Clinical Evidence for the Suitability of Current Bacterial Endotoxin Testing

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Over the last three decades, the sciences of immunology and cell biology have been revolutionized by discovery of complex networks of cell receptors and signals. There is now a rich literature describing interaction between the human immune system and lipopolysaccharides (LPS) which are ubiquitous molecules found embedded in the outer leaflet of the outer membrane of Gram-negative bacteria. At a 30,000 foot level, a unique signaling cascade between Lipid A and LPS Binding Protein (LBP), both soluble and cell surface CD-14, as well as cell surface receptors MD-2 and TLR4, define the basic innate and adaptive human immune response to Gram-negative bacterial endotoxin (Vesy, et al.., 2000). In light of the recent publications regarding potentially "invalid" LAL tests and concerns about patient safety, (PDA, 2019), it is important to reflect on the the human interaction with Gram-negative bacteria as we go through our daily lives and what that means to the establishment of analytical limits for endotoxin testing for parenteral drugs and medical devices.

One of the earliest applications of the LAL test in the 1960s and 1970s, was to detect endotoxin in human blood. The objective of this research was to determine the role that endotoxin played in septicemia (Levin, 1970; Levin, et al.., 1972; Levin 1973). Initially, the focus of these clinical research efforts was to measure endotoxin levels associated with infection primarily in septicemia caused by *E. coli*. Today, sepsis, which is currently referred to as Systemic Inflammatory Response Syndrome (SIRS), is most often caused by *Streptococcus pyogenes* and *Staphylococcus aureus*, both Gram-positive bacteria (Opal, 2007) although SIRS can be caused by a wide variety of microbial infections and non-infectious SIRS is also observed (Constedt et al. 2009). SIRS, a major cause of global death and suffering, has increased by 9% annually from the early 1990s through 2013 and has only in the last few years begun to plateau. SIRS is a multi-step process that results in an uncontrolled and devastating inflammatory response, which can result in multiple organ failure. In the United States 240 people per 100,000 in the general population had SIRS in 2013 and it is currently the 10th highest cause of death in high-income countries (Kadri et al.., 2017, Rhee et al. 2017).

To put endotoxin levels measured during a systemic or whole blood stream infection in context, scientists look to compare baseline endotoxin levels in normal or asymptomatic human subjects to patients suffering from SIRS. In 2002 Nadhazi, et al.. et al. studied the plasma level of endotoxin in 116 healthy blood donors using a chromogenic LAL assay and found that detectable levels of endotoxin were found in all human plasma evaluated, with a recovery range from 0.01-1EU/mL, suggesting that humans do not go through daily life endotoxin-free; they do indeed have an endotoxin "baseline".

If we consider that the average human has about 5000mL of blood in circulation, these healthy subjects had between 50 and 5000 EU in circulation at any given point in time. Please bear in mind that these were *healthy asymptomatic subjects* found suitable for the donation of blood. Subsequent studies have confirmed these findings and in general, control populations of humans studied at random showed endotoxin levels of roughly 0.3 to >10 endotoxin units/mL of blood (Hurley, 2015, Ahola et al. 2017 Hare). In a review article published in 2015, Hurley, et al. reviewed metadata from four decades of evaluations on endotoxin levels in SIRS caused by Gram negative organisms and found that on average the levels of endotoxin present in septic patients was 100-fold higher than normal background levels of endotoxin.

The discovery that humans living and functioning normally could have endotoxin levels as high as 10 or more EU/mL of blood has led us into somewhat of a conundrum. If 350 EU/person is the parenteral threshold pyrogenic dose for the average 70kg person, how can people be walking around with between 50-5000 EU in circulation at any given time? Over the last decade it has been reported by a number of clinical researchers that many people have a sub-clinical, persistent, low-grade inflammation called "metabolic endotoxemia" resulting from higher than expected levels of circulating endotoxin. Metabolic endotoxemia is now thought to impact about 30-40% of residents of western countries (Harte et al. 2012). However, there has also been considerable discussion as to whether metabolic endotoxemia is real

and whether or not it is clinically relevant (Boutagy et al.., 2016). It is important to note that measurement discrepancies of endotoxin detection in blood due to interferences and sample preparation have made it difficult to standardize on a measurement protocol; which is to say from a diagnostic perspective it is not possible at this point in time to affirmatively state at what endotoxin level metabolic endotoxemia can be diagnosed with certainty (Awoyemi et al.), and how metabolic endotoxemia might (or might not) affect the threshold pyrogenic dose.

Hugon et al. (2013) quantified 10^{10} prokaryotes (bacteria) in the healthy human gut. They found that depending upon the subject, the percentage of prokaryotic cells that were Gram-negative bacteria varied between about 16-70%. This means that normal humans have on the order of 10^9 endotoxin producing bacterial cells in their gut at all times. Whittle, et al. (2019) describe the presence of Gram negative bacteria in the blood as part of the human circulating biome., Given the fact that nutritional material passes through the gut into the human blood it should not be surprising that some endotoxin travels the same pathway, and it is likely that the human gut is the main source of circulating endotoxins in humans. Fortunately, LPS is efficiently eliminated by the liver via Kupfer cells and high density lipoprotein (Yao, et al.., 2016).

The underlying causes associated with metabolic endotoxemia involve disease or behaviors that impact gut permeability. It is now known that significantly increased levels of endotoxin are commonly observed in patients who are clinically obese, suffer from chronic diseases such as diabetes or other stress factors. (de Punder and Pruimboom, 2015). Diabetes itself is known to be associated with chronic low-level inflammation. Patients who can be diagnosed as morbidly obese have extremely high levels of circulating endotoxin at all times. Patients with significant metabolic endotoxemia risk factors can have endotoxin levels on the order of 60 to 75 EU/mL with normal blood pressure and body mass indices (BMI) of roughly 25-30 (Pussinen et al. 2011). BMIs in the range of 25-30 are considered overweight rather than obese, which can mean that the numerical quantity of endotoxin circulating in at least 50% of the western population is much higher than previously imagined. In addition, correlations have been reported in spikes in endotoxin level as a result of excessively high caloric, high fat content meals, and alcohol consumption. However, these spikes do not necessarily result in metabolic endotoxemia.

What do these data suggest? First, that many healthy humans very often have a baseline circulating endotoxin level that far exceeds the current maximum allowable endotoxin dose of 5 EU/kg per human. That revelation is perhaps stunning, but it's a strong indication that our endotoxin limits for parenteral products are, in our opinion, about where they should be which is to say safe and very conservative. It also begs the question of possible differences in immunological reactivity between purified LPS that was used to determine the TPD in rabbits and humans (Griesman and Hornick, 1969; Hochstein, 1994), and "natural" endotoxins that may cross the gut and result in low level metabolic endotoxemia (Ahola 2017).

It is difficult to discern from the clinical literature the frequency with which clinical fever results from the injection of parenteral drugs, biologics or even vaccines. This is understandable given how commonly patients exhibit clinical fever. In some reports up to 30% of patients seeking health care professional assistance exhibit fever, which is generally reported only when an oral temperature exceeds 37.7°C or 100°F. Fevers below this temperature are not diagnostic of infection or disease and are within the range considered normal Nall, 2018). Complicating the issue further is that many drugs, vaccines and biologics may themselves induce transient low-level fever. Sometimes we see septicemia mentioned in pharmaceutical literature discussing endotoxin contamination in products. Realistically, given the current state of endotoxin control in the drug, device and biologics industries it can be easily calculated that the 5 EU/kg limit for general parenteral administration would be expected to have a very low risk of impact on a normal patient at all. Of course, most products contain endotoxin levels well below this limit.

These data raise the question of whether it makes sense to require endotoxin limits lower than the calculated value. One EU of endotoxin activity is about 0.2ng of endotoxin This is one billionth of a gram; we are currently releasing Water for Injection at levels below 0.25 x 10^{-9} gram of endotoxin per mL. It takes between 2000-50000 cells of Gram-negative bacterial to result in one endotoxin unit. Compare that to the baseline level of endotoxin for a normal human, not the 40% who are obese, or have diabetes. Any suggestion that endotoxins contributed by drugs, devices or biologics can produce immunological reactions with anything in common to those arising from SIRS must be viewed with a healthy dose of skepticism. For SIRS to have been caused by a biological, device or drug, that product would have been grossly contaminated somewhere along its manufacturing process with considerable numbers of viable pathogenic Gram-negatives and that lot of product would fail both the compendial sterility test and the BET. So, we believe that the use of the word septicemia, which is a profoundly serious condition, is an exaggeration of risk in any discussion of endotoxin contamination of parenteral products manufactured under GMP conditions.

Interestingly, a special case among injectable drugs is vaccines. The US Code of Federal Regulations (CFR) 610.13 states that "the test for pyrogenic substances is not required for the following products: Products containing formed blood elements; Cryoprecipitate; Plasma; Source Plasma; Normal Horse Serum; bacterial, viral and rickettsial vaccines and antigens; toxoids; allergenic extracts; venoms; diagnostic substances and trivalent organic arsenicals. Brito and Singh (2010) reported that many common vaccines against both viral and bacterial disease contain between 100 and 100,000 EU/mL. Diptheria/Tetanus/acellular Pertussis (Dtap or Tdap) had endotoxin levels of 0.288-1390 EU/dose. These vaccines are first administered to infants in a single bolus dose followed by booster vaccines at regularly scheduled intervals. An older study from NIH in 1978 found that vaccines commonly had about 10^3 "bacterial cell wall equivalents" per mL of the injectable preparation (Geier, et al.., 1978).

We can't leave the subject of inflammation without discussing Innate Immune Response Modulating Impurities (IIRMI), A review of the IIRMI literature traces the majority of activity in this field of study to United States Food and Drug Administration laboratories and recently it has been highlighted in publications and presentations as a regulatory concern for endotoxin testing (Haile, et al. 2015; Hughes 2015). The hypothesis is that therapeutic products can contain impurities, some of which are substances related to product, others of which are process or cell substrate related impurities. Although there are no specifications

proposed for these potential IIRMIs, It has been speculated that these impurities have the potential to activate innate immunity cells stimulating the expression of cytokines and leading to immunogenicity and hence inflammation. One of the substances which has been a focus of IIRMI concerns is endotoxin. Considering the levels of endotoxin present in normal human blood and the clinical data indicating that subjects with sub-clinical manifestations of inflammation can and often do have circulating endotoxin levels thousands of times higher than the potential worst case contributions that might be made by drugs, devices and biologics, we must question the relevance of endotoxin-generated "IIRMI" to public health and healthcare product safety.

Discussion

We find no reason to think that the methods currently used to control or monitor for endotoxins in drug, device and biological product manufacturing are insufficient or carry with them any inherent risk to patients. We also find that the current testing requirements *vis-a-vis* endotoxin target levels for various routes of administration are reasonable, prudent and conservative. We also suggest that the final product endotoxin test, by whatever analytical method it is conducted, is effectively a backstop or safety net test with the primary purpose of confirming that a validated cGMP process consistently produces the low, safe endotoxin levels mandated by the BET and in the regulatory approval of each individual product. This is consistent with the understanding that organizations cannot test quality into product, they must instead use the principles of sterility (or quality) by design, validation and where possible statistical assessment of process capability robustly build quality into the process.

We believe that if drugs, devices and biologicals were the source of clinical adverse effects we would certainly know it. If there were a relationship between the mode of action/administration of a drug or biological and clinical fever, it would be identified during clinical trial use before that product's final approval. In fact, some biological products are known to cause a febrile reaction (Doessegger and Banholzer, 2015) and a warning to this effect can be found in the product insert. We concede that in theory, the contamination of product with endotoxins could cause a low grade fever but for that to go unreported it would have been clinically insignificant or go unnoticed clinically which seems highly unlikely. Should a "rogue" lot of product go to market with clinically relevant levels of endotoxins that take fevers above the baseline, physicians are accountable for registering those adverse events. However, we find no evidence of lot-related or overt endotoxin contamination in the scientific literature, the adverse event literature, or the warning letter literature available on FDA's website that is due to an invalid BET assay (PDA, 2019). Thus, it appears that the widespread use of an effective test for bacterial endotoxin (LAL) in combination with the low incidence of bacterial contamination in modern products have combined to result products which are reliably low in bacterial endotoxin and therefore not a significant clinical risk for pyrogenicity.

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