Opioid Analgesics Stop the Development of Clostridial Gas Gangrene

Anjana Chakravorty, Milena M. Awad, Thomas J. Hiscox, Jackie K. Cheung, Jocelyn M. Choo, Dena Lyras,* and Julian I. Rood*

Department of Microbiology, Monash University, Clayton, Australia

Gas gangrene is a potentially fatal disease that is primarily caused by the ubiquitous, anaerobic bacteria Clostridium perfringens and Clostridium septicum. Treatment is limited to antibiotic therapy, debridement of the infected tissue, and, in severe cases, amputation. The need for new treatment approaches is compelling. Opioid-based analgesics such as buprenorphine and morphine also have immunomodulatory properties, usually leading to faster disease progression. However, here we show that mice pretreated with buprenorphine and morphine do not die from clostridial myonecrosis. Treatment with buprenorphine after the onset of infection also arrested disease development. Protection against myonecrotic disease was specific to C. perfringens–mediated myonecrosis; buprenorphine did not protect against disease caused by C. septicum infection even though infections due to both species are very similar. These data provide the first evidence of a protective role for opioids during infection and suggest that new therapeutic strategies may be possible for the treatment of C. perfringens–mediated myonecrosis.

Keywords: Clostridium perfringens; Clostridium septicum; clostridial myonecrosis; gas gangrene; bacterial pathogenesis; opioids; host-pathogen interactions; buprenorphine; morphine; disease suppression.

Clostridium perfringens and Clostridium septicum cause human gas gangrene, or clostridial myonecrosis [1, 2]. Disease is toxin mediated, and onset is rapid, usually within 24–48 hours after infection [2]. C. perfringens produces the essential virulence factor α-toxin, a zinc metallophospholipase C [3], and perfringolysin O (PFO), a cholesterol-dependent cytolsin, which functions synergistically with α-toxin [4, 5]. C. septicum produces a β-barrel pore-forming α-toxin, which is essential for disease but functionally distinct from C. perfringens α-toxin [6].

Unique hallmarks of clostridial gas gangrene are a paucity of polymorphonucleocytes (PMNs) in infected tissues and vascular leukostasis [4, 5]. PMN influx is an essential part of the host innate immune response and facilitates bacterial clearance [7]. Clostridium-mediated vascular leukostasis blocks PMN diapedesis and reduces bacterial clearance. C. perfringens α-toxin, assisted by PFO, induces vascular cellular occlusions, which block efficient blood flow and leads to the formation of an ischemic niche. α-toxin and PFO upregulate adhesion molecules on PMNs, platelets, and the vascular endothelium [8–10]. The mechanism of C. septicum α-toxin–induced leukostasis is unknown [6].

The rapid progression of gas gangrene limits treatment options. Disease management involves antibiotic treatment followed by debridement of infected tissue or amputation [11, 12]. The administration of analgesics is important in pain management, with morphine the opioid most commonly used. Buprenorphine, a semisynthetic opioid, is used in some circumstances. Opioids also can interact with the immune system, generally in an immunosuppressive manner [13–15, 16]. For example, morphine-treated macaques show decreased neutrophil chemotaxis [17], whereas mice treated with morphine and infected with Streptococcus pneumoniae display reduced macrophage killing [18]. Opioid treatment of rodents is also immunosuppressive following infection [18–20].

This study was designed to understand the effect of opioid-based analgesics on the host response to
clostridial myonecrosis. We found that mice pretreated with buprenorphine or morphine and subsequently infected intramuscularly with *C. perfringens* did not die from the infection. These mice did not display vascular leukostasis and developed minimal blackening of the infected limb. Administration of buprenorphine after infection provided significant therapeutic protection against disease due to *C. perfringens* infection. Buprenorphine also was found to modulate the chemokine response induced during *C. perfringens* infection.

**METHODS**

**Bacterial Strains and Growth Conditions**

The wild-type *C. perfringens* strain was JIR325 [21]. *C. perfringens* strain JIR12226, or Cp(ΔAT), is an α-toxin (plet) mutant containing the *C. septicum* α-toxin (csa) complementation vector pJIR2502 [22]. *C. perfringens* strains were grown in TYPG broth, FTG broth, or nutrient agar supplemented with rifampicin (10 μg/mL) and nalidixic acid (10 μg/mL) for strain JIR325 and with erythromycin (50 μg/mL) for strain Cp(ΔAT) [4, 5, 23]. *C. septicum* strain BX96 was grown as described elsewhere [6]. Agar cultures were grown at 37°C in 10% (v/v) H2 and 10% CO2 in N2.

**Opioids**

Buprenorphine hydrochloride (Reckitt Beckinson) and morphine sulfate (David Bull Laboratories) were obtained and stored in accordance with the Australian Controlled Substances Act, Substance 8 (S8) Licensing Restrictions. Naltrexone hydrochloride (Sigma Aldrich) was resuspended in lipopolysaccharide-free deionized water, in accordance with the manufacturer’s instructions. All drugs were diluted in saline.

**Mouse Clostridial Myonecrosis Model**

Mouse virulence trials were conducted as described previously [4, 24]. The trials accorded with Victorian State Government regulations and were approved by the Monash University MARP 2 Animal Ethics Committee. Briefly, wild-type *C. perfringens* strains were grown overnight on nutrient agar with appropriate antibiotic selection. The cells were then placed in Dulbecco’s phosphate-buffered saline (DPBS) [6], washed, and resuspended in a volume of DPBS that was 3 times that of the cell pellet. Female BALB/c mice were injected subcutaneously with either buprenorphine (0.1 mg/kg), morphine (5 mg/kg), or saline (vehicle control) into the left leg 15 minutes before infection. Mice were also injected every 6 or 4 hours with either buprenorphine or morphine, respectively, until the end of the experiment, to ensure drug bioavailability. Analogic doses and readministration conditions were determined in accordance with the Australian National Health and Medical Research Council (NHMRC) guidelines for animal analgesia. Mice then were injected intramuscularly with 50 μL of bacterial cell suspension (approximately 109 colony-forming units) into the right hind thigh muscle and monitored every 30 minutes for 12 hours. Blackening and swelling of the thigh and footpad—the characteristic signs of pathology—were scored as 0.5 (moderate disease) or 1 (severe disease) [25]. Longer periods of observation were not permitted by the Animal Ethics Committee. Mice scoring 1 in any parameter except swelling of the thigh were humanely euthanized.

For naltrexone pretreatment, naltrexone hydrochloride (1 mg/kg) was injected subcutaneously. After 15 minutes, mice were injected with either buprenorphine (1 mg/kg) or morphine (5 mg/kg), rested for 15 minutes, and subsequently infected with *C. perfringens*. For dose-response studies, mice were injected with varying concentrations of either buprenorphine or morphine and monitored as before. The effect of buprenorphine on *C. septicum*-mediated disease was assessed using the same model, as previously described [6, 22, 26].

Viable counts were performed both before and after infection, using agar media with appropriate selection. Infected thigh tissues were collected immediately into 10% formalin for subsequent histological analysis. Tissues were sliced into 5-μm sections and stained with hematoxylin and eosin. Stained sections were analyzed using an Olympus BX51 microscope. PMN influx was quantified in hematoxylin-eosin–stained sections of muscle tissue obtained 3.5–4.5 hours after saline injection from control mice, 3.5–4.5 hours after infection from mice infected with wild-type *C. perfringens*, and 3.5–4.5 hours after infection from mice pretreated with buprenorphine or morphine and subsequently infected with *C. perfringens*. Tissues were scored by investigators blinded to study group, by counting the number of PMNs present in 3–6 images per sample, represented as PMNs/field, where a field is defined as 400 μm2. All statistical analysis was performed using GraphPad Prism. Statistical significance was determined using a log-rank (Mantel-Cox) test for all survival curves and a Mann–Whitney U test for other data.

**Quantitative Polymerase Chain Reaction (qPCR) Array Analysis of Immune Gene Expression**

Infected thigh tissues were obtained 3.5–4.5 hours after infection from 3 mice infected with *C. perfringens* strain JIR325, 3 mice pretreated with buprenorphine (1 mg/kg) and then infected with JIR325, and 3 control mice injected with saline, and specimens were stored immediately in RNA Later (Ambion). RNA was isolated and analyzed separately from these 9 samples using Trizol reagent as described by the manufacturer (Invitrogen), except that tissues were homogenized in Trizol, using a sonicator (Branson) at setting 4 with pulsation for 30 seconds. Contaminating DNA was removed using a DNase treatment kit (Ambion). RNA quality was determined using a Nanodrop spectrophotometer, and only RNA with an A260/280 of >1.7–1.8 was used for complementary DNA (cDNA) preparation.
Mouse Custom StellArray (Lonza) qPCR 96-well plates were designed using the Lonza StellArray software, with each well containing a different gene-specific oligonucleotide. Genes were chosen specifically to analyze the expression of chemokines and cytokines that are involved in cellular trafficking and/or the host’s response to infection. Isolated cDNA was diluted 1 in 15 in DEPC water (Invitrogen) and mixed with SYBR Green (Applied Biosystems) to create a master mixture that was added into each well and incubated for 30 minutes at room temperature before analysis using an Eppendorf Mastercycler qPCR machine. All cumulative threshold (Ct) values were standardized using Calqplex (Eppendorf). Data sets (saline tissues, compared with either wild-type or buprenorphine-pretreated wild-type tissues) were analyzed on the Lonza Web site (available at: http://array.lonza.com/gpr), using Global Pattern Recognition analysis software designed by Bar Harbor Biotechnology (available at: https://www.bhbio.com/BHB/dw/home.html), as previously described [27, 28], and as recommended by the manufacturer. This algorithm internally normalizes the real-time qPCR data set and generates a list of genes that are ranked on the basis of the difference between the test and control expression levels and the consistency of the data between the biological replicates. This proprietary software calculates both the fold-change data and the respective P values. The results are displayed as fold-change with respect to genes that showed minimal changes, which were defined and selected based on Ct values obtained using the Global Pattern Recognition analysis software.

RESULTS

Opioid-Based Analgesics Protect Against C. perfringens–Mediated Clostridial Myonecrosis

To assess the role of opioid-based analgesics in the development and progression of clostridial myonecrosis, mice were pretreated with buprenorphine (0.1 mg/kg) 15 minutes before infection with C. perfringens strain JIR325. This concentration is the maximal amount of buprenorphine allowed in mice, according to NHMRC guidelines [29]. Mice were monitored for the characteristic parameters of clostridial disease, as described elsewhere [4, 6, 22, 25, 26]. The results unexpectedly showed that pretreatment of mice with buprenorphine was protective against C. perfringens infection (Figure 1A and 1B). Mice infected with JIR325 had a mean survival time (±standard error of the mean [SEM]) of 6.5 ± 0.6 hours, compared with 11.5 ± 0.4 hours among mice pretreated with buprenorphine and then injected with JIR325 (P = .0001). Mice injected with saline survived for the duration of the experiment (12 hours), as did mice injected with buprenorphine alone.

To determine whether protective effects were observed with other morphine analogues, we analyzed the effect of morphine (5 mg/kg) pretreatment on C. perfringens–mediated gas gangrene. A higher concentration of morphine than buprenorphine was used because these drugs have different pharmacodynamic properties [14]. The results showed that morphine also protected mice against C. perfringens–mediated disease (Figure 2A and 2B). Mice pretreated with morphine had a greater mean survival time (±SEM) than mice that were not pretreated (10.6 ± 0.9 hours vs 5.4 ± 0.5 hours; P = .0033; Figure 2B). Control mice injected with saline or morphine alone did not develop disease.

Mice preinjected with buprenorphine or morphine and then infected with C. perfringens also failed to develop the key disease pathologies associated with infection, specifically, limping and blackening of the thigh and footpad. Histological analysis of thigh tissue specimens obtained from mice pretreated with buprenorphine (Figure 1C) or morphine (Figure 2C) showed PMN influx into infected tissues, in stark contrast to the vascular leukostasis seen in tissues of mice infected with the wild type, although some C. perfringens–mediated tissue necrosis was observed. Similar viable counts (P > .05) were observed in infected thigh tissue specimens obtained from mice with (approximately 1 × 10⁶ colony-forming units [CFU]) and mice without (approximately 5 × 10⁶ CFU) opioid pretreatment. The effect on PMN influx was confirmed by quantifying the number of PMNs in muscle tissue sections, with a significantly larger PMN influx detected after C. perfringens infections with opioid pretreatment, compared with equivalent C. perfringens infections without pretreatment (Figure 3).

Buprenorphine Does Not Protect Against C. septicum–Mediated Disease

C. septicum also causes clostridial myonecrosis, but the mechanism is different [6, 30]. To see whether buprenorphine also modulated C. septicum–mediated infection, mice were infected with the virulent C. septicum strain BX-96. These mice died from disease at a rapid rate, with 100% mortality and a mean survival time (±SEM) of 3.4 ± 0.2 hours (Figure 4B and 4C). Preinjection with buprenorphine had no effect on disease progression, with mice dying at a similar rate (mean survival time [±SEM], 4.1 ± 0.4 hours). Histopathological analyses of infected thigh tissue specimens obtained from mice with and mice without buprenorphine pretreatment showed no differences in vascular leukostasis or necrosis (data not shown).

Buprenorphine Modulates the Effects of C. perfringens α-Toxin

To determine whether buprenorphine acts by specifically counteracting the effects of C. perfringens α-toxin, mice were infected with an isogenic C. perfringens strain JIR325 derivative, JIR12226 (Cp[AT]), that no longer produces phospholipase C but instead produces the C. septicum β-pore-forming α-toxin (AT) [22]. This strain continues to produce other putative C. perfringens virulence factors, such as perfringolysin O, and was previously shown to induce fulminant disease in a mouse.
Mice preinjected with buprenorphine and then infected with the wild-type *Clostridium perfringens* strain survived 12-hour duration of the experiment (Figure 4B and 4C), whereas mice that were not pretreated had a mean survival time of 6.8 hours. By contrast, pretreatment with buprenorphine had no significant effect on the survival of mice infected with the *Cp*(*AT*) strain (Figure 4B and 4C), with mean survival times (±SEM) of 5.6 ± 0.8 hours (*Cp*(*AT*)) and 7.3 ± 0.1 hours (*Cp*(*AT*)) plus buprenorphine; *P* > .05). These data suggest that the protection conferred by buprenorphine primarily involves counteracting the effects of *C. perfringens* α-toxin.

**Effects of Buprenorphine and Morphine Are Dose Dependent**

Dose-response experiments were performed to determine the lowest doses of buprenorphine and morphine that protected against *C. perfringens* myonecrosis. Mice were injected with different amounts of buprenorphine (Figure 5A) or morphine (Figure 5C) before infection with *C. perfringens*. Mice that were not pretreated died from disease at a normal rate, with a mean survival time (±SEM) of 6.6 ± 0.6 hours, whereas a buprenorphine dose of 0.01 mg/kg provided 100% protection against disease (Figure 5B). Mice pretreated with lower doses of buprenorphine were not protected and died from disease at the same rate as infected mice that were not pretreated (Figure 5A and 5C).
Morphine provided effective protection at a dose of 5 mg/kg, with a 95% survival rate and a mean survival time (±SEM) of 11.2 ± 0.6 hours. Buprenorphine and morphine were not protective at 0.0001 mg/kg. Mice pretreated with lower levels (0.0001 mg/kg) of either buprenorphine or morphine died from disease at the same rate as wild-type infected mice, with mean survival times (±SEM) of 6.8 ± 0.7 hours and 7.3 ± 0.7 hours, respectively ($P = .0001$ and $P = .0054$, respectively). At this concentration, the disease features were similar to those observed in wild-type-infected mice with no pretreatment (Figure 5D). Examination of the gross pathology of the infected region revealed extensive tissue damage, with hemorrhage and blackening of the infected thigh and gas production, whereas mice pretreated with either buprenorphine or morphine showed minimal tissue hemorrhage or blackening but had signs of muscle breakdown (Figure 5E). These data correlated with the histopathological findings.

**Buprenorphine Treatment After Disease Onset Is Also Protective**

To determine whether treatment with buprenorphine after the onset of infection would stop disease progression, mice were infected with *C. perfringens*, and disease was allowed to progress for 3.5 hours, at which time the surviving mice were treated with buprenorphine (0.1 mg/kg). The results showed that these mice had a higher survival rate than infected mice that were not...
treated with buprenorphine; 60% of the treated mice survived until the end of the experiment, compared with none of the untreated mice (P = .003; Figure 6A). The mice treated with buprenorphine after infection had a mean survival time (±SEM) of 9.8 ± 0.9 hours, compared with 4.8 ± 0.5 hours for untreated mice (Figure 6B). However, mice that developed severe disease by 3.5 hours (40%) could not be protected by postinfection buprenorphine treatment and succumbed to disease at the same rate as the nontreated animals (Figure 6A).

**Naltrexone Partially Reverses the Protective Effects of Morphine But Not Buprenorphine**

To determine whether the protective effects of opioids were mediated by an opioid receptor–dependent mechanism, mice were injected with naltrexone (1 mg/kg), an opioid-receptor antagonist, before the administration of either buprenorphine (0.1 mg/kg) or morphine (5 mg/kg), followed by infection with *C. perfringens*. Mice pretreated with naltrexone and then morphine died from disease at a similar rate as mice that did not receive any pretreatment (Figure 6C), with mean survival times (±SEM) of 8.4 ± 0.7 hours and 6.4 ± 0.7 hours, respectively (Figure 6D). By contrast, mice treated with naltrexone and buprenorphine had a mean survival time of >12 hours (Figure 6C and 6D), with no observable pathology.

**Pretreatment With Buprenorphine Modulates the Expression of Genes Involved in the Host Inflammatory Response**

To gain a better understanding of the host factors involved in the buprenorphine modulation of *C. perfringens*–mediated disease, changes in the expression of host immune genes were measured using a high-throughput real-time qPCR array system [27, 28, 31]. RNA was extracted from the thigh tissue specimens obtained from 3 *C. perfringens*-infected mice or 3 mice treated with buprenorphine before *C. perfringens* infection, converted...
to cDNA, and subjected to real-time qPCR analysis. Findings for these samples were compared to those for material isolated from control mice that were injected with saline.

C. perfringens infection was found to significantly \((P < .05)\) downregulate the expression of components of the host chemokine response, particularly \(Ccl3, Ccl20, Ccl5\), and \(Cx3cr1\), which...
encode macrophage inflammatory protein 1α (MIP-1α), MIP-3α, CCL5, and the fractalkine receptor, respectively; Supplementary Table 1 presents the data set for all 96 genes tested. Infection also downregulated the expression of genes encoding the TLR and inflammasome signaling molecules NLRP1, Ttrap, Ticam, and Tollip. The expression level of Il1r1, which encodes the interleukin 1 receptor, was downregulated, whereas tnfα, which encodes the master proinflammatory cytokine tumor necrosis factor α (TNF-α), was upregulated 220-fold (Supplementary Table 1). This observation was in agreement with previous studies that showed that C. perfringens α-toxin induces TNF-α release, although these studies were performed in vitro, using purified toxin [32]. Only one of the downregulated genes, Ccl20, was significantly downregulated when infection was preceded by buprenorphine treatment.

Buprenorphine pretreatment combined with C. perfringens infection significantly upregulated expression of Pglyrp1 (Supplementary Table 1), which encodes the peptidoglycan recognition protein involved in innate immunity [33], and altered the expression of 2 integral chemokine genes, Cxcl2 and Ccl4. Cxcl2, a mouse interleukin 8 homologue, and Ccl4, also known as MIP-1β, are involved in PMN trafficking and activation [34]. Buprenorphine pretreatment also downregulated the expression of Tnfaip1, which encodes a protein that is generally
induced in response to TNF-α and is important in regulating the host response to hepatitis B virus infection [35]. These data provided evidence that C. perfringens actively downregulated key innate signaling pathways and chemokines that are integral to PMN induction and activation. Buprenorphine appears to modulate these effects, thereby allowing active and functional recruitment of PMNs into the infected region and subsequent clearance of the infection.

**DISCUSSION**

In this study, we provide clear evidence that the opioid-based analgesics buprenorphine and morphine modulate the clinical effects of C. perfringens–mediated gas gangrene. Opioid-mediated protection was specific to C. perfringens; disease due to C. septicum infection could not be rescued by opioid administration. These protective effects are most likely due to the vascular leukostatic phenotype characteristic of C. perfringens myonecrosis, which stops PMN trafficking into infected regions. Buprenorphine appears to alter the balance of cellular trafficking by modulating the chemokine and cytokine content of the infected region, thereby allowing effective and efficient bacterial clearance. These conclusions are in direct contrast to those from other studies, in which bacterial infections were enhanced by treatment with these drugs, probably as a result of opioid-mediated inhibition of the immune system [20, 36–42].

It is likely that both classic and nonclassic opioid receptor signaling pathways are involved in mediating disease protection. Opioid-mediated protection was shown to be partially opioid-receptor independent, since naltrexone pretreatment failed to fully rescue the disease pathology. Naltrexone only partially reversed the immunosuppressive effect of morphine in an oral Salmonella Typhimurium infection model [20]. Naloxone, an opioid receptor antagonist similar to naltrexone, also failed to reverse morphine-induced immunosuppression in a murine model of Plasmodium berghei infection [43] and only partially reversed morphine-induced hypersensitivity in mice following Toxoplasma gondii infection [44].

The finding that buprenorphine targets the immunomodulatory properties of C. perfringens α-toxin but not C. septicum α-toxin was unexpected since these diseases manifest in a similar manner. We postulate that C. perfringens α-toxin–mediated vascular leukostasis, rather than necrosis, is the primary target of opioid-mediated protection. Consistent with this hypothesis, C. perfringens–infected tissues demonstrated evidence of tissue necrosis independent of opioid administration, with histological analysis of infected thigh tissue specimens obtained from buprenorphine-pretreated mice showing minimal vascular occlusion and PMN influx, compared with infected but untreated mice. We suggest that buprenorphine enables the innate immune response to contain and clear the infection by subverting the early establishment of cellular and vascular occlusions. This process prevents the establishment of an ischemic environment that fosters the growth of C. perfringens.

The mechanism of opioid action is most likely multifactorial and has significant implications for the chemokine response. Buprenorphine-mediated upregulation of key chemokines involved in PMN trafficking and transmigration has the potential to prevent vascular leukostasis by allowing cellular influx into infected tissues. Indeed, the ability of morphine and buprenorphine to modulate cellular chemotaxis and cytokine production has been observed in several models [41, 45, 46]. Buprenorphine pretreatment upregulated the expression of 2 key mouse chemokine genes, Cxcl2 and Ccl4. The chemokines encoded by these genes regulate the magnitude and duration of PMN activation and trafficking into infected regions. Therefore, our real-time qPCR data provide a mechanistic insight into buprenorphine-mediated immune regulation.

C. perfringens–mediated gas gangrene is a devastating disease. Alternative treatments that take into consideration the cellular interactions that induce disease as opposed to just the pathological manifestations have the potential to offer safer and more versatile treatment options. The results reported in this study have important implications for both the prophylactic and therapeutic control of necrotic C. perfringens infections. However, our findings suggest that the stage of disease at which treatment is administered is critical for the eventual outcome. Since patients are often administered opioid analgesics such as morphine, we consider that any potentially therapeutic effects may be masked because of an inability of the compound to enter into infected regions, owing to a lack of blood flow. We suggest that rapid delivery of buprenorphine treatment to infected patients by topical application or direct infusion into the infected tissues, in conjunction with current treatment regimens, may have considerable therapeutic value for the treatment and management of myonecrotic C. perfringens infections.

**Supplementary Data**

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

**Financial support.** This work was supported by the Australian National Health and Medical Research Council (project grant APP1005991) and Monash University (postgraduate research scholarship to A. C.).

**Potential conflicts of interest.** All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

**References**