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Leading Opinion

Endotoxin: The uninvited guest ☆

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Abstract

In the laboratory environment where biomaterials are synthesized and their biocompatibility assessed, we find that endotoxin contamination is hard to avoid and must not be ignored. In those relatively few cases where endotoxin was known to be present, it has been clearly shown that endotoxin can significantly affect the biological response observed and hence confound any effect of the material. This short review explains what endotoxin is, how to test for it and remove it and what its effect on the biological response to biomaterials is. We advocate routine testing of endotoxin on biomaterials and of reagents used in experimental evaluation of biomaterials and this should be the responsibility of every scientist to ensure the validity of any biomaterial study.

Keywords: Endotoxin; Biomaterials; Cytokines; LPS

Biomaterials specialists are accustomed to the admonition to perform surface characterization studies as part of their effort to understand biocompatibility. Certainly many laboratories are wary of silanes because of the potential for inadvertent contamination with silicon. Endotoxin contamination is a similar issue, but has not yet entered widespread consciousness. Unfortunately many studies of biocompatibility may be compromised by this tiny, detectable, but hard to avoid, contaminant. These compromised studies may teach more about the effect of endotoxin or the interactions between the biomaterial and endotoxin than about biocompatibility. Endotoxin is a very potent stimulus for a wide range of cells (leukocytes, platelets, endothelial cells, epithelial cells) both in vitro and in vivo and since these cells are central to biocompatibility, it is not difficult to see the problem. Some cells such as leukocytes require minute amounts of LPS to be activated [1] while others such as platelets need a larger concentration [2].

Commercially available medical devices need to have low endotoxin levels before they can be approved for sale by the regulatory authorities. Nonetheless, commercially available wound dressings made of natural biomaterials [3] and discs of Titanium and Ti alloy prepared by an orthopedic implant manufacturer in a similar fashion to authentic implants [4] were reported to contain significant amount of adherent endotoxin. Commercially available laboratory reagents used in cell culture such as a collagen solution [5], the recombinant protein rHsp70 [6], and metallic microparticles [4] have also been found to contain endotoxin and induce a significant biological response. The presence of endotoxin contamination represents a serious threat to these products, but the effect on the scientific evaluation of biomaterials should also not be ignored. All these

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products were tested in vitro with cells before and after removal of endotoxin contamination: the presence of endotoxin induced significant production of cytokines [3,6,7] or cell maturation [5] as compared to decontaminated samples.

Reports on endotoxin contamination of medical devices are most often derived from in vitro studies as one does not expect endotoxin to be observed in clinical practice due to the stringent regulation on medical devices. However, there are some examples where during cardiopulmonary bypass and extracorporeal membrane oxygenation, the presence of endotoxins has been observed in vivo [8,9]. They appear to originate mostly from the gut [10–12] rather than from the materials and are believed to be a reaction to the surgical procedure. During hemodialysis, endotoxin contamination is also an issue but the dialysate is usually the source [13,14]. With the use of cardiovascular devices, while endotoxin contamination may be present in vivo in some patients and studies, there has been no investigation showing a significant correlation between the magnitude of endotoxin contamination and postoperative complications [9,15]. In orthopedics, there is now some speculation that endotoxin may play a significant role in aseptic loosening (see Greenfield et al. [16] for review).

This short article explains what endotoxin is, relates our experience as to how to measure and remove contamination, and how we have found or believe material endotoxin contamination may affect and confound the biological response.

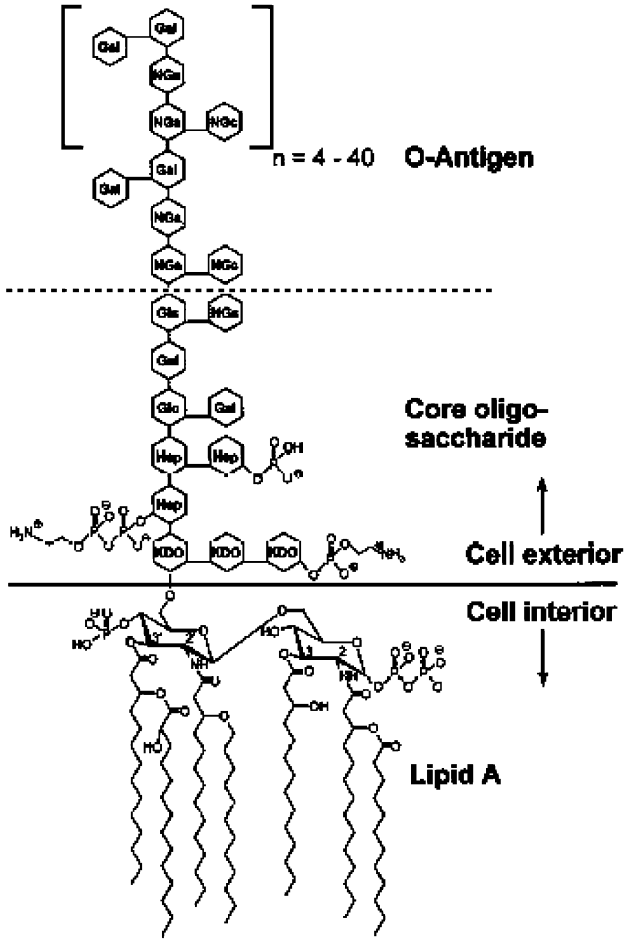

Fig. 1. Schematic view of the chemical structure of endotoxin from *E. coli* 0111:B4 [46].

1. What is endotoxin?

Endotoxins, also called lipopolysaccharides (LPS), are an integral part of the outer cell membrane of Gram-negative bacteria. Endotoxins consist of a lipid component, Lipid A, a core oligosaccharide and a long heteropolysaccharide chain, the O-specific chain representing the surface antigen (O-antigen) (Fig. 1) [17]. The O-antigen is generally composed of a sequence of identical oligosaccharides and is strain specific. Lipid A is the most conserved part of endotoxin and is responsible for most of the biological activities of endotoxin. A single *Escheria coli* contains about 2 million LPS molecules per cell. Endotoxins are shed upon cell death (in large amount) but also during growth and division. They are highly heat-stable and hence are not destroyed under regular sterilizing conditions: a temperature of over 180 °C is necessary to inactivate endotoxins. Endotoxins have a net negative charge in solutions. They can form aggregates (micelles or vesicles) with high stability depending on the solution characteristics (pH, ions, surfactants, etc.); thus the size of endotoxin ranges from about 10–20 kDa (monomer) to over 1000 kDa (vesicles). Due to their hydrophobicity, endotoxins adsorb readily to hydrophobic materials and will also bind cationic materials through their phosphate groups [18] (see Fig. 2).

In humans and animals, endotoxins have very strong biological effects when entering the blood stream with symptoms ranging from fever and shivering to hypotension, adult respiratory distress syndrome, disseminated intravascular coagulation and endotoxin shock. It is its potential to induce fever that has led endotoxins to be also referred to as pyrogens. Most cases of sepsis (a systemic inflammatory response to a local infection) result from Gram-negative bacteria and its pathophysiology is initiated by LPS, which stimulates the synthesis of inflammatory mediators such as the cytokines IL-1β, IL-6 and TNF-α. The critical level of endotoxin leading to sepsis is not clear since it depends on the virulence of the organism, the sites of infection, the host response and some genetic factors. In one study, the median of endotoxin concentration in plasma for septic patients has been reported to be around 2.5 EU/mL (ranging from nondetectable levels to

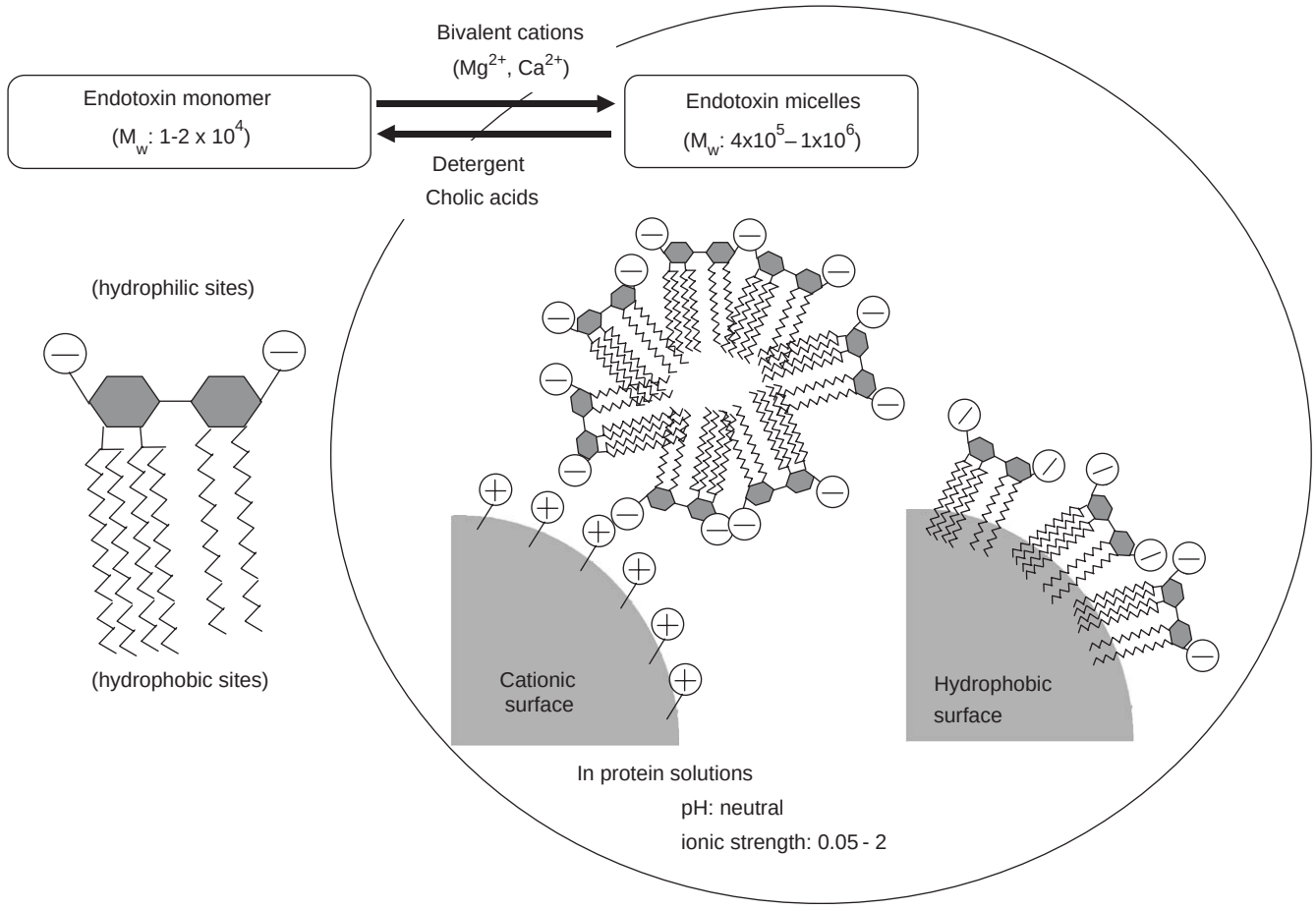

Fig. 2. Schematic diagram showing the adsorption behavior of endotoxin aggregates on biomaterials (adaptated from Hirayama and Sakata [18]).

12 EU/mL) [19]. In children affected by meningococcaemia and admitted for septic shock, the levels were 5 EU/mL (range nondetectable to 40 EU/mL) [20].

Endotoxins have been reported to activate the complement, the coagulation and the kinin systems [21]. LPS also stimulates cells to produce inflammatory mediators. Among others, monocytes and macrophages respond to LPS by releasing cytokines such as TNF-α, IL-1β, IL-6, IL-10 and by expressing Tissue Factor, the primary agonist of thrombin and fibrin formation in many situations. Increased adherence to endothelium, upregulation of integrins and increased respiratory burst activity are hallmarks of leukocyte activation by LPS [21]. In vitro, monocytes and macrophages can be activated to synthesize IL-1β and TNF-α by concentration of LPS as low as 0.01 ng/mL (equivalent to 0.05 EU/mL). In vivo, humans have been known to develop a reaction to endotoxin when injected with endotoxin at 4 ng/kg body weight [22].

Many in vivo models of sepsis or endotoxemia have been developed in animals to better understand the molecular mechanisms of sepsis and develop therapeutic approaches to reduce its toxic risk. As of today there is still no therapy available to effectively treat sepsis.

2. LPS receptors

In the bloodstream, endotoxin binds to the serum protein LPS-binding protein (LBP) and this complex is then able to bind the receptor CD14. CD14 exists in two forms, a soluble form sCD14 and a membrane bound form mCD14 [23]. sCD14 is present in plasma and LPS binding to sCD14 enable cells lacking a membrane bound CD14 receptor (such as endothelial and epithelial cells) to be stimulated by LPS. mCD14 is present on myeloid cells and is a glycosylphosphatidylinositol (GPI)-anchored receptor. Since CD14 lacks transmembrane and intracellular domains, it is unlikely that binding of LPS to mCD14 results in cytokine synthesis. The true receptor for the signal transduction of LPS has been identified recently as being the Toll-like receptor-4 (TLR-4) [24]. TLR-4 also requires an additional molecule MD-2 (a secreted glycoprotein) for effective recognition of LPS [25]. The identification of the true LPS receptor has opened new avenues to design successful therapies to treat sepsis.

It is believed that LPS binds to CD14 and is then transferred to the TLR-4/MD-2 complex. Different intracellular signaling pathways are then activated in

response to LPS. Other receptors such as CD11/CD18 and CD55 also play a role in the mechanisms of LPS signal transduction via TLR-4. Groups of cell receptors that "interact" with CD14 and TLR-4/MD-2 have been described as an activation cluster for different cell types [23]. It is important to note that monocytes, macrophages and granulocytes are not the only cells to possess the LPS-membrane receptors CD14 and TLR-4. Human corneal epithelial cells were recently reported to express both CD14 and TLR-4 [26]. Such a finding further emphasizes the potency of cell activation by LPS and how its presence may interfere with characterizing host response in many areas of biomaterials research.

3. FDA guidelines and endotoxin testing

Due to the serious risks associated with endotoxin, the US Food and Drug Administration (FDA) has set guidelines for medical devices and parenteral drugs. The protocol to test endotoxin contamination of medical devices recommends immersion of the device in endotoxin-free water for at least 1 h at room temperature; this extract/eluate is then tested for endotoxin. Current FDA limits are such that eluates from medical devices may not exceed 0.5 EU/mL, unless the device comes into contact with cerebrospinal fluid where the limit is then 0.06 EU/mL [27]. The classic test for measuring endotoxin used to be the rabbit pyrogen test whereby the sample was injected into the animal and the rise in body temperature observed (an increase of 0.5 °C or more indicated pyrogenicity). Although sensitive (0.5 EU/mL), this test does not determine the endotoxin concentration.

The limulus amebocyte lysate (LAL) assay is now accepted as an alternative for the detection of endotoxin. This assay is based on a cell lysate of the horseshoe crab *Limulus polyphemus* that coagulates in the presence of even very low levels of endotoxins. Different versions of the assay are available: the gel-clot assay or the chromogenic substrate method. LAL kits are available from many companies. The chromogenic substrate assay is more sensitive (0.005 EU/mL) than the older gel-clot assay (0.03 EU/mL). Furthermore, chromogenic substrates can be adapted to assess endotoxin adherent to a biomaterial, resulting in a measure of endotoxin contamination in terms of equivalent endotoxin units/mL (using the calibration curve from solution standards) [4,28,29]. For example, for the studies described below, endotoxin contamination was measured directly on the surface and did not use an extract. A cytokine release method (using whole blood) for endotoxin/pyrogenicity has been developed [30] but it does not appear to be suitable for biomaterials, since it cannot distinguish endotoxin contamination from the effect of the material itself on leukocyte activation; cytokine release is one of those parameters that has been used to assess material effect [31].

While FDA guidelines refer to endotoxin in the extracts from medical devices, we and others [32,33] strongly believe that endotoxin contamination of the material itself should be assessed when performing in vitro and in vivo biocompatibility experiments. Eluting endotoxin from a biomaterial is very difficult, only 40–70% of inoculated LPS were recovered from catheters following a very stringent extraction protocol in ethanol [34]. Even though an extract may be endotoxin free, endotoxin may still be present on the surface [4,33], sufficient to generate a chromogenic substrate effect greater than what is generated by a 0.5 EU/mL solution (the FDA limit). The validity of the 0.5 equivalent EU/mL standard for adherent endotoxin is unproven, although the in vitro evidence (see below) suggests that this does represent an important threshold.

4. How does endotoxin contamination arise?

Since endotoxin is very difficult to remove from a biomaterial (see below), it is important to prevent its contamination in the first place. Since endotoxin is prevalent in tap water, air and people's fingers, it is not difficult to see how endotoxin contamination occurs. In our experience, the most conspicuous source of endotoxin during material synthesis is laboratory water since distillation and deionizing columns do not remove endotoxin: a special column or filtering system is necessary to do so. Commercially available pyrogen free water is a critical necessity in most cases, but even then poor handling of containers can transfer endotoxin to the material during washing procedures.

Chemical reagents, raw materials, and buffers are also all potential sources of endotoxin. It is not uncommon to find high levels of endotoxin in biological products such as albumin, collagen or gelatin (note "endotoxin tested" is not the same as "endotoxin free"). Depending on the source of an antibody or a peptide, endotoxin may also be present. Generally, if the certificate of analysis of a reagent or buffer does not mention endotoxin level, one should assume that it is contaminated. Table 1 shows the level of endotoxin contamination that has been measured in some reagents. Finally, glassware or equipment represents yet another source of endotoxin. Endotoxin can adhere strongly to glassware and may be hard to remove during conventional washing. Washing glassware with a 1% alkaline solution will help remove endotoxin. Since endotoxin is highly heat-stable, standard autoclaving will not destroy endotoxin. On the other hand, 250 °C for more than 30 min or 180 °C for more than 3 h will [35]. Unfortunately few polymers can withstand these conditions.

Table 1
Endotoxin levels of various reagents used in laboratories

Reagents/materials	Endotoxin level
ddH_2O	20 EU/mL
PBS (made from powder reagents)[1]	76 EU/mL
Albumin solution[1]	6EU/mL
Collagen solution (300 μg/mL)[1]	200 EU/mL [5]
Heparin solution (1000 USP/mL)[1]	1 EU/mL
Alginate solution (1%)[1]	10 EU/mL
Agarose gel[1]	64 EU/mL
rHsp70	1EU/mL [6]
Anti-P selectin[1]	1 EU/mL

[1]Prepared with endotoxin-free water or buffer.

As noted above, using endotoxin-free water is strongly recommended. Aseptic technique even outside the laminar flow hood and reducing the amount of time that a material or reagent is exposed to air will also significantly reduce contamination level. Furthermore, regardless of the source, unless a material or a reagent has been specifically tested for endotoxin, one should never assume that it is free of endotoxin (i.e., below the recommended FDA limit of 0.5 EU/mL).

5. How to remove endotoxin contamination?

There are some washing procedures that can effectively remove adsorbed endotoxin, at least from materials that can withstand these strong solutions. To clean metallic particles, Ragab et al., used a cycle of alkali ethanol (0.1 M NaOH in 95% ethanol) at 30 °C followed by 25% nitric acid both for 18–20 h each [4]. Washes in 70% ethanol followed [36] or not [37] by a wash in acetic acid have also been successful. We have used a cycle of washes in NaOH (1 N), HCl (1 N) and then 70% ethanol, all performed in an ultrasonic bath, to remove endotoxin on many polymer particles and films [28,38].

Different methods, such as ultrafiltration, extraction, adsorption, are available to remove endotoxin from contaminated solutions [17]. However, the viscosity of the (polymer) solution or its acidity (such as the case with a collagen solution) prevents the use of many of the available techniques. Finding a supplier that offers an endotoxin-free product is often the only solution.

6. What are the effects of endotoxin on biomaterial biocompatibility?

In analyzing the effect of endotoxin, some researchers have contaminated materials with a purified strain of endotoxin (i.e., using a commercial source of endotoxin such as *E. coli* 055:B5) while others have used materials "as is", which are contaminated by environmental endotoxins. The purified endotoxin contains only the lipid and polysaccharide portions and are much more potent than environmental endotoxins [39]. Some studies reported the adsorbed amount of environmental endotoxin on the materials they tested (levels ranged from the equivalent of 2–29 EU/mL). In other studies, materials (typically particles) were incubated in solution containing pure LPS ranging from 25 EU/mL to 3000 EU/mL, then rinsed and tested; the levels actually adsorbed were never reported. All microparticles [7,32,33,36,37,40] or discs [29] contaminated with endotoxin (purified or environmental) have been reported in vitro to significantly increase the production of TNFα, IL-1β, IL-6 and nitric oxide in macrophages. Adherent endotoxin also induced osteoclast differentiation [32]. In all these studies, removal of endotoxin resulted in levels of cytokine and NO synthesis that were similar to the control (no particles). On the other hand, the presence of endotoxin on particles did not increase the rate of phagocytosis when compared to endotoxin-free particles [7]. In blood, adherent endotoxin on polymer beads (2.5 EU/mL) resulted in increased tissue factor expression and CD11b upregulation in vitro. The presence of endotoxin on the beads masked the effect of surface area on material-induced leukocyte activation: the level of leukocyte activation was the same for all bead concentrations tested with contaminated beads while leukocyte activation was bead concentration (surface area) dependent when beads were cleaned [41]. It is interesting to speculate whether the endotoxin-free biomaterial that activates leukocytes is 'mimicking' endotoxin in some manner, perhaps through the presence of similar "molecular patterns" [42].

Few in vivo studies have been done with some foreknowledge of endotoxin contamination. A significant increase in osteolysis was observed at 7 days [32,43] with deliberately endotoxin contaminated particles, but at 21 days [43] no difference existed between contaminated and clean particles. The presence of endotoxin in alginates may be a factor in the antibody response seen upon implantation in mice. Purification of the alginate, which reduced the endotoxin level by 91%, resulted in the absence of an immune response [44] when two batches of microcapsules were injected intraperitoneally 2 months apart. Endotoxin contamination of alginate has long been considered a major factor in the failure or irreproducibility of alginate–polylysine microencapsulated islet studies [45]. The osteolysis study illustrates how endotoxin contamination may have lead scientists to overlook alternative causes (i.e., endotoxin contamination) of aseptic loosening. It is conceivable that the adsorbed endotoxin would not have been detected by eluate analysis but still present in high enough amounts to activate macrophages upon implantation. One has to

wonder if there other instances of an apparent biomaterial effect where endotoxin contamination was involved.

7. Conclusion

Assessing biomaterial compatibility is already such a complex issue that one does not need to add a factor such as endotoxin contamination, especially when it can be avoided. To ensure that endotoxin does not further compromise an already difficult literature or our understanding of biocompatibility and more importantly to prevent the serious health complications of endotoxin contamination, it is the responsibility of every biomaterials specialist to guard against contamination and conduct proper endotoxin testing. Maybe it is not important in some studies (e.g., platelet activation), but good practice suggests avoiding the development of poor habits.

While it is reasonable to assume that a high degree of endotoxin contamination compromises a study of biomaterial biocompatibility, we admit there is no in vivo evidence to support the contention that slight contamination (say at the equivalent of 1 EU/mL) will result in a different tissue response than contamination at <0.5 EU/mL. However, the in vitro evidence is compelling to us and leads us to the opinion that good biomaterial practice requires (a) regular testing for endotoxin levels using a chromogenic substrate assay (b) reporting of the values, alongside other surface characterization data and (c) great caution in interpreting results when there is even a little endotoxin contamination, especially if the intent is to make some conclusion linking biomaterial properties (other than the propensity to collect endotoxin) on host response. We might even go further and say that much of the biomaterials literature is suspected due to previous failures to test for endotoxin with proper sensitivity. At least that's our opinion and we look forward to being proven wrong.

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