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A bioassay for determination of lipopolysaccharide in environmental samples

Marcus Peters, Petra Fritz and Albrecht Bufe

Abstract

Although the limulus amebocyte lysate (LAL) assay is widely used to determine the concentration of LPS in biological samples, it is known to be susceptible to interference caused by substances of non-bacterial origin. In particular, polysaccharides such as β-glucans and pectic polysaccharides from fungi or plants, respectively, were shown to give higher LPS readings than were actually present in the sample. Here, we describe an assay for the determination of LPS in biological samples based on the stimulation of TLR4/MD2/CD14 transfected HEK293 cells which dose dependently release IL-8 upon stimulation with increasing concentrations of highly purified *Escherichia coli* LPS. The resulting standard curve is used to determine the LPS concentration in unknown samples. We show that the outcome of the LPS stimulation is not affected by the presence of β-glucans or other environmental substances found in dust extracts. Although, we present evidence that the LPS concentration measured with the kinetic chromogenic LAL test correlates with data from the TLR4 assay, the LAL test displays higher LPS readings. We conclude that the described TLR4 assay is a reliable alternative to assess the concentration of LPS in environmental samples without being influenced by polysaccharides such as β-glucans and other environmental substances found in dust extracts.

Keywords

Lipopolysaccharide, bioassay, Toll-like receptor 4, glucans, dust extract

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Introduction

Since the hygiene hypothesis has come into focus of allergologists, several studies have concentrated on the measurement of LPS in environmental samples. Some studies revealed that exposure to high concentrations of LPS in the environment correlates well with the protection from allergic airway diseases, such as asthma.[1] However, LPS is discussed as one reason for the induction of occupational lung disease in farm workers.[2,3] The beneficial or disease-provoking effects of LPS in humans might depend on the concentration of this microbial molecule in the environment.[4,5] The most frequently used method for the quantification of LPS is the limulus amebocyte lysate (LAL) test. However, it is already known from the literature that the LAL test displays virtually higher LPS levels in environmental samples than are actually present. This was shown by comparison of LPS measurements taken by the LAL test with the determination of the concentration of 3-hydroxy fatty acids—a more precise chemical method for the determination of LPS.[6]

The LAL test is critically influenced by polysaccharides, such as β-(1→3)-D-glucan, and pectic polysaccarides from plant origin. These molecules are known to give false-positive results in the LAL assay.[7–10] The reason for this is the activation of the clotting cascade of the LAL reagent not only by lipid A but also by the polysaccharide moiety of the molecule. Owing to the unspecific nature of the LAL test, this assay has already been used for quantification of β-(1→3)-D-glucan in samples.[11] As it is known that environmental extracts contain huge amounts of polysaccharides, the LAL test is likely to be influenced by these contaminants.[12] These problems may be overcome by using other methods for LPS determination, such as the above-mentioned

Ruhr-University Bochum, Germany

Corresponding author:
Marcus Peters, Department of Experimental Pneumology, Ruhr-University Bochum, Bürkle-de-la-Camp-Platz 1, 44789 Bochum, Germany.
Email: marcus.peters@rub.de

determination of 3-OH fatty acids.[13,14] This method uses a mass spectrometry approach to determine exactly the molar concentration of the fatty acids of LPS in a sample. Although this is undoubtedly the most precise method of determining the LPS concentration, this method is cost-intensive and demands complex technical equipment. Likewise, with the newly-developed factor C assay that is more specific for LPS than the conventional LAL-test but requires a kinetic fluorescence reader to evaluate the test.

Given the influence of environmental substances such as β-glucans on the LAL test and the cost-intensiveness of other assays with a higher specificity for LPS, we have developed an assay to determine the concentration of LPS in environmental samples by using HEK293 cells transfected with TLR4/MD2/CD14. We show that this assay gives reliable results with LPS from different bacterial species and that this assay is not affected by environmental substances other than LPS.

Materials and methods

Chemicals

LPS from *Escherichia coli* wild type, *E. coli Nissle* and *Salmonella enterica* sv. Minnesota were purified by phenol/chloroform extraction and subsequently purified by chromatography.[15] Ovalbumin (grade V) and β-(1→3)-D-glucan were purchased from Sigma-Aldrich. TLR2/6 heterodimer-activating diacyl lipopeptide FSL-1 was obtained from EMCmicrocollections GmbH (Tübingen, Germany). Arabinogalactan from *Gummi arabicum* (gum Arabic) was purchased from Biosupplies Inc. (Parkville, Australia).

Cell culture

HEK293 cells stably transfected with a pUNO expression vector for TLR4/CD14/MD2 were purchased from Invivogen (Cayla, France). The cells were cultured in DMEM 10% FCS with normocin, hygrogold, penicillin and streptomycin as antibiotics. The medium was supplemented with Blasticidin for the positive selection of the plasmid-containing cells, as described by the manufacturer. Cells were sub-cultured before reaching confluence by short treatment with DetachinTM from AMS Biotechnology (Frankfurt, Germany).

HEK293-null control cells were transfected with a pUNO vector conferring Blasticidin resistance but lacking coding regions for the receptors. These cells were used as a control in the described experiments. None of the substances used in this work led to stimulation of IL-8 release from these control cells.

Stimulation of the transfected HEK293 cells with LPS

The cells were cultured in a density of $10^6 \times ml^{-1}$ in a 96-depot cell culture plate in 100 µl DMEM medium containing 10% FCS. A standard curve was generated by stimulating the cells with 100 µl of *E. coli* LPS dissolved in DMEM reaching a final concentration of 10 pg/ml to 10 ng/ml for 24 h. Subsequently, IL-8 production was measured in the tissue culture supernatants. This standard curve was used to determine the TLR4-stimulating capacity in the different samples and expressed as *E. coli* LPS equivalent in ng/ml.

Determination of IL-8 in the cell culture supernatants

The supernatants were collected and centrifuged to sediment the cellular debris. The cleared supernatants were measured with an IL-8 OptEIA kit according to the manufacturers protocol (BDbiosciences, San Diego, CA, USA).

Environmental samples

Mattress dust was collected and extracted as described previously.[1,16] To analyze whether substances from the extract had an influence on the assay, we subjected a pool of mattress dust extract to Endotrap blue affinity chromatography to remove LPS. Residual substances were used to spike the assay. Endotrap chromatography was performed according to the manufacturers' protocol (Hyglos GmbH, Regensburg, Germany).

Statistical analysis

To determine the LPS concentration in unknown samples, a standard curve was generated by plotting the IL-8 concentration versus the logarithmic transformed LPS concentration used to stimulate the cells. Graph Pad Prism (Version 5.01) was used to generate a dose-response curve and to extrapolate the LPS concentration from unknown sample.

Results

Characterization of the reactivity of LPS on TLR4/CD14/MD2-transfected HEK293 cells

First, the TLR4 assay was characterized with wild-type LPS from *E. coli*. The minimum detectable concentration giving IL-8 readings above background levels was 10 pg/ml *E. coli* LPS. Concentrations above 1 ng/ml LPS usually resulted in a maximal stimulation of the HEK93 cells with no further increase of IL-8 release (Figure 1A). The standard curve was used in the range of the highest slope to determine unknown LPS concentration in a sample (usually between 10 and 1000 pg/ml). For determination of the coefficient of variation (CV), a sample of 0.1 µg/ml pure *E. coli* LPS was

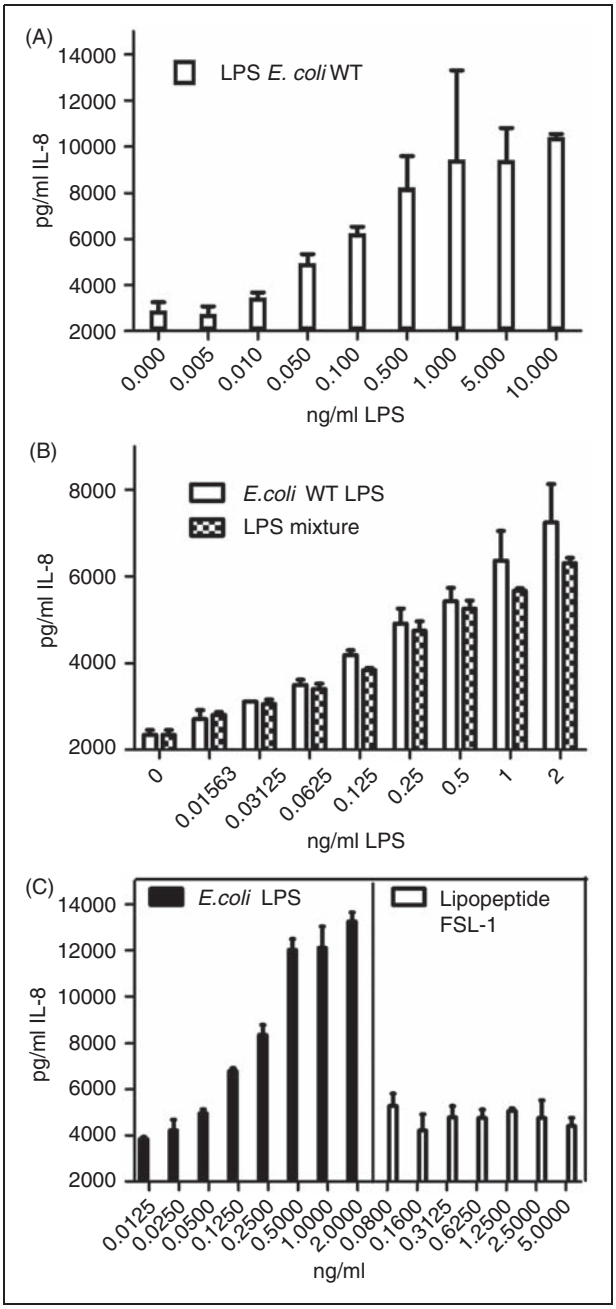


Figure 1. (A) Stimulation of CD14/TLR4/MD2-transfected HEK293 cells with *E. coli* LPS (WT). (B) Stimulation of cells with a mixture of LPS from three different bacterial strains (*E. coli* wild-type, *E. coli* Nissle, *S. enterica* sv. Minnesota) in comparison to stimulation with pure *E. coli* LPS from the wild type strain. (C) Stimulation of cells with FSL-1, a lipopeptide known to activate the TLR-2/6 heterodimer. IL-8 release was measured after stimulation with increasing concentrations of these substances for 24 h. All samples were run in duplicates. Data points represent mean with SEM.

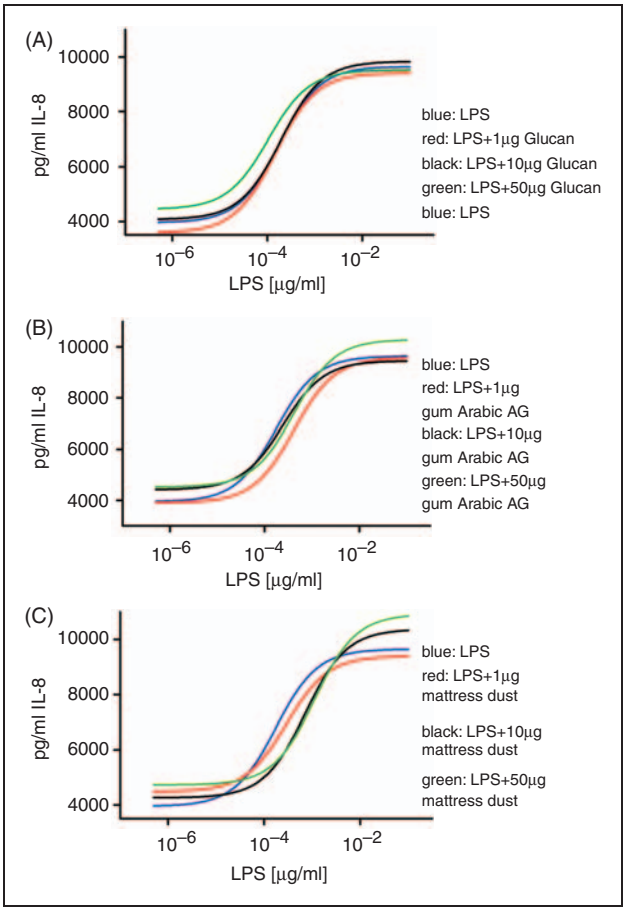


Figure 2. Cells were stimulated with increasing concentrations of LPS in the presence of either different concentrations of β-(1→3)-D-glucan (A), arabinogalactan (B) or mattress dust extract after Endotrap blue chromatography (C). Stimulation of cells with β-(1→3)-D-glucan or arabinogalactan alone did not lead to activation of cells. In contrast, stimulation with mattress dust after Endotrap blue chromatography led to IL-8 production as a consequence of LPS residuals. After stimulation for 24 h supernatants were evaluated for IL-8 concentration. Experiment was repeated twice with similar results.

measured 10 times, resulting in a mean of 0.12 ± 0.02 µg/ml and a CV of 17%.

To analyze the reproducibility of the determination of the LPS concentration in organic samples we measured the LPS concentration in endotoxin-contaminated ovalbumin at three different time points resulting in three independent measurements resulting in a mean concentration of 3.01 ± 0.40 ng/ml.

As environmental extracts contain a mixture of LPS from different bacterial species we next determined whether results would differ from the *E. coli* standard curve when a mixture of different LPS was used. Therefore, IL-8 release resulting from a mixture of LPS, namely *E. coli* wild type, *S. enterica* sv. Minnesota and from *E. coli* Nissle, was compared with stimulation with LPS from *E. coli* wild type alone. As shown in Figure 1B, there is no difference between these two reagents.

As a control for the specificity of the assay, the HEK293 cells that were transfected with the empty vector were stimulated with LPS from different

Table 1. Determination of LPS in mattress dust extract by means of LAL assay and TLR4 assay, respectively.

Dust sample	1	2	3	4	5	6	7	8	9
[ng/mg] LPS_{LAL}	83±26	4±1	15±1	3±1	159±2	100	14±6	14±0	113±16
[%] recovery	132±12	136±4	124±3	124±5	114±0	118	119±2	114±4	111±2
[ng/mg] LPS_{TLR4}	42±20	3±1	10±1	6±1	25±5	22±4	8±3	7±2	10±4

For each sample, LPS concentration is given in ng per 1 mg of extract. For the both tests, the SEM is shown. Additionally, the recovery values of the LPS spiking control in the LAL test are shown.

bacterial species or cells were stimulated with dust extract known to contain different pattern recognition receptor-stimulating substances. Neither TLR4 nor TLR2 agonists, nor β-(1→3)-D-glucan or dust extract induced IL-8 production, showing that IL-8 production depends on the expression of LPS receptor complex encoded by the vector (data not shown).

To analyze whether IL-8 production of the TLR4/CD14/MD2-transfected HEK293 cells is a specific reaction to LPS and not induced by another TLR-activating reagent, we stimulated the cells with the synthetic lipopeptide FSL-1 which is known to lead to strong activation of the TLR2/6 heterodimer. As shown in Figure 1C, stimulation with this lipopeptide did not activate the cells, showing that IL-8 production upon LPS stimulation is a specific reaction to this TLR4 ligand.

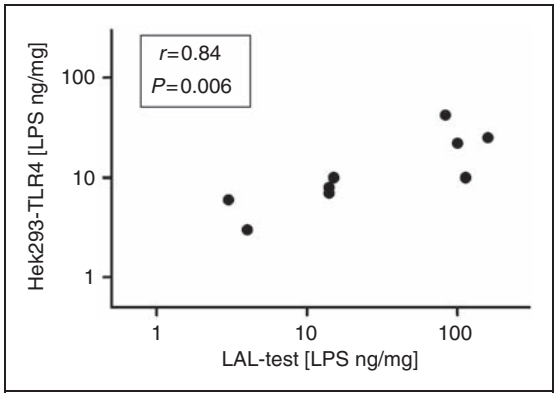


Figure 3. Correlation analysis between the LPS concentration as measured with the LAL test (mean of two independent measurements) or with the TLR4 assay (mean of three independent measurements). Mattress dust was extracted with isotonic NaCl solution. Subsequently, extracts were subjected to either the LAL test or the TLR4 assay. LAL test was carried out as described by the manufacturer. TLR4 assay was done by stimulation of cells for 24 h. Concentration was correlated. Nonparametric Spearman correlation coefficient (r) and the two-tailed *P*-value are shown.

Environmental substances other than LPS do not interfere with the TLR4 assay

Next, it was important to find out whether there are substances in environmental samples that have an influence on the results of the TLR4 ligand assay. For instance, for pectic polysaccharides and β-(1→3)-D-glucan it was already shown that these substances affect the LAL test. Therefore, we have tested polysaccharides in their ability to interfere with TLR activation by spiking during stimulation of cells with LPS. Data presented in Figure 2 show that neither arabinogalactan from Acacia (Figure 2B) nor β-(1→3)-D-glucan (Figure 2A) affects the detection of LPS in a concentration up to 10 μg/ml. Only the 50 μg/ml concentration of β-(1→3)-D-glucan leads to a slight reduction of the LPS sensitivity of the cells.

To further investigate the influence of environmental substances on TLR activation we removed LPS from mattress dust extract by means of Endotrap affinity chromatography. The LPS content of the mattress dust extract used for this study was 44 ng/mg. Following affinity chromatography, the LPS content of the dust extract was reduced to 7 ng/mg; however, it was impossible to get an extract free of LPS by this method. Subsequently, the LPS-reduced extract was used to spike a LPS standard used for stimulation of the TLR4 transfected cells (Figure 2C). Spiking of the LPS standard with the LPS-reduced extract did not lead to alteration of the shape of the standard curve revealing that the presence of substances in mattress dust extract does not have an impact on the quantification of LPS. However, spiking with the 10 and 50 μg/ml concentration of extract resulted in detection of slightly increased LPS levels, likely derived from LPS residues in the dust extract remaining after Endotrap chromatography.

Comparison of the LPS determination in mattress dust extracts by the LAL test and the TLR4 assay

To test whether the LPS determination in dust extracts by using the TLR4 ligand assay correlates with the quantification of LPS by the LAL assay, we subjected nine different mattress dust extracts to the two different assays (Table 1). Measurement of the extracts in the LAL test was performed at the same dilution. Spiking controls in the LAL test were performed with 5 EU/ml LPS for all dust samples. To test whether there was a correlation between the results of both assays, the

determined concentrations were plotted against each other. Figure 3 shows that the results determined by the two different assays show a correlation ($r = 0.84$, $P = 0.006$); however, the LAL test displayed higher values than the TLR4 assay for eight out of nine analyzed mattress dust extract samples.

Discussion

The aim of this study was to develop a sensitive assay for the determination of the concentration of endotoxic LPS in environmental samples with reduced susceptibility to interference by other substances. We present here a reliable assay useful for the sensitive detection of endotoxic LPS in biological fluids and environmental samples.

The widely used LAL test was found to be a valuable tool for measuring LPS in different kinds of samples, for instance in pharmaceutical preparations. However, in more heterogeneous mixtures such as extracts from biological material, it was already shown that some substances interfere with the outcome of the LAL test. One class of substances known to have an influence on the LAL assay are β-glucans.[7–10] Presence of these fungal polysaccharides is known to display virtually higher LPS measurements than actually present in the sample as a result of the activation of the clotting cascade of the LAL reagent. As β-glucans are derived from fungal cell walls, these polysaccharides are common in all environmental samples.[12,17] This may explain why measurement of LPS in environmental dust extracts by means of the LAL test gives higher concentrations in comparison to measurement with the TLR4-transfected cells. We show here that glucans, and other environmental substances, do not have an impact on LPS determination in the TLR4-ligand assay.

Other tests were developed to detect LPS in various samples. For instance, mononuclear cells were stimulated with LPS followed by the measurement of proinflammatory cytokines. However, this assay also seems to be influenced by substances other than LPS as Kikkert et al. have shown that activation of monocytes with β-(1→3)-D-glucan could enhance the response on LPS stimulation.[18] Therefore, this assay seems to be an improper alternative for the LAL assay with respect to the measurement of environmental samples.

One advantage of the described assay is that LPS is measured selectively, as TLR4 is exclusively bound by LPS. There are only a few reports showing that other substances may interfere with the TLR4 receptor, for example taxol from yew trees.[19] Moreover, there are reports showing that heat shock proteins might activate TLR4.[20] However, these substances are unlikely to be found in environmental extracts as we showed here that environmental extracts depleted of LPS do not lead to TLR4 activation.

However, one must keep in mind that the bioassay described here is not detecting all different kinds of LPS. LPS from *Rhodobacter sphaeroides*, for instance, is likely to give negative results in this assay as it is known to be a TLR4 antagonistic molecule.[21] Thus, to determine the exact molar concentration of all kinds of LPS in a sample, a GC/MS approach must be used.[14] However, in most instances it is of interest to quantify LPS molecules with endotoxic potential. For this purpose, the TLR4 assay is a suitable option.

We conclude that the described assay is a valuable tool to detect LPS in samples from various origins with marginal interference by other molecules giving the opportunity to make reliable measurements of LPS in environmental samples where these properties are from particular importance.

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References

1. Braun-Fahrländer C, Riedler J, Herz U, et al. Environmental exposure to endotoxin and its relation to asthma in school-age children. *New Engl J Med* 2002; 347: 869–877.
2. Rylander R, Haglind P and Lundholm M. Endotoxin in cotton dust and respiratory function decrement among cotton workers in an experimental cardroom. *Am Rev Respir Dis* 1985; 131: 209–213.
3. Smid T, Heederik D, Houba R, et al. Dust- and endotoxin-related respiratory effects in the animal feed industry. *Am Rev Respir Dis* 1992; 146: 1474–1479.
4. Eisenbarth SC, Piggott DA, Huleatt JW, et al. Lipopolysaccharide-enhanced, toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. *J Exp Med* 2002; 196: 1645–1651.
5. Peters M, Dudziak K, Stiehm M, et al. T-cell polarization depends on concentration of the danger signal used to activate dendritic cells. *Immunol Cell Biol* 2010; 88: 537–544.
6. Pomorska D, Larsson L, Skórska C, et al. Levels of bacterial endotoxin in air of animal houses determined with the use of gas chromatography-mass spectrometry and Limulus test. *Ann Agric Environ Med* 2007; 14: 291–298.
7. Brandenburg K, Howe J, Gutsman T, et al. The expression of endotoxic activity in the Limulus test as compared to cytokine production in immune cells. *Curr Med Chem* 2009; 16: 2653–2660.
8. Cooper JF, Weary ME and Jordan FT. The impact of non-endotoxin LAL-reactive materials on Limulus amebocyte lysate analyses. *PDA J Pharm Sci Technol* 1997; 51: 2–6.

9. Hirano M, Matsumoto T, Kiyohara H, et al. Lipopolysaccharide-independent limulus amebocyte lysate activating, mitogenic and anti-complementary activities of pectic polysaccharides from chinese herbs. *Planta Med* 1994; 60: 248–252.
10. Vassallo R and Limper AH. Fungal ß-glucan can yield false-positive results with the limulus amebocyte lysate endotoxin assay. *Chest* 1999; 116: 583–584.
11. Foto M, Plett J, Berghout J, et al. Modification of the Limulus amebocyte lysate assay for the analysis of glucan in indoor environments. *Anal Bioanal Chem* 2004; 379: 156–162.
12. Noss I, Wouters IM, Bezemer G, et al. beta-(1,3)-Glucan exposure assessment by passive airborne dust sampling and new sensitive immunoassays. *Appl Environ Microbiol* 2010; 76: 1158–1167.
13. Reynolds SJ, Milton DK, Heederik D, et al. Interlaboratory evaluation of endotoxin analyses in agricultural dusts–comparison of LAL assay and mass spectrometry. *J Environ Monit* 2005; 7: 1371–1377.
14. Saito R, Cranmer BK, Tessari JD, et al. Recombinant factor C (rFC) assay and gas chromatography/mass spectrometry (GC/MS) analysis of endotoxin variability in four agricultural dusts. *Ann Occup Hyg* 2009; 53: 713–722.
15. Güttsches AK, Löseke S, Zähringer U, et al. Anti-inflammatory modulation of immune response by probiotic *Escherichia coli* Nissle 1917 in human blood mononuclear cells. Innate Immun 2011. Epub ahead of print. DOI:10.1177/1753425910396251.
16. Peters M, Kauth M, Schwarze J, et al. Inhalation of stable dust extract prevents allergen induced airway inflammation and hyperresponsiveness. *Thorax* 2006; 61: 134–139.
17. Bertelsen RJ, Carlsen KC, Carlsen KH, et al. Childhood asthma and early life exposure to indoor allergens, endotoxin and beta(1,3)-glucans. *Clin Exp Allergy* 2010; 40: 307–316.
18. Kikkert R, Bulder I, de Groot ER, et al. Potentiation of Toll-like receptor-induced cytokine production by (1–>3)-beta-D-glucans: implications for the monocyte activation test. *J Endotoxin Res* 2007; 13: 140–149.
19. Byrd-Leifer CA, Block EF, Takeda K, et al. The role of MyD88 and TLR4 in the LPS-mimetic activity of Taxol. *Eur J Immunol* 2001; 31: 2448–2457.
20. Ohashi K, Burkart V, Flohé S, et al. Cutting edge: Heat shock protein 60 is a putative endogenous ligand of the Toll-like receptor-4 complex 1. *J Immunol* 2000; 164: 558–561.
21. Rossignol DP and Lynn M. TLR4 antagonists for endotoxemia and beyond. *Curr Opin Investig Drugs* 2005; 6: 496–502.