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Morphine Induces Splenocyte Trafficking into the CNS

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Abstract

Opioids significantly alter functional responses of lymphocytes following activation. Morphine, an opioid derivative, alters the Th1 to Th2 response and modulates functional responses such as cytolytic activity and proliferation. Although there has been extensive research involving morphine's effects on lymphocytes, little is known about the effects morphine has on lymphocyte trafficking. The objective of the study was to use *in vivo* bioluminescent imaging to determine morphine's effect on the trafficking pattern of splenocytes systemically and into the CNS following a neuroinflammatory stimulus. A neuroinflammatory response was induced by intracerebrally administering a DNA plasmid producing IFN- γ in morphine-dependent or placebo wildtype mice. Mice with or without a neurostimulus received adoptively transferred firefly luciferase transgenic splenocytes and were imaged using a charge-coupled device camera. Morphine dependence significantly altered the inherent ability of splenocytes to traffic into the spleen, and lead to non-directed chaotic trafficking throughout the animal, including the CNS. The morphine-mediated effects on trafficking were blocked by naltrexone. Morphine dependence intensified splenocyte infiltration into the CNS following neuroinflammation induced by IFN- γ gene transfer. The study determined that morphine severely altered the ability of non-activated splenocytes to home to the spleen, inducing chaotic extrasplenic trafficking throughout the animals. Following a neuroinflammatory response, morphine exacerbated infiltration into the CNS.

Keywords

Opiates; *in vivo* imaging; Lymphocyte trafficking

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Introduction

Neuroinflammation induced by infectious diseases such as HIV lead to an increase of immune cells trafficking into the central nervous system (CNS) (Wu et al 2000) and (Shacklett et al., 2004). T lymphocytes infiltrate the CNS through three different areas. One pathway is by extravasating across the fenestrated endothelium of the choroid-plexus stroma, migrating through the stromal core and entering the CSF (Carristers et al., 2002) and (Svenningsson et al., 1995). The second pathway into the CNS is via the internal carotid artery, crossing the postcapillary venules at the pial surface of the brain, into the subarachnoid space and the Virchow-Robin perivascular space (Hickey and Kimura 1998), (Hickey et al., 1991), and (Lassmann et al., 1993). In the third pathway, leukocytes enter the CNS directly into the parenchyma through the branching vascular tree of arterioles and capillaries. In this pathway, however, leukocytes are required to cross through the intact blood brain barrier (Sacion et al., 1984).

Lymphocyte trafficking to sites of infection is an imperative function for the initiation of an immune response (Von Andrian et al., 2000). Many substances are capable of inhibiting lymphocyte responses to infections, including drugs of abuse (Friedman et al., 2003, Friedman and Eisenstein, 2004). Opioids act on the immune system through two major pathways: (1) the central pathway, as it can act on the hypothalamo-pituitary-adrenal axis or sympathetic nervous system, or (2) directly on immune cells (Friedman et al., 2003) through opioid receptors such as δ -, μ -, or κ receptors. While the three receptors are able to regulate immune responses, their expression levels are not homogeneous among all cells, and may differ among activation (Benard et al., 2009). The δ - and μ -receptors are absent on resting T cells, but they are upregulated upon activation (Roy et al 1991, Nguyen and Miller, 2002) (Jaume et al., 2007), (Madden et al., 2001) (Kraus et al., 2001) and (Boner et al., 2008).

Chronic treatment with morphine suppresses immune functions such as phagocytosis (Szabo et al., 1995), T- and B-lymphocyte proliferative response to mitogens (Bryant et al., 1991), natural killer activity (Gomez-Flores and Weber, 1999), (Olin et al., 2004) and (Yokota et al., 2000). While the effects of morphine on lymphocyte function have been well characterized, little is known involving morphine's effect on lymphocyte trafficking (Flores et al., 1995).

Recently, researchers have been able to track "*in vivo*" the effects of cancer treatments by using bioluminescent technology based on transgenic mice expressing firefly luciferase (Sweeney et al., 1999) (Rauch et al., 2009). When the animal is injected with d-luciferin (the substrate for luciferase), photon emission will be proportional to the location and amount of viable cells, including cells within the CNS (Ohlfest et al., 2005). The overall goal of this study was to use bioluminescent imaging to determine the effect of morphine dependence on splenocyte trafficking, with or without a neuroinflammatory stimulus. Morphine dependence significantly alters extrasplenic trafficking throughout the animal, including the CNS.

Materials and Methods

Mice

Transgenic FVB mice containing a CMV-[beta]-actin promoter (Niwa et al., 1991) and a bicistronic gene consisting of two reporter genes, firefly (fFL) luciferase and enhanced green fluorescence protein (eGFP) (Ohlfest et al., 2004) was used as source splenocytes. Wildtype FVB mice were purchased from the Jackson Laboratory (Bar Harbor, Maine). All animals were housed and cared for under specific pathogen free conditions in accordance with the University of Minnesota Institutional Animal Care and Committee.

Morphine

Mice were implanted subcutaneously with either a placebo pellet or a 75 mg morphine sulfate pellet. In experiments testing opiate specificity, the opiate mu-receptor antagonist naltrexone (34 mg) pellet was implanted in conjunction with a morphine pellet as previously described (Royet et al., 2005).

IFN γ plasmid inoculation

Animals were deeply anesthetized with a ketamine/xylazine cocktail solution (53.7mg/ml ketamine, 9.26 mg/ml xylazine) delivered at a concentration of 1ml/kg. Gene delivery of pKT2/CLP-mIFN- γ / polyethylenimine (PEI) complexes, pKT2/CLP empty vector / polyethylenimine (PEI) complexes control, were inoculated into the lateral ventricles was accomplished by slow infusion into the coordinates; 1 mm to the right, 0.5 mm posterior of the bregma, and 3.3 mm deep from the cortical surface of the brain. A final volume of 5 μ l was infused over 20 min (flow rate: 0.1 μ l/min) using a microinjection pump (CM 100, Carnegie Medicine, Stockholm, Sweden). DNA/PEI complexes were prepared as described (Wu et al., 2007); seven equivalents of PEI were used per μ g of DNA to generate the complexes. A total dose of 2.5 of DNA was administered, as described by (Sweeney et al., 1999) and (Wu et al., 2007).

In vivo bioimaging

Spleens were removed aseptically from FVB transgenic mice, single-cell suspensions were made by forcing the tissue through a cell strainer with a sterile syringe plunger. Erythrocytes were removed using a RBC lysing buffer. 4×10^6 splenocytes were resuspended in 100 ml of saline and adoptively transferred i.v. into treatment animals. Mice were imaged using Xenogen IVIStm50 system. In order to image live animals, mice were anesthetized by i.p. injection with 230 mg/kg of avertin to allow quick anesthesia and recovery time. Mice were then injected i.p. with 150 μ l of luciferin (28.5mg/ml, substrate for imaging) and imaged using the Xenogen bioimager as described (Ohlfest et al 2005). Luciferase positive splenocytes were quantitated *in vivo* by deriving a gate around the spleen, CNS, or whole animal measuring photon emittance using IgorPro 4.09A software (Alameda CA).

In vitro Luciferase Expression

Under 2 ml/kg of Ketamine/Xylazine anesthesia, mice were cardially perfused with 0.1M phosphate saline buffer solution. Freshly separated brain tissue was homogenized with 1x cell culture lysis reagent (Promega, Madison, WI). Cell debris was removed after centrifugation at 10,000 X g for 5 min. Twenty μ l of supernatant of brain lysate was mixed with 100 μ ls of luciferin substrate (Promega, Madison, WI). Luciferase expression on brain homogenates was measured by luminometer, lumat LB 9507 (Berthold Technologies, Oak Ridge, TN). Protein concentration of the brain homogenates was determined by Quick start Bradford protein assay kit (Bio-Rad, Hercules, CA). Brain homogenate protein levels were normalized in order to assess luciferase activity by measurement by the luminometer.

Results

Opiates have profound immunomodulatory effects on multiple immune parameters, inhibiting the ability to mount an adequate immune response in response to pathogen insult (Roy et al., 2004). While extensive research on the effects of morphine on the immune system has provided convincing evidence on morphine's immunomodulatory effects on immune responses (Roy et al., 2004), (Gomez-Flores and Weber, 1999), (Olin et al., 2007) and (Yokota et al., 2000), however, due to experimental limitations, the effects opiates have on immune cell trafficking remains undetermined. Recently, the ability to follow "*in vivo*"

trafficking patterns of luciferase transfected cells (Sweeney et al., 1999) (Rauch et al., 2009, Ohlfest et al., 2005) made it possible to conclusively determine potential effects of morphine on lymphocyte trafficking.

Morphine's effects on splenocyte trafficking

We first examined "*in vivo*" effects of morphine dependence on splenocyte trafficking. One?? and 24 h post adoptive transfer, fFL⁺ splenocyte homed directly into the spleens in mice administered a placebo pellet (Fig 1A). In contrast, morphine significantly modulated the trafficking of fFL splenocytes back into the spleen (Fig 1B). Moreover, morphine dependence induced diffuse trafficking of fFL splenocytes throughout the animal (n=10). To quantify the alterations in extrasplenic trafficking, photon measurements were taken from the spleen and were subtracted from measurements taken from the remainder of the animal. Morphine dependence significantly suppressed splenocyte homing into the spleen (P=0.01) (Fig 1C) and increased extrasplenic trafficking (P = 0.004) (Fig 1D) throughout the animals.

Morphine's effect on CNS infiltration

With the random trafficking observed in morphine dependent mice, we next wanted to determine if splenocytes are capable of infiltrating the CNS. In this experiment, morphine dependence significantly increased splenocyte infiltration into the CNS (P=0.002) compared to non-dependent mice (Fig 2A & B). To verify that photon emission was occurring from cells in the brain parenchyma, mice were perfused, brain lysates were made from non-morphine and morphine dependent mice and the lysates were assayed for luciferase activity. Morphine significantly induced non-splenocyte??? infiltration into the CNS (P=0.002)(Fig 2C).

Morphine's effect on splenocyte infiltration is mu-receptor mediated

While there are three different opioid receptors, the majority of immunosuppressive effects of morphine or endogenous opioid release is mediated through the μ -receptor (Shavit et al., 1984), Gavériaux-Ruff et al., 1998), (Yin et al., 1999, 2000), (Mace et al., 2002), (Wang et al., 2002a, 2002b), and (Philippe et al., 2003). To determine if the alterations in splenocyte trafficking were morphine mediated, mice were implanted with the opiate antagonist naltrexone in conjunction with morphine. Blocking the mu-receptor with naltrexone significantly increased homing into the spleen (P=0.02) and inhibited splenocyte trafficking into CNS (P=0.02) compared to morphine treatment group (Figs 3A&B). To verify that photon emission was occurring from cells in the brain parenchyma, mice were perfused, brain lysates were made from placebo, morphine, and naltrexone+ morphine mice and lysates were assayed for luciferase activity(Fig 3C). Morphine significantly increased luciferase activity in the CNS (P=0.001), while blocking the mu-receptor with naltrexone decreased luciferase activity (P=0.04). This experiment concluded that morphine induced CNS infiltration was mu-receptor mediated.

Morphine exacerbates CNS infiltration following neuroinflammation

Endogenous and exogenous opioids exert physiologic effects on the CNS, via opioid receptors expressed within the CNS (Chen et al., 1993), (Heagy et al., 1990), (Wang et al., 1994), (Li et al, 1993) and (Yasuda et al., 1993). We next investigated morphine's effect on splenocyte trafficking into the CNS following a neurostimulus. To accomplish this, placebo or morphine-dependent mice were intracerebrally injected with plasmid DNA/PEI complexes encoding IFN- γ . Morphine-dependent mice inoculated with empty plasmid were used as a control. To validate that the splenocytes were entering the CNS, mice were perfused and brains were assayed for luciferase activity. An increase in luciferase activity was detected from the CNS of morphine dependent mice(Fig 4A-E).

Discussion

Overwhelming evidence exists from *in vitro*, *in vivo* animal models, and from human clinical cases, that drugs of abuse profoundly affect nearly every component of the immune system with corresponding impact on the pathogenesis of infectious disease processes (Eisenstein and Hilburger 1998). While it is well accepted that the increase in disease following injection drug use is primarily due to their modulatory effects on immune functions (CHANGE REFER\??), little is known on the effects of opiates on immune cell trafficking.

The study presented is the first to use *in vivo* imaging to delineate the possible effects opiates have on immune cell trafficking. We first sought to determine morphine's effects on nonspecific trafficking of splenocytes. In these experiments, morphine dependence significantly reduced splenocyte homing to the spleen following adoptive transfer. In fact, morphine dependence led to extrasplenic trafficking throughout the animal, noticeably, into the cervical, inguinal, and mesenteric lymph nodes.

It has been hypothesized that HIV attaches to and is carried in monocytes and CD4 T cells as a passenger of a "Trojan horse" (Wiley et al., 1986) and (Jordan et al., 1991). While the Trojan horse theory remains a distinct possibility, entry across the highly regulated blood brain barrier is still required. In the presented study, there was a significant infiltration of splenocytes into the CNS in morphine dependent mice without a neurostimulus. The non-directed splenocyte infiltration into the CNS may be particularly relevant for tuberculosis, HIV, and other pathogens, providing a vehicle for entry into the CNS. While the mechanism of the non-antigen directed CNS infiltration remains unknown, one possible mechanism may be due to sepsis, which has been previously demonstrated in animal models (Hilburger et al., 1997) and (Roy et al., 1999).

Opiates have profound modulatory effects on nearly every cell of the immune system (Donahoe and Falck et al., 2009). While it has been demonstrated that opiates affect trafficking in an antigen non-specific manner (Hilburger et al., 1997) and (Roy et al., 1999), the vast majority of opiate dependent effects alter immune function following an antigen stimulus. In the presented study, a neuroinflammatory response was implemented using an *in vivo* transfection system (Wu et al., 2007) using a plasmid engineered to continuously produce IFN- γ . A significant infiltration of fFL splenocytes was detected in IFN- γ treated animals. To control for injection alone mediating an inflammatory response, a group of mice were inoculated with a non-IFN- γ producing plasmid.

Splenocyte infiltration following a neuroinflammatory response allowed us to answer the question of whether opiates modulate splenocytes trafficking into the CNS. While there was a statistically significant splenocyte infiltration into the CNS following morphine dependence, this response was exacerbated following induction of IFN- γ production. In conclusion, the study presented was the first to use an *in vivo* imaging system to delineate the trafficking pattern of splenocyte homing in morphine dependent mice, revealing that morphine dependence had a profound effect on splenocyte infiltration into the CNS. Morphine dependent mice had a dramatic infiltration of splenocytes into the CNS without the need of a neurostimulus. This infiltration is one possible mechanism for viral entry into the CNS initiating a neuroinflammatory response in opiate drug abusers.

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A. Placebo treatment group

B. Morphine treatment group

Figure 1. Morphine effects on splenocyte trafficking

Placebo or morphine pellets were implanted 48 h prior to adoptive transfer with 4×10^6 fFL positive splenocytes. Mice were bioimaged for luciferase expression 1 and 24 h after adoptive transfer using *in vivo* bioluminescent imaging. Dorsal and ventral image were taken from placebo (panel A) or morphine (panel B) treatment groups. Graph of luciferase expression measured from the spleen (panel C) and extrasplenic (panel D) locations in the animal from panel A and B. Images are representative of a $n=7$, representative images are shown at 1 h post adoptive transfer. * Indicates $p < 0.05$ for Student's t-test.

A. Placebo and Morphine treatment groups

B. Photon emittance from the CNS.

C. Luciferase activity from the CNS.

Figure 2. Morphine's effect on CNS infiltration

Mice were implanted with placebo or morphine pellets 48 h prior to adoptive transfer with 4×10^6 fFL positive splenocytes. Luciferase expression was measured in the central nervous system. Images are representative of a $n=7$. Dorsal image taken from placebo and morphine treatment groups (A), photons release was measures and graphed demonstrating luciferase expression measured form the CNS animals (B). Mice were perfused, brain homogenates were made and assayed for luciferase activity expressed in relative unites (C). Figures are representative of a $n=7$, representative images at 1 h are shown, * indicates $p < 0.05$ for Student's t-test. Morphine dependence significantly induced non-specific fFL splenocyte trafficking into the CNS

Figure 3. Morphine induced chaos is mu-receptor mediated

To determine if the alterations in lymphocyte trafficking was morphine induced, placebo, morphine, or naltrexone + morphine pellets were implanted 48 h prior to adoptive transfer with 4×10^6 luciferase positive splenocytes. Mice were bioimaged for luciferase expression 1 and 24 h post adoptive transfer using in vivo bioluminescent imaging. Graph of photon emittance from luciferase positive splenocytes measured from the spleen (A), and the CNS (B) from images taken from treatment groups. Mice were perfused, brain homogenates were made and assayed for luciferase activity expressed in relative units (C). Figures are representative of a $n=7$ (* indicates $p < 0.05$ for Student's t-test).

A. Placebo / IFN γ vector icv treatment group B. Morphine / IFN γ vector icv treatment group

C. Empty vector control

D. Imaging from the CNS.

E. Luciferase activity from the CNS.

Figure 4. Morphine exacerbates CNS infiltration following a neurostimulus

Mice were administered placebo or morphine pellets 24 h prior to jetPEI™ IFN γ to induce a neuroinflammatory response. Twenty-four h post-IFN γ inoculation, 4×10^6 luciferase expressing splenocytes were adoptively transferred into mice. Luciferase expression was measured in the central nervous system. placebo pellet / IFN γ vector i.c.v. (A) or morphine pellet / IFN γ vector icv treatment groups(B). Dorsal image were taken from morphine pellet/ empty vector control icv (C). Graph of photon emittance from luciferase positive splenocytes measured from in the CNS animals in Figs. 4a–c (D). Graph of luciferase activity detected from homogenized CNS (E). (* indicates $p < 0.05$ for Student's t-test). IFN γ expression in the CNS induced splenocyte infiltration into the CNS.