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Comparison of *In Vivo* Bioluminescence Imaging and Lavage Biomarkers to Assess Pulmonary Inflammation

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Abstract

Gram-negative bacterial endotoxin triggers innate immunity via TLR-4 and NF- κ B signal activation. The aim of this study was to evaluate the use of transgenic mice expressing luciferase as a marker of NF- κ B activation for exploring innate immune responses to pulmonary endotoxin exposure over time thus obviating the need for serial necropsies. Transgenic rNF- κ B-Luc BALB/c mice were exposed to two different types of endotoxin (*N. meningitidis* lipooligosaccharide, and *E. coli* lipopolysaccharide) at multiple doses by nasal instillation. Bioluminescence was quantified *in vivo* at five time points in three separate experiments. In the fourth experiment lungs were imaged *ex vivo* 8 h post exposure and tissue was analyzed for luciferase activity. Non-transgenic BALB/c mice were similarly exposed to lipooligosaccharide and bronchoalveolar lavage was assessed for neutrophil recruitment and IL-6. Non-transgenic BALB/c mice exhibited highly significant increases of IL-6 and neutrophils in bronchoalveolar lavage 4 h after the exposure to instilled doses as low as 30 EU/mouse. In contrast, luciferase imaging of NF- κ B signal activation *in vivo* in transgenic rNF- κ B-Luc mice did not show significant changes over time or over doses from 30 EU to 300,000 EU/mouse of nasally-instilled endotoxin. *Ex vivo* lung imaging 8 h after endotoxin exposure to 3,000 EU demonstrated a strong signal. An intravenous LPS dose of 300,000 EU/mouse produced a measurable luminescence signal *in vivo*. This non-terminal assessment method is useful only with extremely high doses of endotoxin that induce systemic injury and cannot be applied to research of occupational and environmental exposures at relevant levels of endotoxin.

Keywords

endotoxin; bioluminescence; NF- κ B activation; inflammation; transgenic

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1. Introduction

Endotoxin (lipooligosaccharide or lipopolysaccharide) is an ubiquitous environmental contaminant and a potent inflammatory agent of Gram-negative bacteria. It represents one of the activators of Toll-like family receptors and a very potent stimulus for NF- κ B production (Blackwell et al., 1999). NF- κ B activity in various organs has been investigated using transgenic mice carrying a luciferase reporting gene by measuring the bioluminescence signal after introduction of luciferin (Carlsen et al., 2002). Recent studies have explored the use of these mice for the detection of gene activation and measurable bioluminescence signal of NF- κ B activation in various conditions such as hyperoxia (Yang et al., 2004), lung inflammation (Sadikot et al., 2003) proinflammatory responses of prostate tissue (Vykhovanets et al., 2008), endotoxin responses (Yull et al., 2003) or inhibitory effect of basidiomycete fungi on NF- κ B activation stimulated with LPS (Hseu et al., 2010). In addition, it was suggested that bioluminescence signaling can be a useful diagnostic tool for neuroblastoma detection (Dickson et al., 2007), myocardial infarction (Tillmanns et al., 2006) or in measuring the activation of T cell population in lymphoid organs in mice (Azadniv et al., 2007). In these studies, varying signal strengths were detected, but all of them used luciferase transgenic mice as a tool for exploring the development of disease or host responses. One advantage of this approach is the ability to evaluate the progression of outcomes in individual mice over time. Thus, the same mouse is successfully utilized in the assessment of the baseline signal and its alterations at later time points. At the end of the experiment, mouse lungs could be excised for measuring the signal and luciferase activity *ex vivo* (Yull et al., 2003). In the last decade, effort has been expended to develop more sensitive and sophisticated methods for the evaluation of inflammatory responses. Gene regulation of lung inflammatory responses is still not clear and this method could be an important application for these techniques. However, we noted that these studies have resorted to extremely high endotoxin doses. Thus, we sought to use transgenic mice expressing luciferase as an *in vivo* approach for exploring innate immune responses to pulmonary endotoxin at environmentally relevant exposure levels. The aim of this study was to quantitatively assess the relationship between endotoxin administration, inflammatory responses in lungs and the activation of NF- κ B *in vivo* in NF- κ B luciferase (rNF- κ B-Luc) transgenic mice. It was hoped that if sufficiently sensitive, use of the rNF- κ B-Luc mice could reduce the number of animals needed for time course studies. These data were previously presented in abstract form (Thorne et al., 2006), and some control data were drawn from a previous study (Hadina et al., 2008).

2. Materials and methods

2.1. Exposures of rNF- κ B luciferase transgenic mice to endotoxin

Non-transgenic BALB/c (Jackson Laboratories, Bar Harbor, ME) and XEN/Zhang/rNF κ B-Luc-B/C (line rNF- κ B-Luc-B/c) mice on the BALB/c background (Xenogen Corp., Alameda, CA) were used. Transgenic mice were in limited supply and therefore used for as many as four exposures beginning with the lowest dose and with a minimum rest period between trials of 7 days (median=21 days). All exposure protocols were approved by the Institutional Animal Care and Use Committee. Endotoxin exposures used lipooligosaccharide (LOS) extracted from *Neisseria meningitidis* as previously described (Hadina et al., 2008) and LPS from *Escherichia coli* (O111:B4; Sigma-Aldrich, St. Louis, MO). LOS and LPS were nasally instilled and in one experimental setup LPS was administered intravenously into the tail vein. Three different doses of endotoxin were administered (30, 300, 3,000 EU in 50 μ L per mouse) to explore innate immune responses in lungs. Higher doses were also used (30,000 and 300,000 EU/mouse) to establish the sensitivity of bioluminescence detection. Mice that served as controls were administered 50 μ L of sterile, pyrogen-free saline.

2.2. Bioluminescence imaging and luciferase activity

To quantify bioluminescent photon flux, mice were imaged with an IVIS Imaging System (IVIS-100, Xenogen) using an ultrasensitive CCD camera. Mice were anesthetized with 4% and maintained with 2.5% isoflurane in oxygen during imaging. Camera resolution was set at medium sensitivity and D-luciferin (Xenogen) was injected intraperitoneally at 3 mg/mouse 5 min before post-endotoxin imaging time points of 1, 4, 8, 24, and 48 h for the three experimental sets. In the last experiment, 4 and 8 h after endotoxin exposure, high camera sensitivity was used, the abdomen was masked to block interference with the thoracic signal and imaging was performed at 10, 15, 20 and 25 min after luciferin injection (7.5 mg/mouse) in an attempt to maximize sensitivity. Overlay images from each experimental set were loaded as a sequence and analyzed using Living Image Software (Ver. 2.50.1 Xenogen). Measurement regions of interest were selected and evaluated separately as signals from the neck, thoracic and abdominal region. Baseline signals obtained from each mouse 1 d before exposure were used for the comparison with the signals quantified after the exposures. The luminescence values were evaluated as total photon flux and expressed as photons/sec. (p/s). In the fourth experiment immediately after the last *in vivo* imaging (8 h time point) mice were sacrificed, lungs were excised, and imaged within 1 and 5 min at the high sensitivity camera settings. Following imaging, lungs were frozen in liquid nitrogen and stored at -80°C for further analysis. Luciferase activity was measured on the frozen tissue processed according to Promega Luciferase Assay System Protocol (Madison, WI) (Manthorpe et al., 1993). Lung tissue lysate was collected and luciferase activity was measured in relative light units (RLU) using a luminometer (Monolight-3010, Pharmingen, San Jose, CA). Total protein was measured using BCA protein assay (Thermo Fisher Scientific, Inc., Rockford, IL).

2.3. Exposure of non-transgenic mice and evaluation of inflammatory markers in bronchoalveolar lavage

In a parallel experiment, non-transgenic BALB/c mice were exposed to LOS at 30 or 300 EU/mouse by nasal instillation under short term inhalation anesthesia and sacrificed after 1, 4, 8, 24, or 48 h. Lungs were lavaged with 4 mL of saline and bronchoalveolar lavage fluid was analyzed for neutrophils and concentrations of IL-6 using a multiplex immunoassay (BioRad Laboratories, Inc., Hercules, CA) (Hadina et al., 2008).

3. Results

3.1. Endotoxin responses and bioluminescence signal measured *in vivo* in rNF- κ B-Luc mice after nasal instillation and intravenous dosing

Using medium sensitivity camera settings and doses of 30, 300, 3,000 and 30,000 EU/mouse, nasal instillation of LOS from *N. meningitidis* and LPS from *E. coli* resulted in a weak and barely detectable luminescence signal in thoracic region, that was not significantly different from the baseline (Fig. 1). Similar weak signal strengths were observed 1, 4, 8, 24, and 48 hours post exposure. Signals measured over the neck and abdominal regions also did not show significant differences over the time series. In an effort to generate a detectable signal, two mice were exposed to an extremely high dose of LPS from *E. coli* (300,000 EU/mouse) via two different routes of administration: nasal instillation and intravenous injection (Fig. 2A). Comparison of bioluminescence signal with baseline demonstrated a 4.6-fold higher response in the thoracic region at 4 h time point in the mouse treated intravenously. In addition, there was a significant systemic response at four hour time point showing 3.6-fold increase of photon flux in the neck, and an 8-fold increase in the abdominal region of the mouse after the intravenous injection of LPS when compared with the baseline signal. Nasal instillation did not result in any significant changes in the signal over time (Fig. 2B).

3.2. Nasal instillation of endotoxin and assessment with the abdomen masked

To explore the possibility that results obtained in the previous experiments could have been due to a sub-optimal amount of luciferin administered, shorter time period between its administration and imaging, or too low CCD camera sensitivity, we imaged the mice using higher camera sensitivity with the abdomens masked with black paper. In addition we explored the signal strength at four 5- min consecutive imaging times. Using this approach, signals obtained in the thoracic area alone 10 min after the luciferin injection did not show any significant increase at 4 or 8 h time points after nasal instillation of *N. meningitidis* LOS at 3000 EU/mouse (Fig. 3A and B). Bioluminescence signals measured 15 min after luciferin injection showed slightly higher signal strengths than values obtained after 10, 20 and 25 min (data not shown) for the 4 hour post endotoxin time point but were still of little use for this type of research. Augmenting the dose of luciferin from 3 to 7.5 mg/mouse increased the signal strength overall, but points in the neck region were saturated in both, a control mouse and an exposed mouse.

3.3. Lung signals measured ex vivo and luciferase activity in lung homogenates

When we excised and imaged the lungs at the 8 h time point using high camera sensitivity settings, there was a significant difference in the signal strength between control and exposed mouse lungs (Fig. 3C). Comparison of pseudo-images and total photon flux measurements showed a significant difference in measurable signal in excised lungs. Figure 3D and 3E show that total photon flux from imaging excised lungs correlated well with luciferase activity measured in the luminometer. Luciferase activity assayed in the tissue homogenates in half of the lung tissue was 48-fold higher in lungs of mice treated with the dose of 3,000 EU of LOS than in mice treated with saline alone.

3.4. Endotoxin response and inflammatory outcomes in BALB/c mice

In order to compare the sensitivity of bioluminescence as a readout of inflammation to standard biomarkers, we exposed non-transgenic BALB/c mice by nasal instillation to 30 and 300 EU/mouse mimicking exposures in the rNF- κ B-Luc mice. Inflammatory responses in lungs were observed after 1, 4, 8, 24, and 48 hours and characterized by assessing neutrophils and IL-6 in BAL (Fig. 4A and 4B) (Hadina et al., 2008). Significant increases in BAL neutrophils were seen 4 h after these low dose exposures reaching a peak at 24 h when 5- and 15-fold increases were found in comparison with controls. Levels of IL-6 increased dramatically peaking at 4 h post exposure, increased by 9- and 14-fold, and returning to baseline at 48 h. Comparable nasal instillation exposures in the rNF- κ B-Luc mice did not produce differences in measureable bioluminescence signals (Fig.1 top, middle).

4. Discussion

Many studies have evaluated inflammatory responses in mice upon exposure to microorganism associated molecular patterns (MAMPs) involving those acting through the Toll-like receptors, the NOD-like receptor and the RIG-I-like receptors (Sigsgaard et al., 2008). NF- κ B is a protein complex that is a common downstream signal for many pattern recognition receptors and governs cytokine and chemokine production, and neutrophil recruitment (Thorne and Duchaine, 2007). Thus, a murine model in which the induction of inflammation could be monitored over time in a single group of animals would facilitate time course studies with relatively few mice. The alternative is serial-sacrifice studies where typically groups of five to six animals are necropsied at each time point and compared to concurrent sham-exposed controls. Thus, the purpose of this study was to determine if the rNF- κ B-Luc mice yielded a sensitive enough bioluminescence signal upon exposure to endotoxin, a very potent TLR ligand, to allow monitoring of the development of lung

inflammation at environmentally relevant doses. If successful, this approach could be employed for other MAMPs that act through NF- κ B.

Endotoxin inhalation has been shown to induce detectable increases in neutrophil recruitment and cytokine production at doses as low as 5 EU/mouse (500 pg/mouse) in endotoxin-responsive mouse strains such as BALB/c (Hadina et al., 2008) and C3HeB/Fe with 4 h inhalation to airborne concentrations of 0.032 $\mu\text{g}/\text{m}^3$ ($\sim 320 \text{ EU}/\text{m}^3$) (Thorne et al., 1999) or approximately 1 EU/mouse retained dose. After 24 hours, doses of 30 and 300 EU/mouse produced an increase in lavage neutrophils from 121,000 cells/mouse in controls to 608,000 cells/mouse and 1,815,000 cells/mouse, respectively (Hadina et al., 2008). Transgenic rNF- κ B-Luc mice did not demonstrate significant quantifiable endotoxin-induced luciferase signals in the lungs *in vivo* at nasally-instilled doses up to 300,000 EU/mouse. Dosimetry calculations show that this amount would be equal to acute human exposure to 8.4×10^8 EU. This represents an extremely high exposure level that exceeds concentrations measured in the occupational settings with the highest measured levels (i.e. swine confinement facilities). It is known that asthma-like syndrome can develop at exceeding the dose of 200 EU/ m^3 and a daily exposure limit for occupational settings has been suggested at 50 EU/ m^3 (Thorne and Duchaine, 2007). This demonstrates that evaluation of the luciferase bioluminescence signal in the live animals was not useful as a marker of NF- κ B activation at environmentally relevant doses of endotoxin. Intravenous endotoxin application resulted in a strong signal over the whole body including measured regions *in vivo*. Bioluminescence signal strength after intravenous injection peaked 4 h after the exposure in correspondence with cytokine release obtained in BALB/c mice. However, this strong signal measured with an ultrasensitive camera was associated with visible clinical signs of difficulties in breathing and increased breathing rate in exposed mice. It was apparent that these mice experienced respiratory distress and systemic inflammation and thus a bioluminescence method was not required to determine that these mice were adversely affected. Using high camera sensitivity settings we found no significant difference in the signals detected over the thoracic region *in vivo* but excision and lung imaging *ex vivo* resulted in a strong and readily detectable luminescence signal. Although it was reported that the luciferin dose plays a significant role in the amounts of emitted photons and increased signal detection (Yull et al., 2003) we did not see significant differences between signals for luciferin doses of 3 or 7.5 mg/mouse except the image saturation located in the neck region. There are several different studies that successfully evaluated NF- κ B signal in the whole body and separate organs after the exposure to endotoxins (Ho et al., 2007; Yull et al., 2003). However, these studies used significantly higher doses of endotoxin of little relevance to environmental exposures. Yull and coworkers used intraperitoneal injections of 3 $\mu\text{g}/\text{g}$ endotoxin ($\sim 700,000$ EU per mouse) or aerosol exposure with the concentration of 1 mg LPS/mL solution in duration of 30 min (Yull et al., 2003), while Ho et al. intraperitoneally injected 4 mg/kg endotoxin ($\sim 800,000$ EU per mouse) (Ho et al., 2007). Our data showed that measurement of NF- κ B signaling in lungs *in vivo* using rNF- κ B-Luc mice was orders of magnitude less sensitive than measuring neutrophils or cytokines in lung lavage which were markedly and significantly increased at 30 EU/mouse. Thus, this non-terminal bioluminescence method was useful only at very high doses of endotoxin that were irrelevant to occupational environmental exposures.

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Fig.1.

Nasal instillation of different doses of endotoxin (*N. meningitidis* LOS, *E. coli* LPS; 30, 300, 3,000 EU, and 30,000 EU) or saline (control). Quantification of the bioluminescence signal using Living Image Software in the neck, thoracic and abdominal region represented for five rNF- κ B-Luc mice separately.

Fig. 2.

Nasal instillation and intravenous injection of *E. coli* LPS and evaluation of bioluminescence signal at medium sensitivity camera settings. A) *In vivo* images of three rNF- κ B-Luc mice treated with: nasal instillation of saline (control) (left), intravenous injection (middle), and nasal instillation (right) of 300,000 EU LPS (baseline, 1, 4, 8, 24 and 48 h after the exposures). B) Graphs with quantified signals in the neck, thoracic, and abdominal region.

Fig. 3.

Nasal instillation of *N. meningitidis* LOS and *in vivo* imaging using high sensitivity camera settings. A) Bioluminescence images obtained from four rNF- κ B-Luc mice *in vivo* after nasal instillation of (from the left to the right): saline (control, n=2), 3,000 EU of LOS (n=2) with masked abdomen (baseline, 4, 8 h time points). B) Bioluminescence signals measured over the thoracic region. C) Bioluminescence images obtained from rNF- κ B-Luc mice lungs *ex vivo* (controls vs. exposed) at 8 h time point. D) Total photon flux measured in lungs using IVIS-100 system. E) Luciferase activity in the half lung tissue expressed as RLU/ μ g protein.

Fig. 4.

N. meningitidis LOS time course for the exposures of non-transgenic BALB/c mice: saline (controls), 30 and 300 EU per mouse with standard BAL outcomes: neutrophils (PMN) and IL-6 levels (average values of six mice per group).