Endotoxin Activity Assay

Subjects: Biology

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Endotoxin, also referred to as lipopolysaccharide (LPS), is a potent stimulator of the inflammatory cascade which may progress to sepsis and septic shock. The term endotoxic septic shock has been used for patients who have a clinical phenotype that is characterized by high endotoxin activity in addition to a high burden of organ failure; especially a pattern of organ failure including hepatic dysfunction, acute kidney injury, and various forms of endothelial dysfunction. Endotoxic septic shock has been a target for drug therapy for decades with no success. A likely barrier to their success was the inability to quantify endotoxin in the bloodstream. The Endotoxin Activity Assay (EAA) is positioned to change this landscape. In addition, medical devices using adsorptive technology in an extra-corporeal circulation has been shown to remove large quantities of endotoxin from the bloodstream.

Keywords: endotoxin ; sepsis ; endotoxic septic shock ; diagnosis of endotoxemia

1. Introduction

"*Endotoxins possess an intrinsic fascination that is nothing less than fabulous. They seem to have been endowed by nature with virtues and vices in the exact and glamorous proportions needed to render them irresistible to any investigator who comes to know them*".

Dr. Ivan Loveridge Bennett Jr. 1964

Septic shock is a devastating disease with mortality that can exceed 50%. Even recent randomized trials of patients with septic shock in the US [1] and UK [2] found that mortality at 90 days was about 30%. Based on data using the Endotoxin Activity Assay (EAA), Spectral Medical Inc., Toronto, Canada), up to 80% of patients with septic shock have elevated levels of endotoxin, while at least 30% have very high levels, that is, >0.60 EA units [3] and experience mortality at roughly twice the rate of patients with septic shock without high levels of endotoxin [4]. One of the clinical challenges preventing the emergence of successful anti-endotoxin strategies has been to adequately identify patients with endotoxemia [5]. Elevated levels of endotoxin can occur in the setting of culture documented Gram-negative infection, from any source. It can also occur in the setting of Gram-positive infection, fungal infection, or in other cases of septic shock where no microbiologic source is identified as a result of translocation of endotoxin across the gut mucosal barrier in the setting of shock, hypoxemia, and gut hypoperfusion [3][6][7][8]. While it might seem logical to include patients with high suspicion of endotoxemia in clinical studies, lack of clinical diagnostic accuracy has contributed to the inability to prove overall efficacy of the treatment strategy proposed. The EAA is now being recognized as a necessary test for selecting appropriate patients that may respond to therapy [9].

2. Patient Identification for Anti-Endotoxin Therapy: Past and Future

Endotoxin, a lipopolysaccharide component of the cell wall of Gram-negative bacteria, has been a target therapy for sepsis for decades. However, despite promising preclinical data, past efforts targeting endotoxin as a treatment have failed, including human monoclonal IgM antibody against endotoxin, HA-1A [10], as well as its murine equivalent IgM, E5 antibody [11]. Furthermore, various endotoxin neutralizing proteins including bactericidal permeability increasing protein (BPI), analogues of Lipid A (E5564), and detoxifying agents such as alkaline phosphatase have failed [12][13][14]. Although intrinsic problems with these agents themselves may have contributed to their failure, no trial to date has tested an anti-endotoxin drug specifically in patients with proven endotoxemia. Studying a therapy in patients where only the minority can benefit requires large sample sizes and unrealistic effect sizes. For example, if only 30% of patients with septic shock have endotoxic septic shock, a trial of 2000 patients designed to detect a mortality difference would need to achieve at least a 24% absolute risk reduction for patients with endotoxic septic shock and have no adverse effect on mortality for the remaining patients. By contrast, a trial half this size would only require an 11% absolute risk reduction if it enrolled only patients with endotoxic septic shock. Failure to enroll the right patients may have obscured a survival benefit in prior trials.

A current common theme in sepsis research is that better diagnostics are needed. This is because the complexity and heterogeneity of sepsis and the need to better target interventions to the right patient subset, at the right time, at an optimal dose and for an optimal duration, requires better diagnostics to first identify suitable patients to enable timely intervention where an intervention can impact outcome. Whereas the incidence of sepsis in the US is 1.5 million, with 1 in 5 developing septic shock [15], data from screening and enrolment into the Evaluating Use of Polymyxin B Hemoperfusion in a Randomized controlled trial of Adults Treated for Endotoxemia and Shock (EUPHRATES) trial [16] supports the segregated target sub-population with endotoxic septic shock as roughly half of this, or about 150,000 cases per year.

Measuring endotoxin in whole blood or plasma is extremely difficult. Endotoxin is the major component of the cell wall of Gram-negative bacteria, comprising roughly 75% of the surface of the outer leaflet of the outer membrane of the cell wall. It is released into the circulation upon disruption of intact bacteria (death, cell lysis) infecting blood or tissues. It can also enter via translocating from the gastrointestinal tract when barrier function is compromised by hypoperfusion, inflammation, dysregulation of commensal flora, or sepsis from any source. Endotoxin is a glycolipid; it consists of a hydrophobic lipid part, called lipid A, which is anchored in the outer leaflet, and a hydrophilic polysaccharide part, which extends outside the cell. The polysaccharide part is composed of two domains: the core oligosaccharide and the O antigen. The O antigen (also called the O-chain) is a polysaccharide which is composed of several oligosaccharide units and is bound to lipid A through the core region. Lipid A deserves particular attention, as this part of the endotoxin molecule is sensed by the host and is responsible for activating the immune system [17].

Endotoxin that enters the bloodstream is rapidly sequestered by lipoproteins, mainly high-density lipoproteins (HDL) in cooperation with the phospholipid transfer protein (PLTP). Lipoproteins transport endotoxin to the liver, where it is inactivated by the enzymes acyloxy acyl hydrolase and alkaline phosphatase and excreted in the bile [18].

The most commonly used method for detecting endotoxin is the Limulus amebocyte lysate (LAL) assay. It is routinely used in the pharmaceutical industry to detect endotoxin contamination in crystalloid solutions and pharmacological preparations such as vaccines. However, LAL is not practical for use in whole blood and despite alterations to the LAL testing method to improve the test characteristics for blood, no LAL assay is available for clinical use [19][20]. There are numerous reasons that contribute to the difficulty in developing a blood test for endotoxemia. LPS binds to a number of plasma proteins, including lipopolysaccharide binding protein (LBP), high density lipoprotein (HDL), and to cellular blood components such as platelets. In addition, the molecular weight of endotoxin from different strains of Gram-negative bacteria can vary by 100-fold, making quantitative measures challenging. Furthermore, despite the availability of a detection IgM antibody, such as used in the EAA, development of an appropriate antibody immobilization strategy, such as may be used in an enzyme linked immunosorbent assay (ELISA) format, is a critical requirement in analytical performance, and to date has been elusive.

Finally, there is heterogeneity among patients with sepsis not only with respect to endotoxin activity but also in the extent to which endotoxin produces disease [21][22]. Although humans are among the most sensitive species to endotoxin [23] they also possess a host of defenses [24]. In human volunteers, there is a characteristic hyper-inflammatory response such that upon endotoxin administration, there is a distinct, dose-dependent, and highly reproducible increase in plasma cytokine levels, tumor necrosis factor (TNF), and Interleukin-6 (IL-6) as well as the anti-inflammatory cytokine Interleukin-10 (IL-10). A major response is seen in the cardiovascular system with a decrease in mean arterial pressure and increased heart rate. While most patients with endotoxic septic shock are hyperdynamic with increased cardiac index, some patients also exhibit left ventricular dysfunction [25]. Removal of endotoxin therefore can have a dramatic effect on hemodynamics with improved mean arterial pressure and reduced requirement for vasopressors.

In patients with septic shock, as endotoxin levels rise, immune defenses become overwhelmed but the threshold between survivable and lethal endotoxemia can be influenced by age and underlying comorbidity as well as genetic factors, particularly variation in genes regulating complement [26]. Manifestations of endotoxic septic shock such as coagulopathy and hyperlactatemia may help identify patients with lethal endotoxemia [27]. A sequential organ failure assessment (SOFA) score > 7 appears to delineate a high risk of death in patients considered for anti-endotoxin therapy [28][29].

3. The Endotoxin Activity Assay (EAA)

EAA is a homogeneous assay using an IgM antibody with a high affinity for the single epitope of Lipid A that then triggers the formation of an immunological complex including complement [30]. In a whole blood sample, endotoxin combines the IgM and the complex primes the complement receptors on the patient's neutrophils to generate a release of oxyradicals. The test platform is chemiluminescence such that each oxyradical generated a photon of light produced by reacting with luminol, which were then counted in a luminometer.

The EAA is composed of 3 tubes, where Tube 1 is a “blank” and provides information on the baseline chemiluminescence in the whole blood of the individual patient sample. Tube 3 is a 1-point maximum calibrator that represents maximal stimulation with an excess of antibody. Tube 2 is the “sample” containing a fixed concentration of antibody. The EAA result is expressed as a relative value between the sample minus the blank and the maximum calibrator minus the blank [Tube 2 − Tube 1/Tube 3 − Tube 1]. The EAA level is based on a scale of 0 to 1, where 0 = no light emission and therefore no endotoxin activity and 1 represents 100% maximum activity which is based on the concentration of antibody and a fixed concentration of *E. coli* 011B5. EAA results 0–0.39 indicates low endotoxin activity, 0.4–0.59 are intermediate levels, and EAA > 0.6 are high levels.

Given the format of the assay, it is helpful to show the interpretation of the unit-less EAA against a representative dose response curve to approximate an EAA level with a concentration of endotoxin in picogram/mL. An EAA result of 0.6 is approximately 2000 pg/mL and EAA 0.9 is approximately 4000 pg/mL. For EAA levels above 0.9 it is impossible to equate a measurable estimate, but it is >>4000 pg/mL which is a large, often lethal concentration of endotoxin [31]. Therefore, EAA levels in the range of 0.6 to 0.9 is the optimal interval when selecting a patient for treatment with the PMX cartridge.

The clinical utility of the EAA was first established in the Multicenter endotoxin detection in critical illness (MEDIC) trial [3]. Marshall and colleagues performed a multi-center multi-national study of 856 patients upon admission to the intensive care unit (ICU). The results of this research showed that endotoxin activity was significantly correlated with risk of developing sepsis, or septic shock as well as mortality. More recently, Adamik and colleagues found that among patients with septic shock, those with high endotoxin activity experience mortality of approximately 60% while patients with septic shock but without high levels of endotoxin have only a 30% mortality [4]. Thus, using the EAA together with clinical assessment, a patient population with endotoxic septic shock can be identified as an appropriate population for an anti-endotoxin therapy.

Furthermore, direct endotoxin removal strategies that have been tested clinically, such as the neutralizing protein BPI and analogues of Lipid A such as E5564, and others may resurface with the ability to properly select for patients with endotoxemia using the EAA.

While endotoxin has both direct cytotoxic effects as well as effects mediated by the immune system (and complement) an approach of treatment directed at endotoxin may have greater effect in combination with the downstream effects on the immune response. To date, targeting cytokines TNF and IL-1 for example, have not shown a mortality benefit alone although they may have a role when added to treatments directed at endotoxin.

References

1. ProCESS Investigators; Yealy, D.M.; Kellum, J.A.; Huang, D.T.; Barnato, A.E.; Weissfeld, L.A.; Pike, F.; Terndrup, T.; Wang, H.E.; Hou, P.C.; et al. A randomized trial of protocol-based care for early septic shock. N. Engl. J. Med. 2014, 370, 1683–1693.
2. Mouncey, P.R.; Osborn, T.M.; Power, G.S.; Harrison, D.A.; Sadique, M.Z.; Grieve, R.D.; Jahan, R.; Tan, J.C.; Harvey, S.; Bell, D.; et al. Protocolised Management in Sepsis (ProMISe): A multicentre randomised controlled trial of the clinical effectiveness and cost-effectiveness of early, goal-directed, protocolised resuscitation for emerging septic shock. Health Technol. Assess. 2015, 19, i-150.
3. Marshall, J.C.; Foster, D.; Vincent, J.L.; Cook, D.J.; Cohen, J.; Dellinger, R.P.; Opal, S.; Abraham, E.; Brett, S.J.; Smith, T.; et al. Diagnostic and prognostic implications of endotoxemia in critical illness: Results of the MEDIC study. J. Infect. Dis. 2004, 190, 527–534.
4. Adamik, B.; Smiechowicz, J.; Kübler, A. The importance of early detection of endotoxemia. Innate Immun. 2016, 22, 503–509.
5. Marshall, J.C. Endotoxin in the pathogenesis of sepsis. In Endotoxemia and Endotoxin Shock: Disease, Diagnosis and Therapy; Contributions to Nephrolog; Karger Publishers: Basel, Switzerland, 2010; Volume 167, pp. 1–13.
6. Danner, R.L.; Elin, R.J.; Hosseini, J.M.; Wesley, R.A.; Reilly, J.M.; Parillo, J.E. Endotoxemia in human septic shock. Chest 1991, 99, 169–175.
7. Opal, S.M.; Glück, T. Endotoxin as a drug target. Crit. Care Med. 2003, 31 (Suppl. S1), S57–S64.
8. Clark, J.A.; Coopersmith, C.M. Intestinal crosstalk: A new paradigm for understanding the gut as the “motor” of critical illness. Shock 2007, 28, 384–393.

9. Romaschin, A.D.; Klein, D.J.; Marshall, J.C. Bench-to-bedside review: Clinical experience with the endotoxin activity assay. Crit. Care 2012, 16, 248.
10. Ziegler, E.J.; Fisher, C.J.; Sprung, C.L.; Straube, R.C.; Sadoff, J.C.; Foulke, G.E.; Wortel, C.H.; Fink, M.P.; Dellinger, R.P.; Teng, N.N.; et al. Treatment of gram-negative bacteremia and septic shock with HA-1A human monoclonal antibody against endotoxin. A randomized, double-blind, placebo-controlled trial. The HA-1A Sepsis Study Group. N. Engl. J. Med. 1991, 324, 429–436.
11. Angus, D.C.; Birmingham, M.C.; Balk, R.A.; Scannon, P.J.; Collins, D.; Kruse, J.A.; Graham, D.R.; Dedhia, H.V.; Homann, S.; MacIntyre, N. E5 murine monoclonal antiendotoxin antibody in gram-negative sepsis: A randomized controlled trial. E5 Study Investigators. JAMA 2000, 283, 1723–1730.
12. Giroir, B.P.; Scannon, P.J.; Levin, M. Bactericidal/permeability-increasing protein—Lessons learned from the phase III, randomized, clinical trial of rBPI21 for adjunctive treatment of children with severe meningococcemia. Crit. Care Med. 2001, 29 (Suppl. S7), S130–S135.
13. Opal, S.M.; Laterre, P.F.; Francois, B.; LaRosa, S.P.; Angus, D.C.; Mira, J.P.; Wittebole, X.; Dugernier, T.; Perrotin, D.; Tidswell, M.; et al. Effect of eritoran, an antagonist of MD2-TLR4, on mortality in patients with severe sepsis: The ACCESS randomized trial. JAMA 2013, 309, 1154–1162.
14. Pickkers, P.; Mehta, R.L.; Murray, P.T.; Joannidis, M.; Molitoris, B.A.; Kellum, J.A.; Bachler, M.; Hoste, E.A.J.; Hoiting, O.; Krell, K.; et al. Effect of Human Recombinant Alkaline Phosphatase on 7-Day Creatinine Clearance in Patients with Sepsis-Associated Acute Kidney Injury: A Randomized Clinical Trial. JAMA 2018, 320, 1998–2009.
15. Paoli, C.J.; Reynolds, M.A.; Sinha, M.; Gitlin, M.; Crouser, E. Epidemiology and Costs of Sepsis in the United States-An Analysis Based on Timing of Diagnosis and Severity Level. Crit. Care Med. 2018, 46, 1889–1897.
16. Dellinger, R.P.; Bagshaw, S.M.; Antonelli, M.; Foster, D.M.; Klein, D.J.; Marshall, J.C.; Palevsky, P.M.; Weisberg, L.S.; Schorr, C.A.; Trzeciak, S.; et al. EUPHRATES Trial Investigators. Effect of Targeted Polymyxin B Hemoperfusion on 28-Day Mortality in Patients with Septic Shock and Elevated Endotoxin Level: The EUPHRATES Randomized Clinical Trial. JAMA 2018, 320, 1455–1463.
17. Beutler, B.; Rietschel, E.T. Innate immune sensing and its roots: The story of endotoxin. Nat. Rev. Immunol. 2003, 3, 169–176.
18. Steimle, A.; Autenrieth, I.B.; Frick, J.S. Structure and function: Lipid A modifications in commensals and pathogens. Int. J. Med. Microbiol. 2016, 306, 290–301.
19. DuBose, D.A.; Lemaire, M.; Basamania, K.; Rowlands, J. Comparison of plasma extraction techniques in preparation of samples for endotoxin testing by the Limulus amoebocyte lysate test. J. Clin. Microbiol. 1980, 11, 68–72.
20. De Cal, M.; Virzi, G.M. Endotoxin Measurement in Septic Shock. In Endotoxin Induced-Shock: A Multidisciplinary Approach in Critical Care; De Rosa, S., Villa, G., Eds.; Springer Nature: Cham, Switzerland, 2023; Chapter 5; pp. 43–48.
21. Leligdowicz, A.; Matthay, M.A. Heterogeneity in sepsis: New biological evidence with clinical applications. Crit. Care 2019, 23, 80.
22. Cavaillon, J.M.; Singer, M.; Skirecki, T. Sepsis therapies: Learning from 30 years of failure of translational research to propose new leads. EMBO Mol. Med. 2020, 12, e10128.
23. Brinkworth, J.F.; Valizadegan, N. Sepsis and the evolution of human increased sensitivity to lipopolysaccharide. Evol. Anthropol. 2021, 30, 141–157.
24. Pool, R.; Gomez, H.; Kellum, J.A. Mechanisms of Organ Dysfunction in Sepsis. Crit. Care Clin. 2018, 34, 63–80.
25. Charalambous, B.M.; Stephens, R.C.; Feavers, I.M.; Montgomery, H.E. Role of bacterial endotoxin in chronic heart failure: The gut of the matter. Shock 2007, 28, 15–23.
26. Kellum, J.A.; Formeck, C.L.; Kernan, K.F.; Gómez, H.; Carcillo, J.A. Subtypes and Mimics of Sepsis. Crit. Care Clin. 2022, 38, 195–211.
27. Osawa, I.; Goto, T.; Kudo, D.; Hayakawa, M.; Yamakawa, K.; Kushimoto, S.; Foster, D.M.; Kellum, J.A.; Doi, K. Targeted therapy using polymyxin B hemadsorption in patients with sepsis: A post-hoc analysis of the JSEPTIC-DIC study and the EUPHRATES trial. Crit. Care 2023, 27, 245.
28. Fujimori, K.; Tarasawa, K.; Fushimi, K. Effectiveness of polymyxin B hemoperfusion for sepsis depends on the baseline SOFA score: A nationwide observational study. Ann. Intensive Care 2021, 11, 141.
29. Garcia-Ramos, S.; Caamaño, E.; Rodríguez Benítez, P.; Benito, P.; Calvo, A.; Ramos, S.; Power, M.; Garutti, I.; Piñeiro, P. Mortality Risk Prediction in Abdominal Septic Shock Treated with Polymyxin-B Hemoperfusion: A Retrospective Cohort Study. J. Pers. Med. 2023, 13, 1023.

30. Romaschin, A.D.; Harris, D.M.; Ribeiro, M.B.; Paice, J.; Foster, D.M.; Walker, P.M.; Marshall, J.C. A rapid assay of endotoxin in whole blood using autologous neutrophil dependent chemiluminescence. J. Immunol. Methods 1998, 212, 169–185.
31. Romaschin, A.D.; Obiezu-Forster, C.V.; Shoji, H.; Klein, D.J. Novel Insights into the Direct Removal of Endotoxin by Polymyxin B Hemoperfusion. Blood Purif. 2017, 44, 193–197.