res. Comm.* 161:1220-1227 (1989).
23. Tao, S. and McKenna, T.M. *In vitro* Endotoxin Exposure Induces Contractile Dysfunction in Adult Rat Cardiac Myocytes. *Am. J. Physiol.* 267:H1745-H1752 (1994).
24. Weary, M. and Pearson, F. A Manufacturer's Guide to Depyrogenation. *BioPharm.* April, 22-29 (1988).
25. Wille, J.J., Park, J. and Elgavish, A. Effects of Growth Factors, Hormones, Bacterial Lipopolysaccharides, and Lipotechoic Acids on the Clonal Growth of Normal Uretal Epithelial Cells in Serum-Free Culture. *J. Cell. Physiol.* 150:52-58 (1992).
26. —, The United States Pharmacopeia XXIII, U. S. Pharmacopeial Convention, Rockville, MD (1994).

For additional product or technical information, please visit www.corning.com/lifesciences or call 800.492.1110. Customers outside the United States, please call +1.978.442.2200 or contact your local Corning sales office listed below.

CORNING

Corning Incorporated Life Sciences

Tower 2, 4th Floor
900 Chelmsford St.
Lowell, MA 01851
t 800.492.1110
t 978.442.2200
f 978.442.2476

www.corning.com/lifesciences

Worldwide Support Offices

ASIA / PACIFIC

Australia
t 61 2-9416-0492
f 61 2-9416-0493

China
t 86 21-3222-4666
f 86 21-6288-1575

Hong Kong
t 852-2807-2723
f 852-2807-2152

India
t 91-124-235 7850
f 91-124-401 0207

Japan
t 81 (0) 3-3586 1996/1997
f 81 (0) 3-3586 1291/1292

Korea
t 82 2-796-9500
f 82 2-796-9300

Singapore
t 65 6733-6511
f 65 6861-2913

Taiwan
t 886 2-2716-0338
f 886 2-2716-0339

EUROPE

France
t 0800 916 882
f 0800 918 636

Germany
t 0800 101 1153
f 0800 101 2427

The Netherlands
t 31 20 655 79 28
f 31 20 659 76 73

United Kingdom
t 0800 376 8660
f 0800 279 1117

All Other European Countries

t 31 (0) 20 659 60 51
f 31 (0) 20 659 76 73

LATIN AMERICA

Brasil
t (55-11) 3089-7419
f (55-11) 3167-0700

Mexico
t (52-81) 8158-8400
f (52-81) 8313-8589