

Recombinant Factor C Assay for Measuring Endotoxin in House Dust: Comparison With LAL, and (1 → 3)- β -D-Glucans

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Background Measurement of exposure to environmental endotoxin is frequently performed using a *Limulus* amoebocyte lysate (LAL) based assay. Recently, a new method has become available with similar sensitivity and potentially greater specificity using recombinant Factor C (rFC) from the horseshoe crab *Carcinoscorpius rotundicauda*. A preliminary study was carried out to determine the comparability of LAL and rFC in measuring endotoxins in house dust for large scale epidemiologic studies.

Methods House dust samples were collected from family rooms by vacuuming 1 m² of the center of the room. Sixty sieved house dust samples were assayed for endotoxin by LAL (Cambrex, KQCL lysate) and rFC (Pyrogene, Cambrex) and for (1 → 3)- β -D-glucans by ELISA. The resistant parallel line estimation was used for data analysis of LAL and rFC. A four-parameter logistic fit with inverse prediction was used to calculate (1 → 3)- β -D-glucan levels of the samples.

Results The spike recovery was 113.63% (95% CI = 101.69, 125.57%) for LAL and 99.69% (95% CI = 90.14, 109.24%) for rFC assays. The LAL assay gave higher endotoxin estimates compared with rFC. The LAL and rFC estimates were highly correlated ($r = 0.86$, $P < 0.0001$). The difference between LAL and rFC endotoxin estimates correlated with the LAL estimates ($r = 0.51$, $P < 0.0001$). However, the difference was not correlated with (1 → 3)- β -D-glucans.

Conclusion LAL and rFC gave comparable results, hence either assay can be used for studies of endotoxin exposure. The current study shows that (1 → 3)- β -D-glucan is not a major factor interfering with endotoxin measurements in house dust using a Cambrex KQCL LAL preparation. Am. J. Ind. Med. 49:296–300, 2006. © 2006 Wiley-Liss, Inc.

KEY WORDS: endotoxin; LAL; rFC; (1 → 3)- β -D-glucan; house dust

INTRODUCTION

Endotoxins are biologically active lipopolysaccharides, which compose the outer membrane of the gram-negative bacteria. Endotoxins are released into the environment after lysis of the bacterial cells, during active cell growth, and when bacterial cells are taken up by macrophages and processed. Gram-negative bacteria and their endotoxins are ubiquitous colonizers of soil, water, and living organisms.

Endotoxins are potent biological agents associated with bioaerosol-induced respiratory symptoms observed in both indoor [Wan and Li, 1999; Park et al., 2001] and occupational

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environments [Rylander et al., 1985; Milton et al., 1996; Reynolds et al., 1996]. Recent studies show that exposure to endotoxin also appeared to have a protective effect on atopic asthma among children and adults living in farm environments [Riedler et al., 2000; von Mutius et al., 2000; Braun-Fahrlander, 2001, 2002; Eduard et al., 2004; Waser et al., 2004]. (1 → 3)- β -D-Glucans, which are carbohydrate polymers, are cell wall constituents of filamentous fungi and yeasts. Glucans may cause nonspecific inflammatory reactions [Rylander et al., 1992; Rylander, 1996; Wan and Li, 1999; Douwes et al., 2000a,b; Douwes, 2005]. Previous studies have reported dose-response relationships [Rylander et al., 1992] and effects of exposure on lung function [Thorn and Rylander, 1998; Douwes et al., 2000c].

The use of *Limulus* amoebocyte lysate (LAL) for the detection of endotoxin was first reported by Bang [Bang, 1956; Levin and Bang, 1968]. Kinetic turbidimetric and kinetic chromogenic methods are widely used at present to measure endotoxins in environmental samples. The kinetic *Limulus* assay with resistant-parallel-line estimation (KLARE) method developed by Milton et al. [1992, 1997] can be applied to both kinetic chromogenic and kinetic turbidimetric versions of the LAL assay. In the LAL assay endotoxin is detected through a reaction cascade, initiated by the binding of endotoxins to Factor C. However, (1 → 3)- β -D-glucans can interfere in this reaction cascade via Factor G giving false positive results. A new assay, recombinant Factor C (rFC) was recently developed to determine endotoxins in environmental samples [Chen, 2003]. The advantage of using the genetically engineered rFC in measuring endotoxins is that rFC does not contain Factor G that can produce interference from (1 → 3)- β -D-glucans and there is less potential for lot-to-lot variation compared with using LAL. The current study compares a kinetic chromogenic *Limulus* amoebocyte lysate assay and a kinetic fluorometric rFC assay used to measure endotoxin in house dust. Both assays measure only endotoxins that are biologically available to each assay. The objective of this study was to determine the comparability of LAL and rFC estimates and the suitability of rFC assay for large scale epidemiologic studies.

MATERIALS AND METHODS

Reference standard endotoxin (RSE, lot G2B274) was purchased from the United States Pharmacopeia (Rockville, MD) and control standard endotoxin (CSE) was purchased from Associates of Cape Cod (Falmouth, MA). The LALs and the reagents for the rFC assay were provided by the BioWhittaker (Cambrex), Inc. (Walkersville, MD). The endotoxin and glucan free Corning 96-well microtiter plates (Associates of Cape Cod, Falmouth, MA) were used for the assay. For the (1 → 3)- β -D-glucan assay, galactosyl ceramide (Matreya, Pleasant Gap, PA) was used as the coating agent (10 mg in 100 ml absolute alcohol) and 1% bovine serum

albumin (Sigma, St. Louis, MO) in Tris-buffered saline (TBS) (pH 8.0) was used as the blocking agent. Immulon 96-well polystyrene microtiter plates (Thermo Labsystems, Franklin, MA) were used for the immunoassay. Baker's yeast (Sigma) was the standard. The cell line (10C6) used to produce anti (1 → 3)- β -D-glucan IgM monoclonal antibody was obtained from the Collaborative Group (Happauge, NY). The monoclonal antibody was produced at the Lymphocyte Culture Center, University of Virginia, Virginia. The goat anti-mouse IgM-horseradish peroxidase conjugate, anti-rabbit IgG peroxidase conjugate, and tetramethylbenzidine (TMB) were obtained from Calbiochem (La Jolla, CA).

We were provided with house dust samples collected as part of a birth cohort study conducted in Connecticut. The sample collection was described previously [Milton et al., 2001]. The thimbles containing dust were stored at -80°C . The samples were sieved using 425 μm mesh sieves. The sieved dust samples were divided into separate aliquots for various assays and stored them at -80°C until the analysis. The screw top tubes (VWR, Bridgeport, NJ) were used for weighing and storing dust samples. All glassware was baked at 270°C for 30 min to render them free of endotoxin and glucans.

Sample Preparation for Endotoxin and (1 → 3)- β -D-Glucan

Approximately 5 mg of dust was sonicated (model 3200R-1 sonicator, Branson Cleaning Equipment Co., Shelton, CT) with 1 ml of 0.01% triethylamine in 0.05 M potassium phosphate buffer (pH 7.5) for 1 hr, with vortexing every 15 min. After the sonication, 0.5 ml of the suspension was transferred into sterile microcentrifuge tubes for glucan assay and the rest was analyzed for endotoxins by both LAL and rFC assays. The 0.5 ml dust suspension for glucan assay, was autoclaved at 120°C (10^5 Pa) for 1 hr. After being vortexed for 5 min, the autoclaved suspension was centrifuged for 15 min at 4,000g. The supernatant was assayed for glucans as described previously [Milton et al., 2001]. The final estimates were given in nanograms of (1 → 3)- β -D-glucans per milligrams of dust.

Endotoxins (LAL and rFC)

Immediately after the sonication, the dust suspensions were assayed for both LAL and rFC by kinetic chromogenic [Milton et al., 1997] and kinetic fluorogenic assays respectively. Ten dust suspensions were assayed for both LAL and rFC during the same day. Five dilutions of the standards and four dilutions of the samples in replicates were loaded (50 μl) on to a 96-well microtiter plate. All the dilutions were prepared in 0.01% triethylamine 0.05 M potassium phosphate (TAP) buffer (pH 7.0). For the LAL assay, the reconstituted lysate (lot no. 1L034U) was added

(50 μ l) to each well. For all the samples, we used the lysates from the same lot to minimize the variations caused by using different lots. Immediately after the addition, the plate was kept in the microplate reader (BioWhittaker KQCL, Walkersville, MD, BioTek ELX808IU equipped with KC4 software, Winooski, VT) and the kinetic readings (405 nm) were taken at 37°C. Each well was read every 30 s for 2 hr, which resulted in 241 total OD readings.

For rFC assay, the dilutions of the standards and samples were prepared exactly similar to the LAL assay, loaded them (100 μ l) on to a 96-well plate and then pre-incubated the plate for 10 min at 37°C. During the 10 min pre-incubation the rFC reagent was prepared by gently mixing rFC, substrate, and buffer (ratio 1:5:4). After the 10 min duration, the plate was placed inside a biosafety hood and 100 μ l of the rFC reagent was dispensed into each well. The fluorescence readings were taken by an incubating microplate reader (BioTek FLX800IU equipped with KC4 software, Winooski, VT) for 2 hr at 37°C (excitation 380/20 nm; emission 440/30 nm). Each well was read every 90 s, which gave 81 total FU (fluorescent unit) readings. We used the same lot of rFC, substrate and buffer for the whole study. A series of dilutions of standards (20–0.015 EU/ml range) were run with each LAL and rFC assay. The gain or sensitivity setting of the plate reader was adjusted for the lot of rFC reagent used. This was done by dispensing 100 μ l of 1 EU/ml endotoxin standard diluted in TAP buffer into appropriate wells, pre-incubating for 10 min followed by adding 100 μ l of the rFC reagent. The endpoint readings were taken before and after 1 hr incubation at 37°C at different gain settings (30, 35, 40, 45, 50, and 55). The KC4 software allows up to 6 gain settings. We plotted the log of the mean blank corrected RFU (relative fluorescent unit) observed after 1 hr for the 1 EU/ml samples versus gain settings. We chose the interpolated gain setting corresponding to 3.5 log RFU as the sensitivity setting for the machine with TAP buffer and the current lot of rFC.

Recovery Efficiency

The recovery of endotoxin added to dust samples was determined by adding a known amount of CSE to suspensions of house dust in TAP buffer as follows. Approximately 5 mg of dust samples were sonicated with 1 ml TAP buffer, with vortexing every 15 min. After sonication for 1 hr, samples were vortexed for a further 2 min and then divided into 500 μ l portions. An initial dilution of 1:25 was prepared from each portion. CSE ($\sim$ 10 EU) was added to the 1:25 initial dilution of one portion and the 1:25 dilution from the other portion was assayed without added endotoxin. Serial dilutions were prepared from the initial dilution of standard and samples with and without added endotoxin and both the LAL and rFC assays were performed. Both samples and the standard were run on the same microtiter plate.

Data Analysis

Software for resistant-parallel-line estimation [Milton et al., 1992] was used to calculate estimates for both LAL and rFC assays. All endotoxin results were computed in EU of RSE lot G2B274 per milligrams of dust. A four-parameter logistic fit with inverse prediction [Chew et al., 1999] was used to calculate (1 $\rightarrow$ 3)- β -D-glucan levels and the assay-based standard error for each pair of dilutions of the unknown samples. SAS software (version 8.02, SAS Institute, Cary, NC) was used for summary data analysis comparing the various assay results.

RESULTS

The mean spike recovery for the LAL was 113.63% (95% CI = 101.69, 125.57) and for the rFC was 99.69% (95% CI = 90.14, 109.24). The limit of detection for both assays was 0.015 EU/ml. We obtained linear calibration with $r > 0.99$ for both LAL and rFC.

LAL consistently produced higher endotoxin estimates compared with rFC. The geometric mean endotoxins measured by LAL was 37.22 EU/mg where as the rFC estimate was 19.34 EU/mg for the same house dust samples (the geometric mean ratio LAL/rFC = 1.92). The LAL and rFC estimates were highly correlated (Spearman correlation, $r = 0.86$, $P < 0.0001$) (Fig. 1 and Table I). The difference between EU/mg determined by LAL and rFC was moderately correlated ($r = 0.51$, $P < 0.0001$) with LAL but not with rFC estimates. There was no correlation between (1 $\rightarrow$ 3)- β -D-glucan estimates with the LAL-rFC difference.

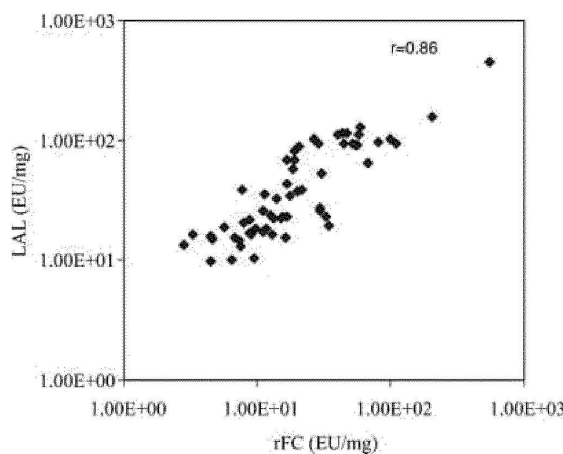


FIGURE 1. rFC (EU/mg) versus LAL (EU/mg) estimates ($n = 60$).

TABLE I. Spearman Correlations Between rFC, LAL and (1 → 3)-β-D-Glucan Estimates

	LAL	rFC	Difference LAL-rFC
	EU/mg	EU/mg	EU/mg
(1 → 3)-β-D-Glucan (ng/mg)	0.25	0.21	0.12
LAL (EU/mg)		0.86*	0.51*
rFC (EU/mg)			0.14

* $P < 0.0001$.

DISCUSSION

LAL and rFC gave comparable results. It has long been recognized that, in the LAL assay, (1 → 3)-β-D-glucans can interfere in the reaction cascade giving erroneously high or low results [Roslansky and Novitsky, 1991]. The presence of other *Limulus* proteins in the lysate may also interfere with responses to endotoxins. For the LAL assays carried out for the present study, we did not use the β-glucan blocker produced by Cambrex, Inc., to block glucan interference in LAL reaction cascade. An advantage of using the rFC method is that there is only one well defined enzyme present so there is no possibility of a Factor G induced response to (1 → 3)-β-D-glucans. We expected that LAL assay would give higher results than rFC due to response to (1 → 3)-β-D-glucans in house dust. Therefore, we expected to find some correlation between the LAL—rFC difference and the dust content of (1 → 3)-β-D-glucans. Failure to find this correlation indicates that (1 → 3)-β-D-glucan in house dust was not a major factor interfering with the LAL reaction cascade. One reason that we did not find any correlation between endotoxin estimates with (1 → 3)-β-D-glucan estimates might be because endotoxins were determined in suspended dust where as glucans were determined in supernatants (house dust suspensions were sonicated at room temp for 15 min, autoclaved for 1 hr at 120°C, centrifuged at 4,000g, separated the supernatant from the precipitate).

LAL estimates were higher compared with rFC estimates for all the samples we assayed, and larger differences between LAL and rFC were significantly correlated with larger LAL estimates of endotoxin content. A possible explanation may be differences in the Factor C of the horseshoe crabs that were the source of reagents. For the LAL assay the reagent is extracted from wild North American horseshoe crabs, *Limulus polyphemus*. The rFC was produced from the cDNA of the Singaporean horse shoe crab, *Carcinoscorpius rotundicauda* cloned in *Baculovirus* expression system in SF9 cells [Ding et al., 1995]. The genetically engineered rFC produced from *Carcinoscorpius rotundicauda* is reported to be 97.7% homologous (sequence identity) to that of *Tachypleus tridentatus* the Japanese horse

shoe crab [Nakamura et al., 1988; Ding et al., 1993, 1995]. An unpublished study found that the Factor C of *Carcinoscorpius rotundicauda* has 89.3% homology with that of *Limulus polyphemus*, North American horse shoe crab Factor C [Lin Chen, personal communications]. Thus, subtle differences in responsiveness of the Factor C from these species may account for their small but systematically different results.

In the rFC assay, rFC is activated by binding to endotoxins, and the activated molecule then cleaves a synthetic tripeptide coumarine substrate releasing a fluorescent compound. The fluorescence produced is directly proportional to the endotoxin concentration of the sample. We observed that the gain or the sensitivity setting of the fluorescent reader was extremely important for rFC assay. The different solvents (TAP, water, LRW) and the different lots of rFC reagents required different sensitivity settings. Therefore, we suggest that it is advisable to carry out sensitivity tests regularly for each solvent and for each lot of rFC and to adjust gain accordingly to obtain consistent results.

This study shows that rFC and LAL are highly comparable, the estimates are highly correlated and either assay can be used for epidemiologic studies of endotoxin exposure. Because the sensitivity of LAL for environmental endotoxin varies from lot-to-lot [Milton et al., 1997], it is possible that subsequent comparisons with other lots of LAL will demonstrate larger or smaller differences between the results of LAL and rFC assays. Because rFC is a cloned protein produced in tissue culture, it is likely to have less lot-to-lot variation than LAL from wild horseshoe crabs and thus may prove to be a more consistent means of measuring environmental endotoxin exposure and with a variety of environmental samples making both house dust and occupational samples from agricultural and manufacturing processes in the future. Additional data on the performance of rFC over many lots are needed to confirm this expectation.

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