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Bioluminescent Detection of Endotoxin Effects on HIV-1 LTR-driven Transcription in Vivo

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SUMMARY We investigated the effects of Gram-negative bacterial lipopolysaccharide (LPS) on luciferase expression in transgenic reporter mice in which luciferase expression is driven by the nuclear factor κB (NF-κB)-dependent portion of the human immunodeficiency virus-1 (HIV-1) long terminal repeat (HIV-1 LTR). Using these mice, we dissected the sources of luciferase activity at the organ level by (a) assessing luciferase activity in organ homogenates, (b) bioluminescence imaging in vivo, and (c) bioluminescence imaging of individual organs ex vivo. Luciferin dosage was a critical determinant of the magnitude of photon emission from these reporter mice. Photon emission increased at doses from 0.5–6 mg of luciferin given by intraperitoneal (IP) injection. The differential between basal and LPS-induced bioluminescence was maximal at 3–6 mg of luciferin. Luciferase expression was highly inducible in lungs, liver, spleen, and kidneys after a single IP injection of LPS, as assessed by luciferase activity measurements in organ homogenates. Luciferase activity was also induced in the forebrain by treatment with IP LPS. In contrast, aerosolized LPS produced a response localized to the lungs as assessed by both bioluminescence and ex vivo luciferase assay measurements. These studies demonstrate the utility of luciferase reporter mice for determining organ-specific gene expression in response to local and systemic stimuli. (J Histochem Cytochem 51:741–749, 2003)

Bioluminescence is the emission of visible light from living creatures mediated by an enzyme-catalyzed reaction of molecular oxygen with luciferin. A variety of different bioluminescent systems have been identified in nature, each requiring a specific enzyme and substrate. Firefly luciferase produces photons in a reaction that requires ATP, magnesium, and a benzothiazoyl–thiazole luciferin (Wilson and Hastings 1998). Light emission from the firefly luciferase-catalyzed luciferin reaction is broad band (530–640 nm) and peaks at 562 nm (Rice et al. 2001). This emission spectrum, coupled with the optical properties of biological tissue, allows light (especially with spectral content above 600 nm) to penetrate through several centimeters of tissue. Therefore, it is possible to detect light emitted from internal organs in mice that express luciferase as a reporter gene. Current bioluminescent imaging techniques offer the potential to investigate contributions of specific molecules to disease pathogenesis in genetically engineered mice in models of human diseases. This powerful technique can reduce the number of animals required for experimentation because each animal can be used as its own control and multiple measurements can be made in the same animal over time, minimizing the effects of biologic variation.

We have recently reported a line of transgenic reporter mice [referred to as HLL (HIV-LTR/Luciferase)
mice) that are engineered to carry the proximal 5′ human immunodeficiency virus (HIV-1) long terminal repeat (LTR) driving the expression of Photinus luciferase complementary DNA (cDNA) (Blackwell et al. 2000). The proximal HIV-LTR is an extensively characterized NF-κB-responsive promoter (Kretzschmar et al. 1992) that contains a TATA box, an enhancer region between nucleotides −82 and −103 with two NF-κB motifs and three Sp1 boxes from nucleotides −46 to −78. NF-κB activation is absolutely required for transcriptional activity of the proximal HIV-LTR (Moses et al. 1994; Alcami et al. 1995), and luciferase expression in these mice closely correlates with independent measurements of the NF-κB activation pathway and NF-κB-dependent gene expression (Blackwell et al. 2000).

In this study, we explored bioluminescent detection of HIV-LTR-driven luciferase production after injection of E. coli endotoxin (lipopolysaccharide, LPS), a known stimulus for NF-κB activation and HIV-LTR promoter activation. Previously, we have shown that 30–60 min is the optimal time for bioluminescence measurement after a single IP injection of luciferin (Sadikot et al. 2001) and that chest bioluminescence is tightly correlated with lung luciferase activity in two different models of lung inflammation (Sadikot et al. 2001, 2002). In the present study, we report the kinetics of luciferase activity as determined by bioluminescence in individual mice after IP injection of LPS. We performed detailed dose–response studies to identify the relationship between luciferin dose and detectable bioluminescence in HLL mice. We have dissected the source of inducible and basal expression of luciferase and have evaluated the systemic nature of the endotoxin response by determining the effect of treatment with LPS on luciferase expression in the lungs, liver, spleen, kidney, large intestine, and brain. In addition, the systemic effects of IP LPS on activation of the HIV-LTR are contrasted to the localized pulmonary effects of exposure to aerosolized LPS.

Materials and Methods

Animal Model

Transgenic mice weighing 20–30 g and expressing Photinus luciferase cDNA under the 5′ HIV-LTR (C57B6/DBA background) were used for all experiments. The mice used in these studies were hemizygous for the transgene. E. coli LPS (serotype 055, B5; Sigma Chemical, St Louis, MO) was given as a single IP injection (3 μg/g in 200 μl normal saline) or by aerosol (1 mg/ml solution in sterile normal saline). For treatment with aerosolized LPS, each cage was placed in a closed chamber and LPS solution was delivered by ultrasonic nebulization over 30 min. The endotoxin solution was delivered as a continuous aerosol with a driving flow rate (8 liters/min) that was generated by a small volume nebulizer (Resigard II; Marquest Medical, Englewood, CO) over a standardized 30-min interval. According to the manufacturer, the average generated particle size is 1.67 μm. The optimal dose and duration of the LPS aerosol were determined in previous studies (Sadikot et al. 2001). All studies were approved by the Vanderbilt University Institutional Animal Care and Use Committee.

In Vivo Bioluminescent Determination of Luciferase Activity

Mice were anesthetized with ketamine/xylazine before imaging to immobilize them for the duration of the integration time of photon counting (3 min). Mice were shaved over the chest and abdomen before imaging. Luciferin (1 mg/mouse to 12 mg/mouse in 200 μl isotonic saline) was given as an IP injection and mice were imaged in a supine position with an intensified charge-coupled device (ICCD) camera (C2400-32; Hamamatsu, Bridgewater, NJ). Based on our previously reported results (Sadikot et al. 2001), all bioluminescence measurements were done 30 min after IP injection of luciferin. For the duration of photon counting, mice were placed inside a light-tight box. Light emission from the mouse was quantified by photon counting using Argus image processing software (Hamamatsu). A digital false-color photon emission image of the mouse was generated for illustration purposes and captured photons were counted over a standard area (500 pixels) corresponding to the region of the thorax and head overlying the midlung zone and brain, respectively. Baseline photon counts were obtained before LPS challenge so that each mouse could be used as its own control.

In some experiments, a skin flap excision over one side of the chest was made under anesthesia before bioluminescent imaging. In these studies, mice were sacrificed after imaging and before recovery from anesthesia.

Ex Vivo Bioluminescent Determination of Luciferase Activity in Individual Organs

Various organs were excised from sacrificed control and LPS-treated mice 30 min after IP luciferin injection. Organs were placed in RPMI 1640 cell culture medium and immediately imaged as described above.

Luciferase Activity Assays in Organ Homogenates

Mice were asphyxiated with CO2 and organs were removed, homogenized in 1 ml of reporter lysis buffer (Promega; Madison, WI) using a Dounce homogenizer, and stored at −20°C. Luciferase measurements were made by adding 100 μl of freshly reconstituted luciferase assay buffer (Promega) to 20 μl of organ homogenate. Luciferase activity was measured as relative light units (RLU) using a standard luminometer (Monolight 3010; Analytical Luminescence Laboratories, San Diego, CA). Luciferase activity was normalized for total protein content, which was measured with the Bradford assay (Bradford 1976).

Statistical Analysis

Our statistical analyses were performed with GraphPad InStat version 3.01 for Windows NT (GraphPad Software; San Diego, CA) using an unpaired t-test and unpaired ANOVA test.
Results

Time Course for Bioluminescence After LPS

Initial studies were done to determine the time point of maximal bioluminescence of HLL mice after a single IP injection of 3 μg/g of LPS. In these studies, 1 mg luciferin was administered 30 min before each imaging procedure. Although increases in bioluminescence were noted at 1–2 hr in the abdomen, chest, and head, peak photon emission occurred between 4–8 hr and values returned to baseline by 24 hr (Figure 1). Repeat injections of luciferin into animals that were not treated with endotoxin were not appreciably different from the baseline value (data not shown). On the basis of these results, subsequent measurements of bioluminescence were made 4 hr after treatment with IP LPS.

Dose–Response Relationship Between Injected Luciferin and Detected Bioluminescence

To determine the dose–response relationship between the dosage of luciferin and photon emission in control mice (not treated with LPS), we treated HLL mice with progressively higher doses of luciferin (Figure 2). In this experiment, an escalating dosage of luciferin, from 0.5 mg to 12 mg, was administered every 2 hr to the same mouse (n=4) and bioluminescence was measured and quantitated over the head and chest (Figure 2B). We have previously shown that bioluminescence returns to background by 2 hr after a single dose of luciferin (Sadikot et al. 2001). In this experiment, we found a marked increase in bioluminescence with increasing doses of IP luciferin from 0.5 to 6 mg. Photon emission from the head and chest reached near maximum after injection of 6 mg of luciferin, indicating that luciferase activity in HLL mice is saturable with delivery of sufficient luciferin (Figure 2B). On the basis of this experiment, 1 mg, 3 mg, or 6 mg of luciferin was administered as a single IP injection to control and LPS-treated mice (Figure 3). Although differences were observed between the pretreatment and post-LPS treatment values at all luciferin dosages, the magnitude of the bioluminescence response was dependent on the exact luciferin dosage. Over the chest, 2.6-fold, 7.3-fold, and 7.6-fold increases in bioluminescence were found at the 1 mg, 3 mg, and 6 mg dosages of luciferin, respectively (Figure 4A). Only a relatively modest increase in bioluminescence was detected over the head, and this was best detected with the 3-mg dosage of luciferin (2.8-fold increase at the 3-mg luciferin dose and 1.8-fold increase at the 6-mg luciferin dose) (Figure 4B). In contrast to these values for in vivo bioluminescence, the luciferin dosage that was administered to these mice did not affect the ex vivo measurement of tissue luciferase activity in organ homogenates from either the lungs or the brain (Figures 4C and 4D). These data indicate that, although the administration of luciferin to the mice increases bioluminescence, there is no effect on tissue luciferase gene expression.

Analysis of the Source of Bioluminescence in the Chest

To determine the specificity of bioluminescence over the chest for measuring the lung’s response to LPS, a skin flap excision was performed over the right side of the chest wall while the left side of the chest remained intact (Figure 5A). For these studies, bioluminescence was measured independently directly over a standardized area on the left and right side of the chest at 4 hr after IP or aerosolized LPS. Luciferin was injected IP
at a dose of 3 mg 30 min before imaging. Removal of the skin overlying the chest wall attenuated basal and LPS-induced bioluminescence. However, the fold induction of bioluminescence over the chest (LPS treated/untreated control) was markedly increased by skin flap excision (Figures 5B and 5C). In these studies, fold induction of photon emission (treated/control) was markedly greater from the right (skin removed) chest (21.9-fold following IP LPS and 15.0-fold after aerosolized LPS) than from the left (skin intact) chest (3.7-fold after IP LPS and 3.9-fold after aerosolized LPS).

To further dissect the organ source of luciferase in HLL mice at baseline and after LPS treatment, the various components of the chest were excised and ex vivo tissue luciferase activity was measured (Figure 5D). The chest wall and skin had relatively high levels of luciferase activity that were not changed by treatment with LPS (IP or aerosolized). Luciferase activity in the heart was modest and unaffected by LPS treatment. In contrast, only a low level of basal luciferase activity was detected in lungs, and this was markedly induced by exposure to IP or aerosolized LPS. These data indicate that basal luciferase activity in the chest of HLL mice is primarily derived from skin and chest wall components, whereas LPS-inducible luciferase activity is primarily of lung origin. Therefore, external measurements of bioluminescence over the chest in HLL mice can be used as a surrogate marker for HIV-1 LTR gene expression in the lungs in this model.

Induction of Luciferase Activity in Visceral Organs and Brain by LPS Treatment

In addition to investigating the sources of luciferase in the chest of HLL mice at baseline and after LPS treatment, we measured tissue luciferase levels in the visceral organs and brain. In the abdomen (Figure 6A), increased luciferase activity was found in tissue homogenates of liver, spleen, and kidney after IP LPS. Interestingly, the highest values were seen in the large intestine, and this was not affected by LPS treatment. The high basal values for luciferase activity in the intestines in these mice markedly attenuated our ability to detect the LPS response of abdominal organs by bioluminescence. Aerosolized LPS did not bring about increased luciferase activity in any of the abdominal organs tested, demonstrating that this treatment provides a local, compartmentalized stimulus for HIV-LTR promoter activity at the doses used in this study.

Because a small increase in bioluminescence was detected over the head after treatment with IP LPS (shown in Figure 4), we measured luciferase activity in the forebrain (frontal lobe and cortex) and hindbrain (cerebellum) (Figure 6B). These data indicate that a
change in HIV-1 LTR-driven gene expression occurs in the forebrain in response to systemic LPS. As with the abdomen, no changes in brain luciferase activity were identified after aerosolized LPS.

Examination of Ex Vivo Bioluminescence
We excised various tissues from control and LPS-treated mice after injection of luciferin and examined bioluminescence ex vivo (Figure 7A and 7B). Abun-

![Figure 3](image)

Figure 3  Three mice were treated with 1 mg, 3 mg, or 6 mg of IP luciferin and bioluminescence was measured at baseline (BL) and 4 hr after a single IP dose of 3 μg/g of LPS.

![Figure 4](image)

Figure 4  Bioluminescence was measured over the chest (A) and head (B) of the mice shown in Figure 3 at baseline and after treatment with IP LPS. After death, luciferase activity was measured in lung (C) and brain (D) tissue. Luciferase activity is reported as relative light units (RLU) per μg protein (* indicates \( p<0.05 \) compared with baseline). Values represent mean ± SEM (n=3).
dant ex vivo photon emission was detected in the skin, and this was not increased in response to LPS treatment. Bioluminescence was detectable in the excised lung tissue after exposure to either IP or aerosolized LPS. In addition, bioluminescence was detected in the kidney, liver, and spleen in mice that were treated with IP LPS but not from mice treated with aerosolized LPS. These studies demonstrate that ex vivo bioluminescence is a feasible strategy to identify inducible luciferase activity in intact organs. This approach is particularly useful in the abdomen of HLL mice, because high basal luciferase activity is present in this area.

Discussion
Using a transgenic reporter mouse in which luciferase expression is determined by the NF-κB-dependent regulatory portion of the HIV-1 LTR, we have dissected the sources of luciferase activity at the organ level and have optimized noninvasive detection of luciferase activity by bioluminescence after delivery of luciferin to HLL mice. Our experiments show that luciferin dosage is a critical determinant of the magnitude of photon emission that results from the luciferase–luciferin reaction. In HLL mice, IP luciferin injection of 6–12 mg maximizes light output without affecting tissue lu-
ciferase activity as measured ex vivo in organ homogenates by a standard luciferase assay. The chest wall and skin express a relatively high level of luciferase in untreated control HLL mice. In contrast, lungs have an extremely low level of basal luciferase gene expression that is markedly upregulated by exposure to either IP or aerosolized LPS. Interestingly, whereas the response to aerosolized LPS is compartmentalized to the lungs, a single IP injection of LPS markedly increases luciferase activity in liver, spleen, and kidney. However, LPS treatment does not substantially affect high levels of basal HIV-LTR-driven luciferase gene expression.

Figure 6  Tissue luciferase measurements for the liver, spleen, kidney, and intestine (A) and for the forebrain and hind brain (B) after a single IP injection of 3 μg/g of LPS (IP LPS) or exposure to aerosolized LPS (inhaled LPS) (* indicates p<0.05 compared with control). Values are mean ± SEM (n=3).

Figure 7  Mice were treated with a single IP injection of 3 μg/g of LPS (IP LPS) or exposure to aerosolized LPS (Inh LPS). (A) Thirty min after treatment with 3 mg luciferin, the lung, kidney, liver, spleen, and skin were removed and placed in RPMI medium for ex vivo measurement of bioluminescence. (B) Photon emission was quantitated in organs from three separate HLL mice (mean ± SEM).

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expression in the large intestine and skin. Bioluminescence and tissue luciferase activity (as measured by standard luciferase assays in tissue homogenate) are induced in the forebrain by treatment with IP LPS, indicating that the cerebral inflammatory signals are a part of the response to systemic LPS. Finally, ex vivo bioluminescence is shown to differentiate the effects of LPS on various intra-abdominal organs. These studies demonstrate the utility of bioluminescence detection in luciferase reporter mice for determining organ-specific gene expression.

Firefly luciferase was cloned in 1985 (de Wet et al. 1985). Three years later, an assay to measure luciferase in mammalian cell lysates was developed (Nguyen et al. 1988), which enabled the luciferase gene to become a useful tool for in vivo studies of gene regulation. Luciferase is an excellent marker for gene expression because of its lack of post-translational modifications and an in vivo $t_{1/2}$ of approximately 3 hr (Lipshutz et al. 2001). An early report employed transgenic mice in which the HIV-1 LTR promoter drove expression of firefly luciferase (Morrey et al. 1993). In this study, topical luciferin application was used to examine luciferase expression in skin. Subsequently, improved methodology to track light emission non-invasively from the luciferase–luciferin reaction was developed (Contag et al. 1997; Wu et al. 2001). The ability to track light-emitting cells in small laboratory animals has opened up a wide range of applications in biomedical research, including the in vivo monitoring of tumor growth and metastasis (Contag et al. 1998, 2000; Edinger et al. 1999; Sweeney et al. 1999; Rehemtulla et al. 2002; Vooijs et al. 2002; Wetterwald et al. 2002), transplantation (Koransky et al. 2001), and gene therapy (Lipshutz et al. 2001; Wu JC et al. 2002). In addition, a variety of studies have been performed that employ bacteria expressing luciferase to investigate the pathogenesis of infections caused by Staphylococcus, Mycobacterium, E. coli, and Salmonella (Contag et al. 1995; Hickey et al. 1996; Siragusa et al. 1999; Francis et al. 2000, 2001; Rocchetta et al. 2001).

To date, studies that examine expression of promoter-luciferase gene expression in transgenic mice using whole-animal measurement of bioluminescence are few (Sadikot et al. 2001, 2002; Carlsen et al. 2002; Wu N et al. 2002). Here we have examined bioluminescence in LPS-treated mice that express firefly luciferase driven by the NF-κB-dependent regulatory portion of the HIV-1 LTR. Our findings using HLL mice are consistent with and complementary with a recent report of Carlsen and associates (2002), who examined whole-animal and regional expression of luciferase in transgenic mice where NF-κB-binding sites from the Ig κ light chain promoter were coupled to firefly luciferase. Together, these studies indicate that NF-κB activation can be monitored in vivo by bioluminescent imaging to detect luciferase activity as a surrogate marker for NF-κB-dependent gene transcription. An advantage of this technology is that it allows detection of molecular end points, such as activation of NF-κB, in the context of animal models of disease. Using bioluminescence as a surrogate end point of a molecular event allows detection of the entire evolution of activation in a single animal. In terms of study design, this enables fewer animals to be included in experiments to achieve statistically valid results and could potentially be applied to high-throughput studies that examine interdiction. Clearly, this technology can be applied to the examination of important gene regulatory regions and specific trans-activating factors that determine transcriptional rates of specific genes in vivo. Because dysregulation of gene expression has been proposed as a mechanism for the pathogenesis of a variety of human diseases, bioluminescent detection of promoter function using luciferase expression constructs in vivo is likely to become an important tool to investigate gene regulation in mouse models of human diseases.

The current methodology of simple two-dimensional projections of a three-dimensional, spatially distributed light source inside the animal is a powerful method that allows (semi)quantitative assessment of gene expression in a single animal over time. However, several improvements in the imaging technology are being pursued to overcome current limitations in spatial resolution and sensitivity of photon detection. A possible approach could involve improvements in the imaging method, e.g., by performing multiplane imaging combined with 3-D tomographic reconstruction algorithms. In addition, several groups are investigating the possibility of transiently reducing the scattering properties of tissue (in particular skin) to improve both sensitivity and spatial resolution of this technology (Pickett et al. 2002). Although there are some present limitations, this emerging technology can provide insight into complex gene regulation in intact organs that cannot be obtained by studies in cell culture systems.

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