METHODS IN MOLECULAR MEDICINE™

Septic Shock

Methods and Protocols

Edited by

Thomas J. Evans

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Cover illustration: Human renal proximal tubular cells following pro-inflammatory ctyokine injury. The cells have been stained for the epithelial marker cytokeratin (green) with a DAPI nuclear (blue) counter-stain. They have a highly motile phenotype and show extensive filipodia formation. (P. A. Glynne, unpublished observation. *See* Chapter 18.)

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Preface

Septic shock remains a serious medical condition with high mortality. Despite many advances in intensive care medicine and antibiotic development, this has not changed appreciably in the last 20 years. Frustratingly, over the same period of time, enormous advances have been made in understanding the underlying pathogenic mechanisms of this condition. This has resulted in the development of several novel therapies for septic shock, which, despite excellent theoretical grounds for their efficacy, have failed in altering mortality attributable to sepsis.

The reasons for these failures are multiple, but it is clear that further research is required aimed at increasing our understanding of the basic pathophysiological processes that occur following infection. Research into septic shock draws upon a number of different disciplines, ranging from molecular and cellular biology to physiological measurements on whole animals. *Septic Shock Methods and Protocols* is an attempt to draw together into one volume a number of protocols that are of use in the investigation of the mechanisms of septic shock. I have divided the book into five sections. The first deals with endotoxin, the lipopolysaccharide component of the Gram-negative cell membrane that can mimic many of the features of septic shock. Gram-positive organisms are found increasingly as causes of septic shock, and several aspects of toxins produced from these bacteria are considered in the second section. Cytokines have been a central focus of interest in sepsis research for many years and several aspects of cytokine biology are highlighted in the third section. In the fourth section, methods for studying nitric oxide and other reactive nitrogen intermediates are considered. Finally, the last section describes a variety of methods for studying primary cell cultures, an essential component of developing in vitro methods to study septic shock.

Obviously *Septic Shock Methods and Protocols* cannot provide an exhaustive account of every protocol that might be used in sepsis research. I have therefore carefully chosen those highlights that I believe either are of great intrinsic importance or are poorly covered elsewhere in published protocol manuals. I am very grateful to all the authors who have contributed to this

volume. I believe their collected experience is invaluable and I hope that this book will allow both newcomers and those with more experience to apply successfully the techniques needed for their research.

T. J. Evans

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ENDOTOXIN

1

Assay of Endotoxin by *Limulus* Amebocyte Lysate

Paul A. Ketchum and Thomas J. Novitsky

1. Introduction

Horseshoe crabs fight off infectious agents with a complex array of proteins present in amebocytes, the major cell type in their hemolymph. These amebocytes contain both large and small granules *(1)*. When exposed to bacteria or other infectious agents the amebocytes release proteins into their surroundings by exocytosis. The small granules of *Limulus* amebocytes contain antibacterial proteins, including polyphemusins and the big defensins *(2)*. The large granules contain the *Limulus* anti-lipopolysaccharide factor (LALF) and the clot-forming group of serine protease zymogens. Exocytosis is initiated by the reaction of amebocytes with lipopolysaccharide (LPS) from Gram-negative bacteria or other microbial components. LPS is also called endotoxin because it is found in the outer membrane of the gram-negative bacterial cell wall. A solid clot forms in response to the lipid A portion of LPS, thereby walling off the infection site or preventing the loss of blood when the animal is damaged physically *(3)*.

The clot-forming cascade of serine proteases is the basis for the *Limulus* amebocyte lysate (LAL) assay for endotoxin (**Fig. 1**). Factor C is activated autocatalytically by LPS, which in turn activates factor B, which then activates the proclotting enzyme *(4)*. The activated clotting enzyme cleaves coagulogen to coagulin, which forms the firm clot. Clot formation was the basis for the first LAL assay for endotoxin *(5)*. The LAL assay has replaced other tests (e.g., the rabbit pyrogen test) in part because the LAL cascade amplifies the initial signal (LPS) greatly, permitting the detection of picogram quantities of LPS. Clot formation can also be initiated by (1→3)-β-D-glucan (**Fig. 1**) from fungal cell wall, (*see* **Note 7; refs. *6,7***).

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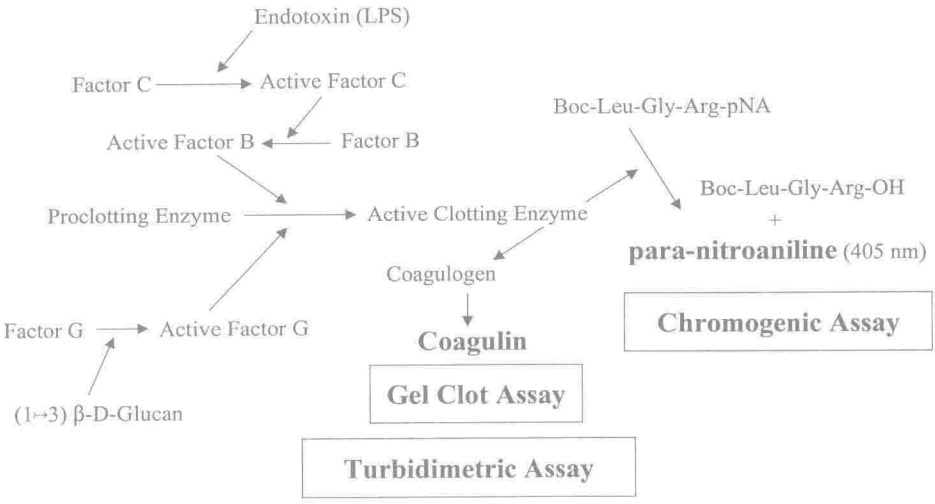

Fig. 1. The *Limulus* blood clotting cascade.

1.1. LAL Method for Measuring Endotoxin

Since the LAL gel-clot assay was first approved in 1977 by the Food and Drug Administration (FDA) for detecting endotoxin, manufacturers have developed two additional LAL methods. The turbidimetric LAL method is an adaptation of the gel-clot to instrumental analysis *(8)*. Turbidity is monitored as an increase in light scattering caused by clot initiation. The LAL reagent is specially formulated to be incubated in a test-tube reader such as the LAL-5000 *(9)*, which measures the turbidity of each tube with time. The computer-based software determines the time for the reaction to reach a predetermined onset optical density (OD). The log of the onset time is linearly related to the log of the endotoxin concentration *(9)*.

The chromogenic LAL method utilizes a peptide substrate that turns yellow when hydrolyzed by the proclotting enzyme *(10)*. One example is the peptide substrate Boc-Leu-Gly-Arg-*p*-nitroanilide shown in **Fig. 1**. Activated proclotting enzyme cleaves the chromophore from the arginine, releasing the yellow-colored *p*-nitroaniline (*p*NA). In the normal end-point assay, the amount of pNA released is determined after a prescribed 37°C incubation by reading the OD or absorbance at 405 nm. This reagent can also be used in a kinetic assay where the time required to attain an onset at OD_{405} (usually 0.03–0.1) is related to the endotoxin concentration.

The LAL assay for blood endotoxin is composed of three basic parts: sample collection and handling; extraction of the blood/serum sample; and testing the extracted sample with the chromogenic LAL assay. Both the chromogenic

end-point assay and the turbidimetric assays are used to detect endotoxin in body fluids; however, here we describe the chromogenic method. For a recent review of the literature see Novitsky *(11)*.

1.2. Interfering Substances in Blood

Animal blood contains soluble enzymes, antibodies, LPS binding proteins, and HDL that interfere with the detection of endotoxin by LAL assays. The serine proteases present in blood can act on the chromogenic substrate in the absence of the LAL reagent and must be inactivated. Moreover, humans possess sophisticated mechanisms for binding, transporting, and eventually processing LPS to remove it from the circulation. LPS binding protein, cationic antibacterial proteins, and bacterial permeability-increasing protein are examples of serum proteins that bind LPS and interfere with endotoxin measurement. The degree of interference varies among patient sera as demonstrated by Warren et al. *(12)* through studies on the plasma samples from blood donors. Some individuals also have high concentrations of serum antibodies against endotoxin *(13)* capable of neutralizing its biological effects. Two methods are available to deal with serum-protein interference: the heat dilution method *(14,15)*, and the acid treatment described in **Subheading 3.2.2.** *(16)*.

Certain blood samples have a yellow color whose absorbance interferes with measuring *p*NA at 405 nm. This interference is avoided by diazo-coupling the *p*-NA, thus forming a purple complex that absorbs at 540–550 nm with a threefold higher extinction coefficient than *p*NA. The diazo-coupling method is useful in the chromogenic endpoint LAL assay *(17)*.

2. Materials

2.1. Equipment Required

1. The end-point chromogenic LAL method requires a microplate reader with a 545-nm filter for measuring diazo-coupled *p*NA and a 640-nm filter for eliminating background interference. The plate reader is connected to a computer with a software package suitable for analyzing the results of the LAL assay.
2. Incubating the plate at 37°C requires either a temperature-controlled microplate reader or a microplate block incubator. Either a water bath or a heating block at 37°C is used during the blood-extraction procedure.
3. A clinical centrifuge capable of 1300–1500*g* is used to prepare blood plasma and perform the blood-extraction protocol.

2.2. Laboratory Reagents and Materials

1. All materials used directly in the assay must be essentially free of endotoxin.
   a. LAL reagent-grade water (LRW), glass pipets (Fisher, Pittsburgh, PA), certified microtiter plates, Rainin pipet tips, and Eppendorf combitips are recommended.

b. Depyrogenated blood extraction tubes (10 × 75-mm) and any other glassware is wrapped in aluminum foil and baked at 240°C for at least 4 h.
c. Purple-top ethylenediaminetetraacetic acid (EDTA) Vacutainer (Fisher) tubes are used for blood collection. Heparin Vacutainer tubes certified to be endotoxin-free may be substituted.
d. The gloves worn during blood handling and performing the assay must be powder-free, because the powder contains endotoxin and can contaminate the assay.

2. The chromogenic LAL reagent kit with endotoxin standard and diazotization reagents is available from Associates of Cape Cod (Falmouth, MA). This kit contains Pyrochrome LAL, Pyrochrome Buffer, endotoxin standard and the diazo-coupling reagents. Other manufactures supply the chromogenic LAL reagent suitable for the assay and an endotoxin standard. If not purchased, the diazotization reagents are made according to information in **Table 1**.
3. The blood-extraction reagents are 0.5% Triton X-100 prepared in LRW, 1.32 *N* HNO_3 diluted from concentrated HNO_3 in LRW, and 0.55 *N* NaOH prepared by dissolving solid NaOH in LRW. These reagents are stable at room temperature.

2.3. LAL Product Insert

1. The product insert provided with each lot of LAL reagent contains valuable information on how to reconstitute the LAL reagent, storage of the reconstituted LAL, testing methods, volumes of reagent to use, sensitivity of the reagent, and recommended endotoxin standards. Because LAL is a biological product, the conditions of storage and stability of the reconstituted reagent are critical to success.
2. LAL reagents are licensed by the FDA and other regulatory bodies for detection of endotoxin in pharmaceutical preparations. They are not licensed for the detection of endotoxin in blood and other body fluids. When used for this purpose, the results are for research use only.

2.4. Endotoxin Standard

1. The reference standard endotoxin (RSE) is made from *Escherichia coli* 0113 and known as EC-6. Other endotoxin standards are related to RSE and their potency documented in a certificate of analysis (Control Standard Endotoxin; CSE).
2. The quantity of endotoxin is recorded as an endotoxin unit (EU): one EU is equivalent to 100 pg of RSE. Endotoxin is routinely reported as EU/mL.
3. The endpoint assay with diazo-coupling is sensitive over the range of 0.25–0.015 EU/ml. Reconstituted endotoxin standards are stable for >1 wk at 4–8°C.
4. Because endotoxin forms micelles and binds to glass surfaces, solutions of reconstituted endotoxin are vortexed for 5 min or longer. Each dilution made in a test tube should be vortexed for 0.5–1.0 min before use or further dilution. Endotoxin standards are usually diluted in LRW or in diluted (1/10) pyrogen-free

Table 1
Reagents for the Chromogenic LAL Assay

	Composition	Storage
Blood Extraction Reagents		
Nitric acid	1.32 *N*	Room temperature
Triton X-100	0.5% (v/v)	Room temperature
NaOH	0.55 *N*	Room temperature
Pyrochrome Reagents		
Pyrochrome (lyophilized)	Reconstitute with 3.2 mL buffer	4–8°C
Pyrochrome reconstitution buffer	0.2 *M* Tris HCl pH 8.0 (23°C)	Room temperature
Endotoxin		
Endotoxin standard (lyophilized)	Make 0.25 EU/mL	4–8°C
LAL reagent water		Room temperature
Diazo-coupling reagents		
Sodium nitrite (lyophilized)	0.417 mg/mL in 0.48 *N* HCl (below)	Room temperature
Hydrochloric acid	0.48 *N*	Room temperature
Ammonium sulfamate (lyophilized)	3 mg/mL	Room temperature
n[1-naphthyl]-ethylenediamine dihydrochloride (NEDA)	0.7 mg/mL LRW	Room temperature

plasma. Dilutions can be performed in pyrogen-free test tubes or in the microtiter plate.

3. Methods

3.1. Sample Collection and Handling

1. Blood samples can be drawn from lines or fresh sticks into the Vacutainer purple-top tube *(18)*. The sample is placed in ice and transported to the laboratory for making plasma (*see* **Note 1**).
2. When present, blood endotoxin levels in sepsis patients tend to remain elevated over a period of days *(18)*. Unless one is looking for a specific event, timing of blood collection within the 12 h following onset of sepsis is not a critical factor in detecting blood endotoxin.
3. Plasma samples can be subdivided and tested before freezing, or stored at –80°C for months before doing the assay (*see* **Note 2**). Storage at –80°C and transportation on dry ice is advised.

3.2. Protocol for the Chromogenic LAL Method for Endotoxin Detection

3.2.1. Setting Up the LAL Assay

Set up the LAL assay in a biosafety cabinet or laminar flow hood. If this is not possible, the technician should take special precautions to ensure that the work space is free of dust and the reagents and materials do not become contaminated. Perform the assay in an isolated area with restricted traffic and minimal interference. *Do not* lean over the microplate when adding samples to the wells. Keep the microplate lid closed unless adding samples or performing dilutions. Always use aseptic techniques when pipeting.

3.2.2. Preparing the Blood Sample

Wear nonpowdered gloves and observe the safety regulations for blood handling as directed by your institution. These instructions apply for each blood sample.

1. Place two sterile endotoxin-free 10 × 75-mm test tubes on ice and label them "A" for acidification and "B" for neutralization.
2. Add 200 µL of nitric acid and 200 µL of Triton X-100 to the "A" tube (use within 30 min, do not store mixture).
3. With a separate pipet tip, add 200 µL of sodium hydroxide to the "B" tube.
4. Thaw frozen samples at room temperature, then vortex them for 1 min prior to transferring 100 µL of blood to tube "A." Cover the tube with the nonexposed side of parafilm and vortex for 30 s.
5. Immediately incubate tube "A" at 37°C for 5 min.
6. Again vortex tube "A" for 30 s and centrifuge at 1300–1500*g* for 5 min. Remove tube "A" and place on ice.
7. Using an endotoxin-free pipet tip, transfer 200 µL of the supernatant fluid from tube "A" to tube "B" (containing sodium hydroxide).
8. Vortex tube "B" for 5 s then store on ice until assayed. This represents a 1/10 dilution of the blood sample.

3.2.3. Setting up the Microplate

Before thawing the samples, turn on the plate-heating block or the plate-heating reader, and prepare the tube heater.

1. Set up the OD plate reader as follows:

Temperature	37°C
Automix	Off
Wavelength	540 or 550 nm
Background wavelength	630 nm
Calibration	on