

25. Evaluation of endotoxin–biocide interaction by the *Limulus* amoebocyte lysate assay

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SUMMARY

Previous reports of neutralization of endotoxin pyrogenicity and systemic shock by a formaldehyde condensate biocide suggested that the interaction might be a generalized one related to formaldehyde activity. This study utilized *Limulus* amoebocyte lysate (LAL) as the indicator system for endotoxin activity. Purified *Escherichia coli* endotoxin was mixed with several levels of commercially available industrial biocides at doses comparable to those in actual use. Of those tested, compounds with known available aldehyde neutralized LAL endotoxin effect (i.e., no gelling) while those with no aldehyde had no effect.

INTRODUCTION

Endotoxins, the lipopolysaccharide portion of the outer membrane of gram-negative bacteria, are capable of eliciting a large number of pathophysiological effects in their hosts including decreased spirometry, pulmonary inflammation, fever, septic shock, and a range of hyper-immune responses. Considering the ubiquitous nature of gram-negative bacteria, the detection and neutralization of endotoxins are of significance.

In 1964, Levin and Bang described the ability of endotoxins to coagulate the lysate of amoebocytes of the horse shoe crab, *Limulus polyphemus* [8, 9]. Since that time, the *Limulus* amoebocyte lysate (LAL) assay has become the 'method of choice' in detecting the presence of endotoxins [10]. Although there is no certainty that the LAL assay measures the same aspects as the rabbit pyrogen bioassay (RPB), the official test of endotoxin screening, excellent correlation has been shown between these

two assays [5]. The U.S. Food and Drug Administration has established guidelines for the LAL assay to be used as an alternative method for the RPB [11]. This is of considerable advantage since the LAL assay is simpler, more rapid, reproducible, and economical as a method of endotoxin screening.

In a series of studies by Brown et al. [1–3], two substances – noxythiolin (oxymethylene methylthiourea) and taurolin (*bis*-[1,1-dioxo-perhydro-1,2,4-thiadiazinyl-4] methane) – have been effective in the treatment of bacterial peritonitis. The chemotherapeutic effect of these substances has been attributed to their ability to undergo slow decomposition, releasing free formaldehyde.

Pfirrmann and Leslie [12] investigated changes in the mean rectal temperature of rabbits injected with endotoxin and determined that the pyrexic and lethal effects of endotoxins can be dramatically reduced if administered with taurolin. In the same article, the mitigation of endotoxin lethality in mice

was evaluated; the survival times of the mice were significantly prolonged and the number of mortalities decreased.

The role of microbial contamination as a potential occupational biohazard is presently receiving much attention [4, 6, 7]. However, progress is urgently needed in establishing methods for the inactivation of hazardous biological agents. In this study, we evaluated the application and significance of the LAL assay in the determination of endotoxin neutralization by EPA-registered biocides, particularly formaldehyde-based biocides.

MATERIALS AND METHODS

Utmost care must be used when performing the LAL assay since the test is very sensitive and contamination by gram-negative bacteria in any step may lead to erroneous results. Strict adherence to the sequence of mixing the reactants (endotoxin/biocide/LAL test system) and to incubation conditions is extremely important.

Glassware

All glassware used in this study was depyrogenated by baking at 180°C for 4 h.

Water

Water used to reconstitute the lysate, endotoxin, and used as negative controls, was purchased from Abbott Laboratories and labeled as sterile and non-pyrogenic.

LAL

PYROTELL brand (Associates of Cape Cod,

Table 1

Biocides in study

HHT	1,3,5-Tris (2-hydroxyethyl) hexahydrotriazine
OXA	4,4-Dimethyloxazolidine
GLA	Glutaraldehyde
IT	5-Chloro-2-methyl-3(2H) isothiazolone + 2-methyl-3(2H) isothiazolone
NM	4,4'-(2-Ethyl-2 nitrotrimethylene) dimorpholine + 4-(2-nitrobutyl) morpholine

Table 2

Neutralization of control standard endotoxin (CSE) by selected biocides at pH 7

Biocide	Biocide concentration (ppm)	LAL reaction with CSE (ng/ml)			
		0 (Control)	0.1	1	10
HHT (9.9) ^a	1000	—	—	—	—
GLA (4.0) ^a	1500	—	—	—	—
IT (4.4) ^a	100	—	+	+	+
OXA (10.7) ^a	1000	—	—	—	—
NM (7.9) ^a	1000	—	+	+	+

^a Pre-buffered pH.

Inc.) – Lot Nos. 99-43-386, 99-60-403, and 99-88-431 LAL was used. Each vial was reconstituted with 5 ml of pyrogen-free water with a resulting sensitivity of 0.03 EU/ml.

Endotoxin control

Escherichia coli Control Standard Endotoxin (CSE) – Lot No. 44 (Associates of Cape Cod, Inc. #0113, PPE-E-434).

Escherichia coli cultures

ATCC 8739 and 11229 cultures were grown for 24 h in nutrient broth at 37°C. Heat-killed cultures were boiled for 10 min, then allowed to cool before performing the LAL test.

Table 3

Neutralization of cell-linked endotoxin by selected biocides determined by LAL assay

Biocides (pH 7.0)	Biocide concentration (ppm)	Extinction dilution of <i>E. coli</i> ^a	
		ATCC 8739	ATCC 11229
Control (heat)	—	10 ⁻⁵	10 ⁻⁵
GLA	1500	UD ^b	UD
IT	100	10 ⁻⁵	10 ⁻⁶
OXA	1000	UD	UD
NM	1000	10 ⁻⁵	10 ⁻⁶

^a 24-h culture ~ 10⁹ cfu/ml.

^b UD = LAL-negative with undiluted culture.

Table 4
Titration of endotoxin activity (LAL) with formaldehyde

Endotoxin concentration (ng/ml)	Formaldehyde molarity (mM)								
	1200	600	300	150	120	75	60	38	30
10	-	-	+/-	+/-	+/-	+	+	+	+
1	-	-	-	+/-	+	+	+	+	+
0.025	-	-	-	-	-	+/-	+/-	+	+
0.0125	-	-	-	-	-	+/-	+	+	+
0.006	-	-	-	-	-	-	-	+/-	+/-
0.003	-	-	-	-	-	-	-	+/-	+/-
Control	-	-	-	-	-	-	-	-	-

Biocides

The active agents of each industrial biocide are listed in Table 1. Biocides were used at their actual working concentrations at pH 7.0 or as noted in Tables 2 and 3.

Performing the assay

Serial dilutions of the CSE or cultures were made from which 50 μ l was added to 50 μ l of specified concentrations of biocides in 10 \times 75 mm glass test tubes. Each tube was gently vortexed and allowed to sit at room temperature for 30 min before addition of 100 μ l of LAL reagent. The tubes were gently mixed once more before incubating in a water bath at 37°C for 60 min. Each test was done in triplicate. Tubes were covered with pyrogen-free parafilm while incubating at room temperature or in a water bath. Tubes were carefully removed from

the water bath after 60 min for observation. A positive reaction was characterized by a firm gel that remained when the tube was carefully inverted through 180 degrees. A negative result was characterized by the absence of a gel or by a viscous gel that did not remain intact when inverted. Sensitivity of CSE (positive control) must meet labeled LAL reagent sensitivity and the negative controls must show no gel for the assay to be determined valid.

RESULTS AND DISCUSSION

Since it has been reported that the formaldehyde residues of taurolin and noxythiolin were responsible for their ability to neutralize endotoxins, formaldehyde was used in the first step of our study. Formaldehyde reduced the effectiveness of endotoxins to cause gelling of the LAL reagent in a dose-response manner (Table 4).

Treatment of purified endotoxin (Table 2) and cell-bound endotoxin (Table 3) with commercially available biocides substantiates the claim that formaldehyde-releasing biocides are endotoxin neutralizers. However, it also suggests that other aldehydes are capable of neutralizing endotoxins since glutaraldehyde also neutralized endotoxins at the concentrations used. Preliminary studies (results not shown) indicate that several aliphatic and aromatic aldehydes neutralize endotoxins. In some cases, the extent of neutralization appears related to

Table 5
Chemical detection of formaldehyde release from formaldehyde adducts^a

Adduct	Formaldehyde in chemical structure based on synthesis	Number of formaldehyde molecules detected (Nash reagent)
HHT	3	2.9167
OXA	1	0.939-1.04
NM	1	0.05-0.08

^a Modified from Rossmoore and Sondossi [14]. Assays were conducted at pH 7.0 in water by the Nash method (1953).

the aldehyde content of the molecule; in others, the reactive properties are not readily explained. Additional studies on a large series of aldehydes will be done in order to elucidate the mechanism of action.

Not all of the formaldehyde condensate biocides used in our study were effective against endotoxins. The ineffectual biocides in this study do not appear to release formaldehyde under the conditions of this assay. The finding concurs with results obtained with Nash reagent [14] which suggest that compounds whose equilibrium reaction strongly favors the formaldehyde adduct have minimal effectiveness as endotoxin neutralizers. This may explain the results obtained with 4,4'-(2-ethyl-2 nitrotrimethylene) dimorpholine + 4-(2-nitrobutyl) morpholine (NM) where formaldehyde was not detected appreciably at the pH of the Nash reaction (Table 5).

The LAL reaction was performed with biocides that had pH values ranging between 4.5 and 10 when diluted to their working concentrations. The LAL reaction was not inhibited, although the sensitivity may have shifted from that obtained at its optimum pH (7.0).

Knowledge of the mode of action of formaldehyde neutralizing endotoxin is necessary for the development of safe, yet effective, biocides for lowering the risk of work-related morbidity [13]. The synergistic and antagonistic effect of reaction conditions must be investigated carefully.

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