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ORIGINAL ARTICLE

CR3 (CD11b/CD18) activation of nasal neutrophils: a measure of upper airway endotoxin exposure

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Abstract

Inhaled endotoxin (lipopolysaccharide, LPS) initiates an inflammatory response and leads to the expression of CR3 (CD11b/CD18) receptors on polymorphonuclear leukocytes (PMNs). We determined if PMN activation in nasal lavage fluid (NLF) is a possible biomarker of occupational endotoxin exposure. Seven subjects exposed to endotoxin provided NLF samples that were split into three aliquots (negative control – 1 M nicotinamide; sham; positive control – 11 ηg of exogenous LPS) and PMN activation was measured using a chemiluminometer. Differences in mean PMN activation were apparent, negative control: 548 ± 15.65 RLU 100 μl^{-1}; sham: 11469 ± 2582 RLU 100 μl^{-1}; positive control: 42026 ± 16659 RLU 100 μl ($n = 7$; $p < 0.05$). This technique shows promise as a diagnostic method for measuring upper airway LPS exposure.

Keywords: *Chemiluminescence; CR3; endotoxin; lipopolysaccharide; polymorphonuclear leukocytes*

Introduction

Bacterial endotoxin or lipopolysaccharide (LPS) is composed of a toxic lipid A moiety and two immunogenic polysaccharide components. Endotoxin embedded in the cell wall of gram-negative bacteria can cause an inflammatory response once it is inhaled into the upper airways. Endotoxin is also highly ubiquitous in both indoor/outdoor environments and exposure to endotoxin results in a heterogenic response depending on age and the level of exposure (Singh & Schwartz 2005). Early exposure to environmental endotoxins during childhood may protect children from developing allergies and/or asthma (Singh & Schwartz 2005). However, acute exposures in occupational settings have resulted in symptoms including chest tightness, myalgias, fevers and dyspnoea (Singh & Schwartz 2005). Similarly, studies investigating long-term endotoxin exposure in occupational settings have also reported adverse respiratory symptoms (Thorn 2001, Wang et al. 2005).

Endotoxin exposure in low concentrations activates the Th2 response in which the adaptive immune system facilitates the development of allergic asthma/rhinitis (Singh & Schwartz 2005). However, chronic exposures trigger the Th1 response, which activates the innate immune system. This results in the release of interleukins (IL)-2 and IL-12 and interferon (IFN)-γ which facilitate the development of airflow limitation (Singh & Schwartz 2005), enhanced airway hyperactivity, decreased forced expiratory volume and forced vital capacity (Singh & Schwartz 2005, Thorn 2001). Inhaled LPS in the upper airways binds lipopolysaccharide-binding protein (LBP) forming an LPS–LBP complex. This complex then induces the transfer of LPS to the membrane-bound CD14 receptor, a differentiation antigen and toll-like receptor (TLR)-4 on polymorphonuclear leukocytes (PMNs). The activation of TLR-4 by LPS triggers the innate immune response resulting in the release of proinflammatory cytokines. In addition, LPS stimulates the expression of adhesion receptors such as complement receptor 3 (CR3), a heterodimer (CD11b/CD18) essential for PMN adhesion and transmigration (Zhou et al. 2005). LPS can also stimulate CD14 on monocytes and macrophages (Tobias et al. 1993) resulting in LPS

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binding with other leukocytes as well. The soluble form of CD14 (sCD14) can compete with membrane-bound CD14 (mCD14) and the formation of sCD14–LPS complexes can reduce the effects of an endotoxin response (Haziot et al. 1995, Schutt et al. 1992).

Currently endotoxin exposures are assessed by air sampling using impingers (bioaerosol/liquid) or air filters (Bello et al. 2002, Duchaine et al. 2001, Oppliger et al. 2005, Pacheco et al. 2003, Thorne et al. 1992, 1997) and subsequently measuring endotoxin levels using the *Limulus* amoebocyte lysate (LAL) assay. The structural heterogeneity of LPS serotypes and unique sampling, extraction and storage techniques causes great variability in LAL results (Douwes et al. 1995, 1997, Liebers et al. 2007, White 2002). Furthermore, these methods to assess endotoxin exposures may not accurately reflect personal exposure as workers generally wear respiratory protection. Therefore, a non-invasive personal biomarker of endotoxin would be valuable in determining inhaled endotoxin exposures. Previous studies have investigated personal biomarkers of endotoxin exposure; however there have been no reports of markers that have successfully detected inhaled endotoxin exposure in human nasal lavage fluid (NLF) (Borm et al. 2000, Keman et al. 1998, Mueller-Anneling et al. 2006). Systemic endotoxin and PMN activation in whole blood of patients in an intensive care unit has been measured using an endotoxin activity (EA) assay (Romaschin et al. 1998). In this study, the EA assay was modified to create a luminol-enhanced chemiluminescence assay (Figure 1) to determine if PMN activation is a possible biomarker of inhaled endotoxin exposure.

Methods

Subjects

Seven subjects (laboratory animal handlers) with occupational exposure to endotoxin provided a nasal lavage sample after a work shift near the end of a working week. Formal written consent was obtained from each volunteer for the collection of NLF and the protocol was approved by the research ethics board at the University of Toronto.

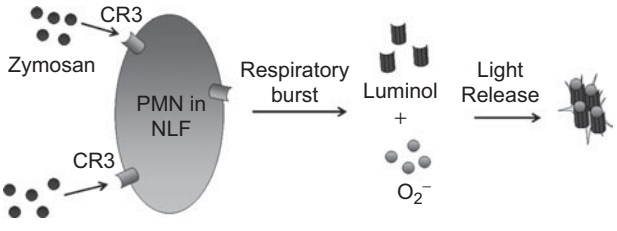

Figure 1. Chemiluminescence in nasal lavage fluid (NLF) samples. Zymosan binds activated CR3 receptors on a polymorphonuclear leukocyte (PMN), resulting in a respiratory burst releasing reactive oxygen species. Oxygen radicals then combine with luminol causing light release, which is measured by the chemiluminometer.

Nasal lavage sampling

Nasal lavage samples were collected by instilling 5 ml of 0.9% saline into each nasal cavity using a 10 ml syringe with a cannula attachment (Becton-Dickinson, Franklin Lakes, NJ, USA). The saline solution was retained in the nasopharyngeal region by palatal pressure for 10 s and then expelled into a sterile non-pyrogenic collection vessel (50 ml BD Falcon tube; Becton-Dickinson). The sample was rapidly transferred into another sterile non-pyrogenic 15 ml tube and centrifuged at 2200 rpm for 15 min under 22°C (model 5810R; Eppendorf, Hamburg, Germany). The cellular pellet was resuspended in 1.25 ml of 0.9% saline and separated into three aliquots (100 µl/aliquot).

PMN processing and luminol-enhanced chemiluminescence measurements

The first aliquot served as a negative control and was treated with 1 M nicotinamide (VWR, Mississauga, Canada) (Ungerstedt et al. 2003); the second aliquot was not treated; the third aliquot served as a positive control in which 11 ηg of exogenous LPS was added with 100 µl of serum protein. As previously reported by Wright et al. (1991), serum protein causes an increase in the adhesive capacity of CR3 receptors on PMNs. Thus to stimulate the expression of additional CR3 receptors the complement protein, C3b, found in serum binds to the LPS-anti LPS (IgM) complex and then attaches to PMNs, priming them for a respiratory burst (Romaschin et al. 1998). NLF samples from three subjects were incubated for 15 min and the remaining four NLF samples were incubated for 30 min to determine time-dependent effects. After this incubation period, lyophilised luminol and zymosan were added into all three aliquots and incubated for another 15 min. The tubes were then removed from the incubator and inserted into the chemiluminometer (AutoMat LT, Bad Wildbad, Germany). The chemiluminescence was measured for 20 min and the PMN activation was calculated by the software, which integrated the chemiluminescence values over the 20 min.

Air sampling

Area sampling at the research facility was performed for 8 h (length of work shift) using SKC Aircheck PCXR8 samplers (SKC, Eighty Four, PA, USA). The flow rate was calibrated to 3.00 l min^{-1} using a Bios Dry-Cal DC-1 Calibrator (SMG/Interlink, Plano, TX, USA). Endotoxin levels were measured using the limulus amebocyte lysate (LAL) assay.

Statistical analysis

All data are expressed as means with the standard error of the mean (SEM). The data were analysed using GraphPad Prism Software version 5.0 (San Diego, CA, USA). Differences between treatments were evaluated using the one-way ANOVA test for variance.

Results

Chemiluminescence measurements

All three treatments, negative control, sham and the positive control were measured for 20 min in the chemiluminometer, and are represented in Figures 2 and 3. Figure 2 represents NLF samples that were incubated for 15 min. The positive control in all three graphs shows an exponential increase depicting CR3 receptor saturation on PMNs. The mean values of the three treatments: negative control, sham and positive control were statistically significant from each other as determined using the one-way ANOVA test (graph I: $p = 0.0001$; graph II: $p = 0.0001$; graph III: $p = 0.001$). We further investigated a longer incubation period to determine time-dependent effects of PMN activation. NLF samples in Figure 3 were again split into three treatments: negative control, sham and positive control, which were incubated for 20 min. In these samples the initial rise to saturation is not present for the positive control due to the increased incubation time. The difference between the three treatments in each sample is statistically significant as determined using the one-way ANOVA test (graph IV: $p = 0.0001$; graph V: $p = 0.001$; graph VI: $p = 0.0001$ and graph VII: $p = 0.001$).

Polymorphonuclear leukocyte activation

PMN activation was determined by integrating the chemiluminescence values over the 20 min. The negative control was 548 ± 15.65 RLU 100 µl, sham was 11469 ± 2582 RLU 100 µl, and the positive control was 42 026 ± 16 659 RLU 100 µl. The mean values of the three treatments were statistically significantly different from each other as determined by the one-way ANOVA test ($p < 0.05$) (Figure 4).

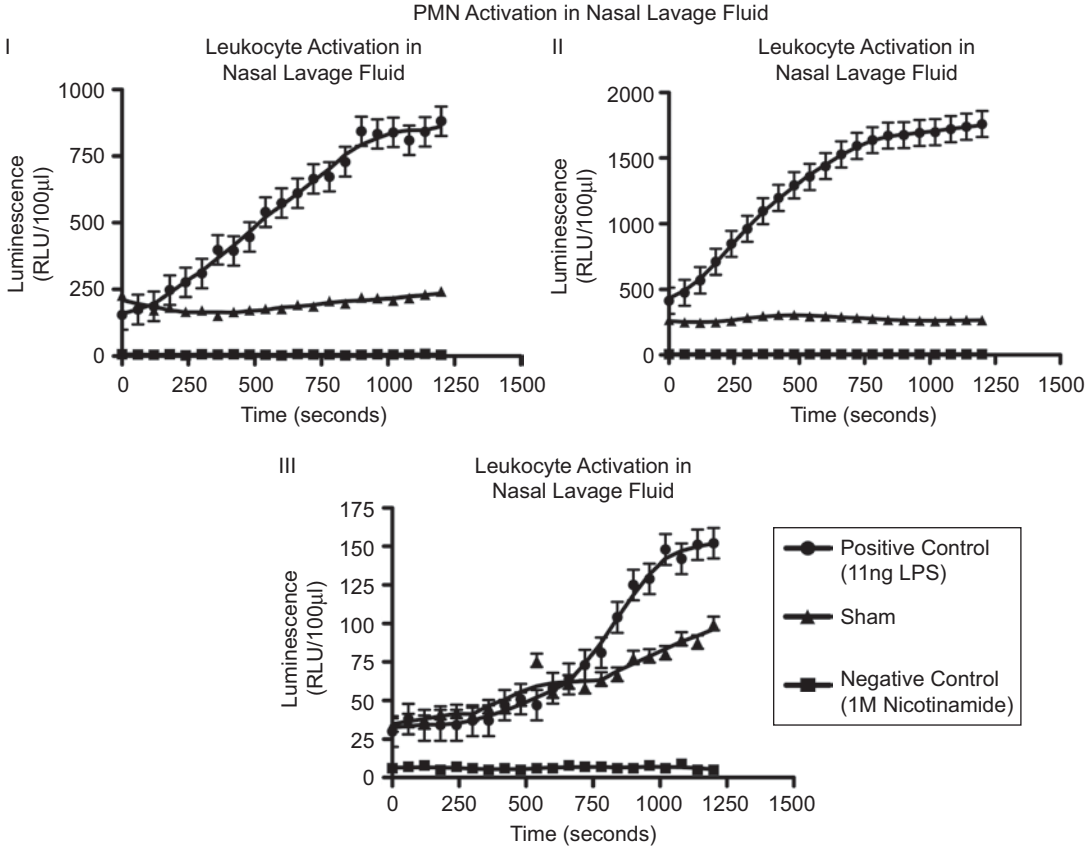

Figure 2. Graphs I, II and III represent polymorphonuclear leukocyte (PMN) activation in nasal lavage fluid (NLF). NLF samples (I–III) were incubated for 15 min and the chemiluminescence was measured for 20 min. The positive control increases exponentially and plateaus at approximately 13 min. The sham treatment represents basal chemiluminescence levels. The negative control produced very low chemiluminescence as CR3 receptors were blocked. The difference between the three treatments for graph I was $p = 0.0001$, graph II $p = 0.0001$ and graph III $p = 0.001$ as determined by a one-way ANOVA test.

Airborne endotoxin

Ambient levels in the facility ranged from 0.4 to 13.6 EU m^{-3} as determined through the LAL assay.

Discussion

Sampling of the upper airways provides a useful approach for assessing airway pathology, as the nasal passages are the initial location for inflammatory reactions against inhaled pathogens. Nasal secretions using lavage techniques are relatively non-invasive and the ability to obtain multiple samples pre/post-exposure can reflect physiological perturbations resulting from pathogen exposure. Inhaled LPS binds LBP in the upper airways, which stimulates the expression of CR3 (CD11b/CD18) on PMNs (Alexis et al. 2003, Moreland et al. 2002, Ungerstedt et al. 2003, Wright et al. 1991, Zhou et al. 2005).

Once CR3 is expressed, PMNs are activated and ready for a respiratory burst. In our assay zymosan binds CR3 receptors resulting in an oxidative burst thus forming reactive oxygen species (ROS). The ROS species then combine with luminol and cause the release of light photons, which are measured by the chemiluminometer. The seven subjects exposed to occupational endotoxin provided NLF samples containing primed neutrophils. Using the NLF samples we measured basal expression of CR3 on PMNs via chemiluminescence (sham treatment). Moreover, NLF samples treated with exogenous LPS (positive control) showed increased chemiluminescence. Conversely, chemiluminescence was inhibited in the negative control by blocking CR3 receptors with

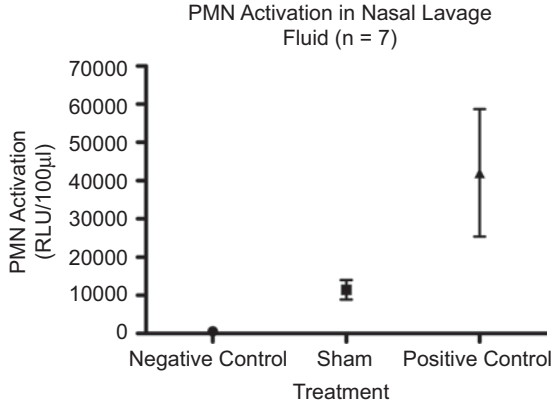

Figure 4. Polymorphonuclear leukocyte (PMN) activation in nasal lavage fluid. The graph represents the mean ± SEM PMN activation at the three treatments ($p < 0.05$).

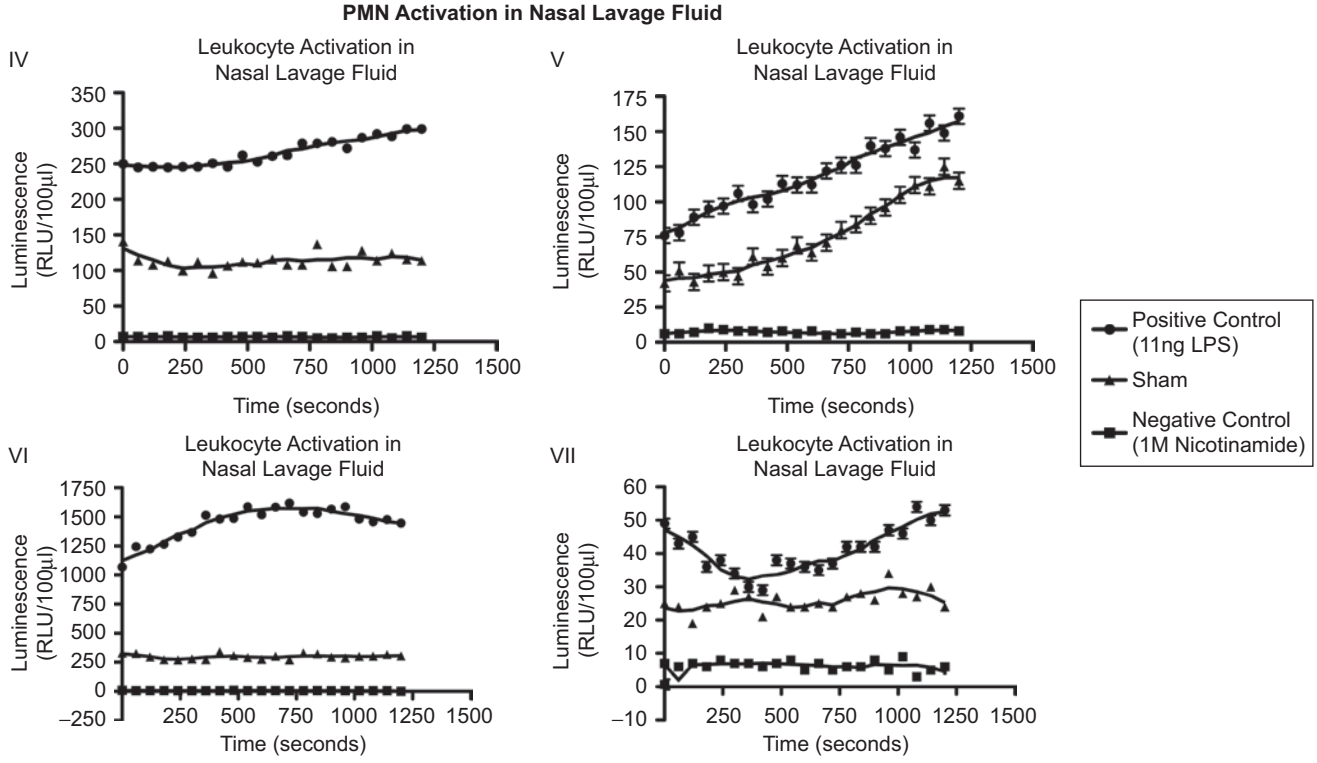

Figure 3. Graphs IV, V, VI and VII represent polymorphonuclear leukocyte (PMN) activation in nasal lavage fluid (NLF). NLF samples (IV–VII) were incubated for 20 min and the chemiluminescence was measured for 20 min. The sham treatment represents basal chemiluminescence levels. The positive control is approximately twofold larger than the sham treatment for all four NLF samples. The negative control produced very low chemiluminescence as CR3 receptors were blocked. The difference between the three treatments for all four graphs was statistically significant as determined through a one-way ANOVA test (graph IV $p = 0.0001$, graph V $p = 0.001$, graph VI $p = 0.0001$ and graph VII $p = 0.001$).

nicotinamide. These findings were apparent in all seven subjects over the entire 20 min with a high degree of statistical significance (Figures 2, 3). PMN activation in NLF was determined by the chemiluminometer software, which integrated the raw chemiluminescence values for each treatment over the 20 min (Figures 2, 3). The mean PMN activation for the negative control was 548 ± 15.65 RLU 100 µl, sham: 11 469 ± 2582 RLU 100 µl and 42 026 ± 16 659 RLU 100 µl for the positive control ($n = 7$; $p < 0.05$) (Figure 4). Mean PMN activation levels determined using chemiluminescence indicates the assay is able to successfully detect inhaled endotoxin exposure as it can quantify sham PMN activity, increased PMN activity in samples spiked with LPS and decreased PMN activity in samples with a CR3 inhibitor.

The sham chemiluminescence levels of the subjects ($n = 7$) varied between 25–250 RLU/100 µl PMN priming is dependent on CD14 and therefore levels of sCD14 vs mCD14 will cause a varied response to LPS (Troelstra et al. 1997). Although sCD14 enhances the LPS response it is not as effective as mCD14 (Troelstra et al. 1997); thus higher levels of mCD14 would cause increased CR3 expression, which in turn would increase chemiluminescence levels. Furthermore, the samples were collected and analysed in three consecutive days with three samples on the first day, three samples on the second day and one sample on the third day. Samples were also analysed within 1 h of collection each day. Therefore, the variation in chemiluminescence between the seven subjects is due to interindividual variation.

This assay measures the expression of CR3 receptors, which is directly induced by LPS exposure (Ungerstedt et al. 2003, Wright et al. 1991). Previous investigations to determine a successful biomarker for endotoxin exposure have been largely unsuccessful; however, a study by Borm et al. (2000) reported that increased endotoxin exposure correlated with an increased ratio between bactericidal permeability protein (BPI) and LBP in NLF (Borm et al. 2000). Although the study by Borm et al. (2000) was able to determine a marker of endotoxin exposure, the subjects involved in their study had been exposed to endotoxin at levels greater than 2000 EU m^{-3} in an occupational environment (Borm et al. 2000). Endotoxin exposures at these levels are considered to induce an adverse systemic response (Rylander 1995). Moreover, a study conducted by Heederik and Douwes (1997) determined that endotoxin exposures from 10 to 100 Eu m^{-3} induced airway inflammation resulting in adverse respiratory health effects which has also been confirmed in other studies (Yang 2004). In our study the subjects were exposed to endotoxin between levels of 1 and 15 EU m^{-3}, approximately 150-fold lower than the subjects in the Borm et al. study (2000). At these exposures we were able to quantify a marked response in PMN activation determined through chemiluminescence measurements. Using NLF samples from individuals who had primed PMNs due to occupational endotoxin exposure we were able to induce increased PMN activation with exogenous LPS and decrease PMN activation by using a CR3 inhibitor. Furthermore, another study investigating airborne endotoxin from laboratory animals, determined that levels of 0.12–14.63 EU m^{-3} induced respiratory symptoms in scientists and technicians (Pacheco et al. 2003). The current gold standard to quantify endotoxin exposure is through the use of air sampling, which determines ambient levels of endotoxin only and fails to consider *in vivo* exposure. It is thus important that a suitable marker is developed to measure endotoxin exposure at lower levels (10–100 EU m^{-3}), which can induce airway inflammation (Rylander 1995).

A limitation in studies that use the nasal lavage technique is the dilution factor between lavage samples (Borm et al. 2000, Frischer & Baraldi 2000). In order to correct for variable rates of recovery between NLF samples, total protein concentration is often measured in the NLF samples and the results are adjusted accordingly (Frischer & Baraldi 2000). However, the luminol-enhanced chemiluminescence assay used in this study to quantify endotoxin exposure determines the activation per neutrophil thus not requiring any additional dilution adjustments. As the assay measures the activation of integrin receptors (CR3) there may be other sources that can potentially activate CR3. One of the most common sources in occupational exposures is the family of β-(1,3)-D-glucans, a cell wall component of fungi, plants and certain bacteria (Thorn et al. 2001). A study by Thorn et al. determined that although glucans increased the release of inflammatory cytokines in blood, the amount of cytokines released was not statistically different from exposures conducted with saline (Thorn et al. 2001). Although there are other sources in occupational environments that may activate PMNs, exposure to LPS from the autolysis of gram-negative bacteria is still the most prevalent. Lastly, a limitation inherent in the technique is the route of breathing of the subject. PMN activation in NLF samples would provide a lower threshold of exposure if subjects employ the oral route of breathing during occupational exposure. This would cause LPS to enter the lower airways directly and bypass the nasal passages.

This is the first successful assay, which is capable of detecting endotoxin exposure at levels that can cause airway inflammation. In addition, the luminol-enhanced chemiluminescence assay is a rapid diagnostic test requiring only 30 min from sample collection to the endpoint of determining PMN activation. Most importantly, the assay quantifies *in vivo* exposure to endotoxin, which is ubiquitously found in many occupational settings (Borm et al. 2002, Christiani et al. 1993,

Dennekamp et al. 1999, Gillespie et al. 2006, Liebers et al. 2006, Madsen, 2006, Milton et al. 1996, Pacheco et al. 2003, Wang et al. 2005, Zock et al. 1998).

Occupational exposure to endotoxin can adversely affect respiratory health (Borm et al. 2002, Keman et al. 1998, Liebers et al. 2006, 2007, Liu 2004, Michel et al. 1997, Pacheco et al. 2003, Singh & Schwartz, 2005, Wang et al. 2005, Zock et al. 1998, 1999) and currently there is no standard for measuring inhaled endotoxin exposure (Liebers et al. 2007). Our findings indicate that a modified EA assay using luminol-enhanced chemiluminescence to determine PMN activation may provide a better means to measure endotoxin exposure.

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