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Morphine Impairs Host Innate Immune Response and Increases Susceptibility to *Streptococcus pneumoniae* Lung Infection[1]

Jinghua Wang,* Roderick A. Barke,† Richard Charboneau,† and Sabita Roy[2]*†

Chronic morphine use impairs host innate immune response and increases susceptibility to bacteria and virus. In this study a novel mouse model of chronic morphine treatment, followed by intranasal inoculation with *Streptococcus pneumoniae*, was used to investigate microbial events and host innate immune response. Our results show that chronic morphine treatment markedly delayed neutrophil recruitment and increased bacterial burden in the lung, spleen, and blood with a subsequent increase in mortality. In morphine-treated animals, before neutrophil recruitment, a significant decrease in TNF-α, IL-1, IL-6, MIP-2, and KC was observed both in bronchoalveolar lavage fluids and in lung tissue. In the early phase of infection, we found that accumulation of galectin-3 in the alveolar space of streptococcus-infected lungs was decreased after morphine treatment. The transcription factor NF-κB in lung resident cells was also inhibited after morphine treatment. Taken together, these results suggest that chronic morphine treatment in an *S. pneumoniae* infection model suppresses NF-κB gene transcription in lung resident cells, which, in turn, modulates the transcriptional regulation of MIP-2 and inflammatory cytokines. The decreased synthesis of MIP-2 and inflammatory cytokines coupled with the decreased release of galectin-3 result in reduced migration of neutrophils to the site of infection, thereby increasing susceptibility to *S. pneumoniae* infection after morphine treatment. *The Journal of Immunology,* **2005, 174: 426–434.**

Drug use and abuse have generated serious health concerns (1, 2). There exists a well-recognized relationship between addictive drugs and increased incidence of infection (3–5). Drug abuse has been determined to be a significant risk factor for the development of community-acquired pneumonia (6). However, the mechanism by which drugs of abuse affect host resistance to pneumococcal infection has not been well characterized in either humans or an experimental animal model. *Streptococcus pneumoniae* is one of the most common diagnoses among opiate abusers (7); it is responsible for >25% of all cases of pneumonia (8, 9) and is associated with an overall mortality rate of 23% among hospitalized patients (10).

An important step in the clearance of an infectious agent is the recruitment and activation of neutrophils to the infected site (11). Neutrophil recruitment involves communication among the responding neutrophil, vascular endothelium, and resident cells of the infected tissues. This communication is brought about by signals that direct neutrophils from the vascular compartment to the infected tissue and is dependent on intercellular signaling molecules, such as adhesion molecules, cytokines, and chemokines. Movement of neutrophils across blood vessel walls to the site of infection first requires that the migrating neutrophils firmly attach to the endothelial walls. Adhesion molecules have been shown to facilitate this process (12). In Gram-negative bacteria-induced acute lung infection and injury, the classical adhesion molecules, β_2 integrins, and selectins have been implicated as being involved in the adhesion process. However, in the case of Gram-positive bacteria such as pneumococcal pneumonia, a series of studies conducted by Doerschuk et al. (13–16) clearly demonstrates that migration of neutrophils to the alveolar space is independent of these two classical adhesion molecules. Pulmonary infection with *S. pneumoniae*, in contrast, seems to induce an alternative or nonclassical neutrophil diapedesis pathway. This observation was supported in a recent study by Sato et al. (17, 18), which demonstrated that galectin-3, a novel adhesion molecule, plays a significant role as an adhesion molecule in the process of extravasation of neutrophils in a mouse model of streptococcal pneumonia.

C-X-C chemokines such as MIP-2, cytokine-induced neutrophil chemoattractant, and murine KC (19), are predominantly stimulatory and induce neutrophil chemotaxis. *S. pneumoniae* infection induces a rapid, coordinated, time-dependent expression of MIP-2 and KC chemokines in lung tissue and bronchoalveolar lavage (BAL)[3] fluid (20). Furthermore, the early response cytokines, TNF and IL-1, may amplify, propagate, and prolong the expression of these chemokines. Increased expression of the NF-κB family of transcription factors has been implicated as the molecular mechanism by which these early response cytokines modulate chemokine expression (21–24).

Interestingly, morphine has been shown to alter many of the steps involved in neutrophil migration from the intravascular compartment to the site of infection. Alterations include 1) modulation of proinflammation cytokines, 2) inhibition of chemokine synthesis and chemokine receptor expression, and 3) inhibition of NF-κB activation (25–27). The present study was designed to investigate

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[3] Abbreviations used in this paper: BAL, bronchoalveolar lavage; MPO, myeloperoxidase.

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the mechanisms by which chronic morphine treatment affects neutrophil migration and its consequence on infection susceptibility using an *S. pneumoniae* model of lung infection.

Materials and Methods

Mice

$CB6F_1$ male mice, 8 wk old, were obtained from Harlan Breeders and maintained under specific pathogen-free conditions. Mice were exposed to a standardized 12-h light, 12-h dark cycle and had free access to food and water. All animal experiments were performed in accordance with the institutional animal care and use committee's guidelines at the University of Minnesota.

Pneumococcal pneumonia model and drug treatment protocol

S. pneumoniae, serotype 3, obtained from American Type Culture Collection (ATCC 6303), were grown for 6 h to midlogarithmic phase at 37°C in Todd-Hewitt broth (Difco), harvested by centrifugation at 1500 × *g* for 15 min, and washed twice in sterile PBS. Bacteria were then resuspended in sterile PBS at ~2×10^8 CFU/ml, as determined by plating serial 10-fold dilutions on sheep-blood agar plates. Pneumonia was induced by intranasal inoculation of 1×10^7 CFU. Briefly, mice were lightly anesthetized with isoflurane (Halocarbon Laboratories) and inoculated with bacteria in 50 μl of PBS, applied to the tip of the nose and involuntarily inhaled. The animals were held in a vertical position for 1 min to ensure migration of the inoculum to the alveoli. Animals were implanted with either a 75-mg morphine pellet or a placebo pellet (controls) 24 h before *S. pneumoniae* inoculation. The pellets were obtained from the National Institute on Drug Abuse.

Bronchoalveolar lavage

The trachea was exposed through a midline incision and cannulated with a sterile 22-gauge Abbocath-T catheter (Abbott). Bronchoalveolar lavage (BAL) was obtained by instilling and collecting two 0.5-ml volumes of cold PBS through the incised trachea. A total of 0.9–1 ml of lavage fluid was retrieved per mouse.

Survival studies

Mice given morphine or placebo and intranasally inoculated with *S. pneumonia*e were observed daily, and mortality records were kept for 7 days. The percent survival and mean survival time were calculated.

Pathogenesis studies

Morphine or placebo pellet-implanted animals were infected with 1×10^7 CFU as described above. Mice were killed by CO_2 inhalation at 4, 24, and 48 h postinfection. Weight and health status were monitored before the infection and at the time of death. Blood samples were collected through the heart, and 10 μl was plated on blood agar plates and incubated for 12 h at 37°C to determine the presence of bacteria. BAL fluid was obtained as described above. Lungs and spleens were aseptically removed, cleared of blood with cold saline, and homogenized in 2 ml of cold PBS. Serial dilutions of the homogenates and BAL fluids were plated on blood agar plates. Plates were incubated at 37°C overnight, and *S. pneumoniae* colonies were counted.

Neutrophil recruitment

Total cell numbers in BAL were counted from each sample in a hemocytometer (Hausser Scientific, Horsham, PA). BAL neutrophil count was determined on cytospin preparations stained with a Diff-Quick staining kit (IMEB). The neutrophil population in lung tissue was evaluated by the quantification of myeloperoxidase (MPO) activity. Briefly, a volume of 150 μl of crude lung homogenate was mixed with 150 μl of potassium phosphate buffer containing 1% hexadecyltrimethylammonium bromide. The mixture was sonicated for 20 s, then centrifuged at 5000 × *g* for 30 min. MPO activity was measured by adding 40 μl of the supernatant to 250 μl of reaction solution (0.01 mg/ml *o*-dianisidine and 0.004% H_2O_2 in potassium phosphate buffer). The reaction was stopped by adding 25 μl of 1% sodium azide, and the OD was read at 450 nm against a standard curve generated using commercially available MPO (Sigma-Aldrich).

Neutrophil chemotaxis assay

Normal neutrophil migration toward BAL collected from morphine- and placebo-treated mice was tested in vitro. To obtain enriched populations of neutrophils, the peritoneal cavity was washed with PBS 3 h after peritoneal injection of 2 ml of thioglycolate. The neutrophil purity was >90%, as assessed by light microscopy of Diff-Quik (IMEB)-stained cytospin preparations. After the cells were resuspended in 5.0 ml of RPMI 1640 without phenol red containing 10% FCS-RPMI 1640, calcein AM was added to a final concentration of 5 μg/ml, and the cells were incubated for 30 min at 37°C. Calcein AM-labeled neutrophils were washed twice with FCS-RPMI 1640, counted, and resuspended in 10% FCS-RPMI 1640 to a final concentration of 2×10^6/ml. BAL samples collected from control and morphine-treated mice were also diluted 1/10 with PBS-BSA before chemotaxis assay. Chemotaxis was analyzed using a disposable, 96-well chemotaxis system (ChemoTx; NeuroProbe). BAL samples (29 μl) were loaded in triplicate into wells of the microplate. A serial dilution of fluorescence-labeled cells was also performed (ranging from 1000 to 5×10^4 cells in 25 μl) and loaded directly into wells of the plate to account for any interassay variability in cell labeling. Next, the framed filter (containing 3-μm diameter pores) was installed; 25 μl of neutrophil suspension (2×10^6/ml) was pipetted onto the top side of the filter directly above each well, and the chamber was incubated for 1 h at 37°C and 5% CO_2. After incubation, nonmigrated cells were removed from the top of the filter, and the chamber containing the filter apparatus was centrifuged (400 × *g* for 5 min). Migrated cells in the bottom of the chamber were quantified by measuring calcein AM fluorescence (excitation, 485 nm; emission, 530 nm) with a fluorescence plate reader.

Inflammatory mediators

Inflammatory cytokines, including TNF-α, IL-1β, and IL-6, and the chemokines MIP-2 and KC, which are chemoattractants for neutrophils, were quantified using cytokine-specific ELISA kits (R&D Systems) according to the manufacturer's instructions.

EMSA

Activation of the NF-κB pathway was detected by EMSA for NF-κB. Two hours after pneumonia infection, lungs were aseptically removed and cleared of blood with cold saline. Nuclear protein of lung tissues was isolated using a nuclear extraction kit (Sigma-Aldrich) according to the manufacturer's instructions. Extracts were frozen in dry ice and stored at −80°C. Determination of protein concentration was performed using a protein assay kit (Bio-Rad). Oligonucleotides were labeled, and the binding reactions were performed as described previously (23). Briefly, 10 μg of nuclear extract was incubated with ^{32}P-labeled probe, double-stranded oligonucleotide (5′-AGTTGAGGGGACTTTCCCAGGC-3′) in binding buffer at room temperature for 20 min. Bound complexes were resolved on 5% nondenaturing polyacrylamide gels in 0.5× Tris borate/EDTA buffer, dried, and autoradiographed overnight.

Western blot analysis of galectin-3

Cell-free supernatants of BAL fluids or homogenates of exudated leukocytes (cell fractions) of BAL fluids were fractionated by SDS-PAGE and transferred to a nitrocellulose membrane (Amersham Biosciences). After incubation with StartingBlock (Pierce), membranes were incubated with anti-galectin-3 Ab (Santa Cruz Biotechnology), followed by anti-rabbit IgG-peroxidase (Amersham Biosciences). Ab complexes bound to membranes were detected by exposure to film after incubation with Supersignal Chemiluminescence Detection Substrate (Pierce) according to standard procedures.

RT-PCR analysis of CXCR2 mRNA

Total RNA was extracted from BAL leukocytes at 4, 24, and 48 h after infection by cell lysis in guanidine thiocyanate, followed by phenol acid extraction. RNA (1 μg) was reverse transcribed for 90 min at 42°C with 200 U of superscript Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies) using random hexamers. Five microliters of cDNA was used for each PCR, and amplification was performed with oligonucleotide primers specific for mouse CXCR2 as described previously (25).

Histologic examination

After 24-h fixation of lungs in 10% Formalin and embedding in paraffin, 4-μm-thick sections were stained with H&E, then processed for light microscopy to quantify the percentage of lung involved in the inflammatory process.

Statistical analysis

Data were analyzed for statistically significant differences by two-way ANOVA between animals. Individual group comparisons were made by two-tailed Student's *t* test. Statistical significance was accepted at $p < 0.05$.

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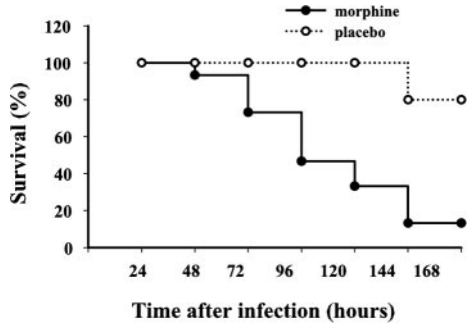

FIGURE 1. Effect of chronic morphine treatment on the survival of mice infected with *S. pneumoniae*. Mice were implanted with morphine and placebo pellets and then inoculated intranasally with *S. pneumoniae*. The survival curves were markedly different between morphine and placebo groups ($p < 0.05$ for morphine vs placebo groups; $n = 15$). Results are representative of three independent experiments.

Results

Morphine increases mortality after pneumococcal lung infection

Chronic morphine treatment markedly increased mortality after pulmonary *S. pneumoniae* infection. Fig. 1 shows the effect of chronic morphine treatment on mortality. There was a time-dependent increase in the mortality rate after morphine pellet implantation. In the morphine-treated group, 60% mortality was observed at 4 days postinfection compared with no significant mortality in the placebo-treated control mice. At 7 days postinfection, morphine treatment resulted in 86.7% mortality compared with 20% mortality in the placebo-treated group.

Morphine potentiates bacterial growth in lung, spleen, and blood

Bacterial burden in infected animals is dependent on a balance between the bacteria's proliferation and the clearance by the host's innate immune response, including phagocytosis and killing by recruitment of neutrophils (28). The effects of morphine and placebo treatments on bacterial dissemination were next investigated. Bacterial burdens in lung tissue and BAL are presented in Fig. 2. At the early time point (4 h), bacterial burdens in the chronic morphine- and placebo-treated groups were similar. However, there was a time-dependent increase in bacterial counts in the morphine-treated group. Conversely, in the placebo group, a time-dependent decrease in bacterial counts was observed.

No bacterial CFU was detected in the blood from placebo-treated control mice. However, significant dissemination of bacteria was observed in the blood from chronically morphine-treated mice. Bacterial dissemination was observed as early as 24 h postinfection in the morphine-treated group, and a significant number became bacteremic at 48 h after infection, corresponding to the time at which significant mortality was observed (Fig. 2*C*).

Although the bacterial counts in the blood of the morphine-treated group 4 h postinfection were not significantly different from those in placebo-treated animals, counts in the spleens of morphine-treated animals were significantly higher than those in placebo-treated control mice. These differences between control and chronic morphine-treated animal spleens were also apparent at 48 h after infection (Fig. 2*D*).

These data suggest that morphine treatment results in a compromised pulmonary host defense during the early stages of infection and that morphine treatment may be the contributing factor in the increased bacterial load and severity of infection seen in the morphine-treated mice.

Histopathologic assessment of lung tissue

Lung tissues were examined at 4, 24, and 48 h after *S. pneumoniae* intranasal inoculation of the mice. Although no pathologic changes in the lung could be found at 4 h postinoculation (Fig. 3, *A* and *B*), pronounced changes were observed at 24 and 48 h after *S. pneumoniae* infection. At 24 h after inoculation, the lung's initial foci of inflammation were restricted to perivascular areas localized close to infected bronchioles. Mice treated with morphine showed an exacerbated inflammation in the lung compared with mice treated with placebo (Fig. 3, *C* and *D*). Histopathological examination of lung from both morphine- and placebo-treated groups at

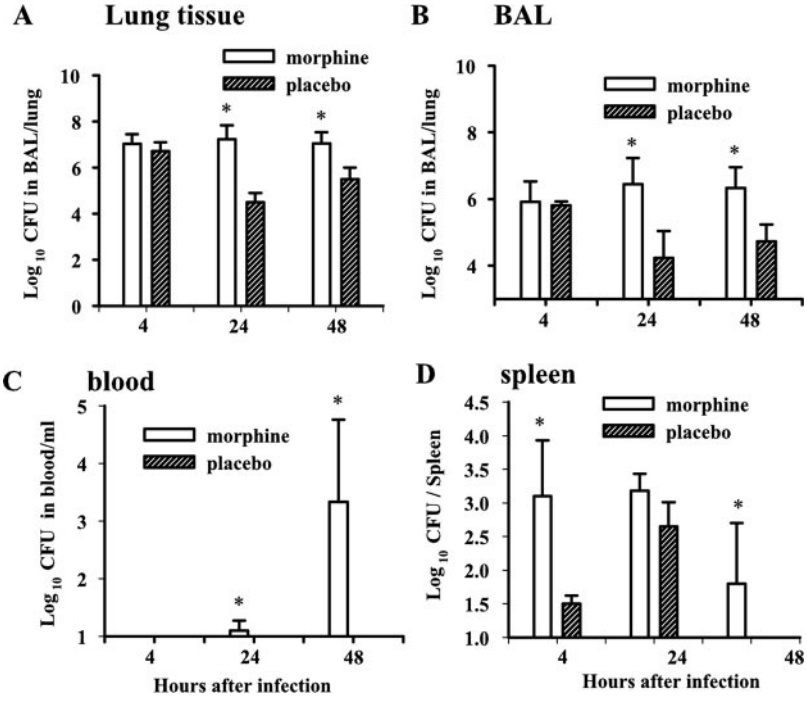

FIGURE 2. Bacterial counts in lung tissue (*A*), BAL fluid (*B*), blood (*C*), and spleens (*D*) of morphine-treated and placebo control mice infected with *S. pneumoniae*. Data are expressed as the mean ± SEM of three independent experiments. The chronic morphine-treated group is significantly different from the placebo control group (*, $p < 0.05$; $n = 6$).

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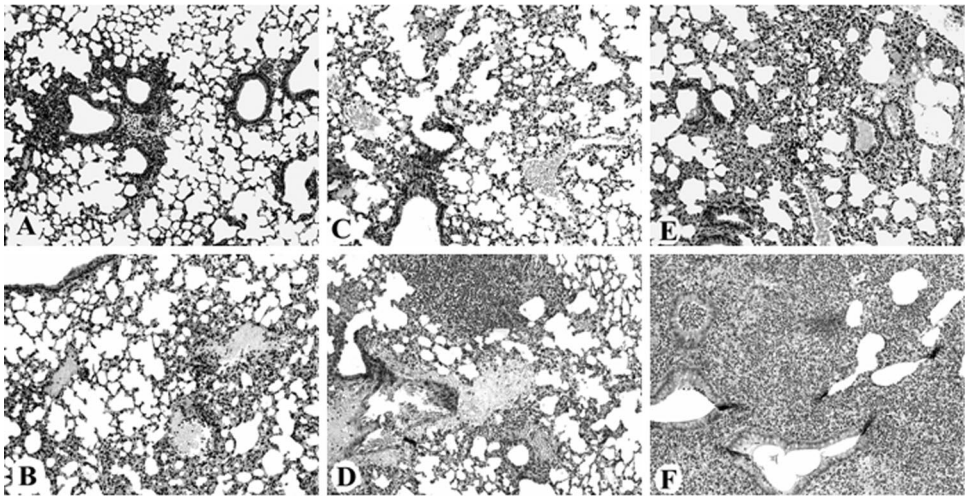

FIGURE 3. Histopathology of the lungs 4, 24, and 48 h after infection with *S. pneumonia*e. Lungs were excised and fixed in 10% buffered formalin, dehydrated in graded alcohol, and embedded in paraffin. Sections (4 μm) were cut from the paraffin-embedded fixed tissue, mounted on poly-L-lysine-coated slides, and stained with H&E. At least 10 random fields were observed in each section at $\times$300 magnification using a Zeiss microscope. The representative slides were photographed and are presented: *A*, section from the lung of a placebo control mouse 4 h after inoculation with *S. pneumoniae*; *B*, section from the lung of a morphine-treated mouse 4 h after inoculation with *S. pneumoniae*; *C*, section from the lung of a placebo control mouse 24 h after inoculation with *S. pneumoniae*; *D*, section from the lung of a morphine-treated mouse 24 h after inoculation with *S. pneumoniae*; *E*, section from the lung of a placebo control mouse 48 h after inoculation with *S. pneumoniae*; *F*, section from the lung of a morphine-treated mouse 48 h after inoculation with *S. pneumoniae.*

48 h after pneumococcal challenge revealed evidence of pneumonia, including multiple parenchymal foci with alveolar inflammation, alveolar epithelial cell hypertrophy, interstitial edema, hemorrhage, alveolar necrosis, and fibrin deposition. Inflammatory cell infiltration containing lymphocytes, neutrophils, and macrophages was also observed. However, the degree of tissue injury in morphine-treated mice was substantially greater than that in placebo-treated mice (Fig. 3, *E* and *F*).

Morphine decreases neutrophil recruitment after S. pneumoniae *lung infection*

To investigate whether compromised neutrophil migration was responsible for the increase in bacterial load and severity of infection seen in morphine-treated mice, morphine- and placebo-treated mice were infected with *S. pneumoniae*, and neutrophil migration was measured in the lungs at various time points. In the placebo-treated group, pneumococcal pneumonia infection induced a rapid, time-dependent accumulation of neutrophils and monocytes/macrophages in the lungs. Neutrophil numbers in control animals increased significantly ~6 h after infection and were highest ~24 h postinfection. However, at 48 h postinfection, a significant decrease in neutrophil numbers was observed compared with levels observed at 6 and 24 h postinfection (Fig. 4*A*). MPO activity measured in lung homogenate correlated with neutrophil recruitment in cells harvested from the BAL (Fig. 4*B*). In the chronic morphine-treated group, there was a significant delay in the entry of neutrophils into the alveolar compartment, resulting in significantly fewer neutrophils in lung tissue and BAL fluid 6 h post infection. However, in contrast to the observation made in placebo-treated animals, neutrophils in lung tissue and BAL fluid from the chronic morphine-treated group continued to increase late in infection, resulting in higher neutrophil counts at 48 h postinfection (Fig. 4, *A* and *B*).

Effects of morphine on MIP-2, KC, TNF-α, IL-1, and IL-6

A decrease in neutrophil migration was observed in the morphine-treated mice during the early phases of infection; therefore, we investigated whether chemokines, which are potent neutrophil attractants, were involved in the morphine-induced inhibition. Our results show that in the placebo-treated group, at 4 h after bacterial challenge, but before any significant neutrophil influx into the lung occurred, there was a significant increase in MIP-2 and KC concentrations in the BAL fluid and lung homogenates. In contrast, in the morphine-treated group, at 4 h after bacterial challenge, a significant decrease in MIP-2 and KC concentrations were observed in both BAL fluid and lung homogenates (Fig. 5).

Inflammatory cytokines have been implicated in the amplification, propagation, and prolongation of chemokine responses after *S. pneumoniae* infection; therefore, the inflammatory cytokines, TNF-α, IL-1, and IL-6 protein concentrations were measured in

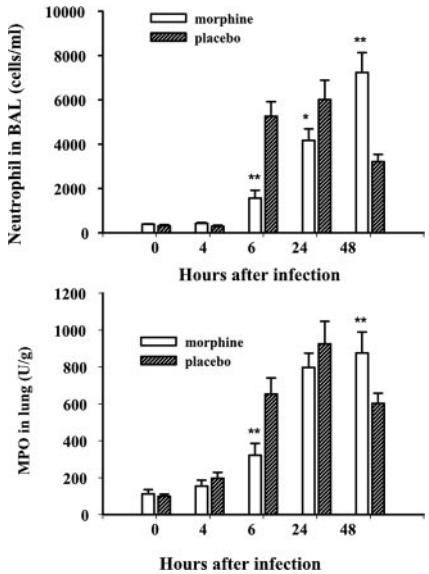

FIGURE 4. Recruitment of neutrophils in BAL (*A*; Diff-Quick stain) and lung tissue (*B*; MPO activity) of chronic morphine-treated and placebo control mice. Data are presented as the mean $\pm$ SEM. Mean values from three independent experiments are shown. **, $p < 0.01$; *, $p < 0.05$ (compared with the placebo control group; $n = 6$).

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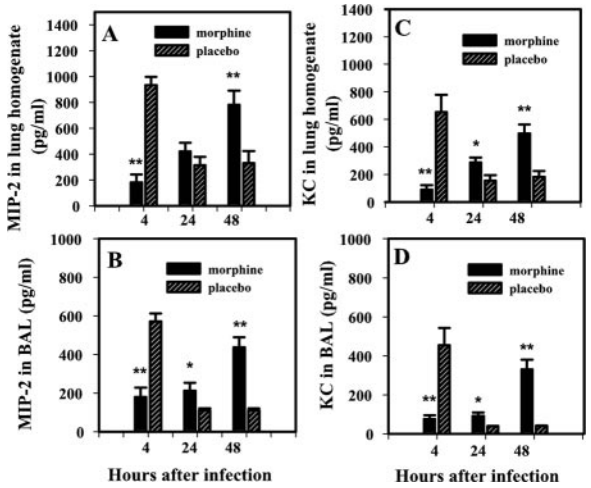

FIGURE 5. The levels of MIP-2 and KC in lung tissue (*A* and *C*) and BAL (*B* and *D*) in morphine-treated and placebo control mice. Data are expressed as the mean ± SEM of three independent experiments. *, $p < 0.05$; **, $p < 0.01$ (compared with the placebo control group; $n = 6$).

BAL fluid at 4, 24, and 48 h after infection. Our results showed that chronic morphine treatment significantly inhibited the synthesis of these cytokines when measured 4 h after infection. However, morphine treatment resulted in a compensatory increase in these inflammatory cytokines when measured 24 and 48 h after infection (Fig. 6).

Effect of morphine on CXCR2

Because neutrophils constitutively express the CXCR2, and MIP-2 interacts with CXCR2 to initiate recruitment during infection, the effect of chronic morphine treatment on neutrophil CXCR2 expression was investigated using RT-PCR. Four hours after *S. pneumonia* infection, there was a significant increase in CXCR2 mRNA expression in cells harvested from the BAL of placebo control mice. In contrast, chronic morphine treatment resulted in a significant decrease in CXCR2 mRNA expression at the early time point of infection (Fig. 7). This result is consistent with the decreased neutrophil recruitment into the alveolar compartment in early stages of infection seen with morphine treatment. Similar results were found for CXCR2 protein expression using FACS analysis (data not shown).

Effect of morphine on galectin-3

Movement of neutrophils across blood vessel walls to the site of infection first requires that the migrating cells firmly attach to the endothelial wall. Galectin-3 is a β-galactoside-binding lectin implicated in inflammatory responses as well as in cell adhesion. To identify whether galectin-3 mediated the impaired neutrophil recruitment induced by chronic morphine treatment, accumulation of galactin-3 in the BAL cell-free fraction and expression of galectin-3 in the BAL cell fraction were determined by Western blotting. At 4 h postinfection a significant decrease in the accumulation of galactin-3 was observed in the BAL cell-free fraction of chronic morphine-treated mice compared with the placebo control (Fig. 8*A*). On the contrary, the synthesis of galectin-3 in BAL cell fractions from morphine-treated mice was similar to that in placebo control mice (Fig. 8*B*). This result suggests that the early observed decrease in accumulation of galactin-3 in chronic morphine-treated animals may be partially responsible for the observed defect in neutrophil recruitment.

Two mechanisms may be involved in the decreased accumulation of gelactin-3 in BAL caused by morphine treatment: inhibition

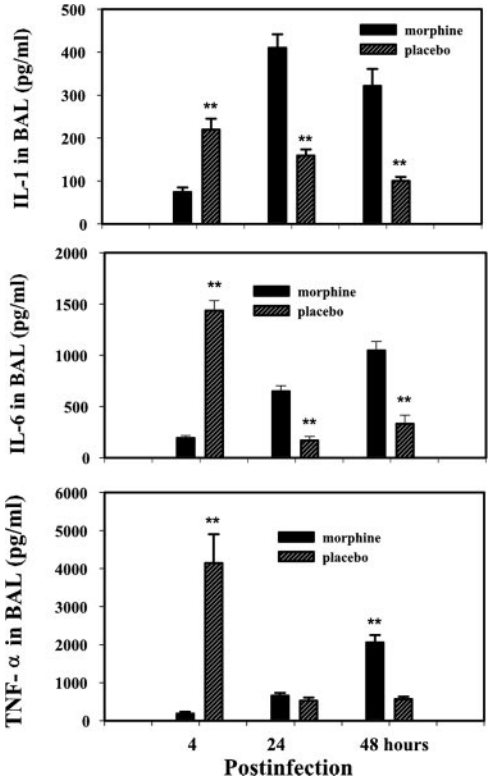

FIGURE 6. Effect of chronic morphine treatment on cytokines, IL-1, IL-6, and TNF-α in BAL fluid from infected mice. Data are presented as the mean ± SEM of three independent experiments. **, $p < 0.01$ (compared with the placebo control group; $n = 6$).

of galectin-3 release or synthesis. We investigated which mechanism contributed to the decreased accumulation of galectin-3 in BAL fluids during *S. pneumoniae* infection and morphine treatment. First, lung resident cells were obtained from untreated mice. The cell suspension was washed twice in cold RPMI 1640 medium, adjusted to a concentration of 2×10^6 cells/ml. Cells were then treated for 24 h in medium containing either vehicle (control) or morphine (500 ng/ml; National Institute on Drug Abuse, Rockville, MD). Cells were then washed twice, and fresh medium was

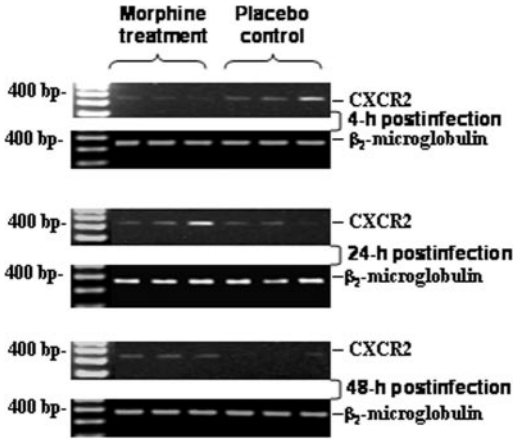

FIGURE 7. Effect of chronic morphine treatment on CXCR2 gene expression in leukocytes derived from BAL fluid. mRNA expression of CXCR2 in leukocyte of BAL fluid was determined by RT and 25 cycles of PCR. RT-PCR results were quantified using Image-Pro Plus software and are presented as a ratio of CXCR2 mRNA/β_2-microglobulin mRNA. The results are representative of three independent experiments.

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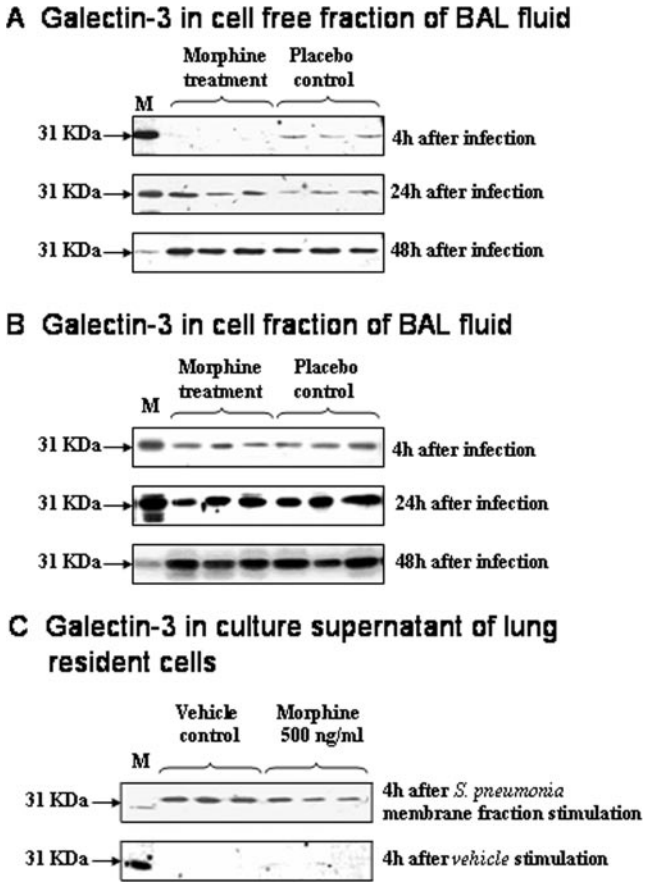

FIGURE 8. Effect of chronic morphine treatment on galectin-3 release in the BAL cell-free fraction (*A*), synthesis in the BAL cell fraction (*B*), and release in the culture supernatant (*C*) of lung resident cells. Levels of galectin-3 were compared by Western blotting. To verify equality of loading, blots were reprobed with anti-tubulin Ab. Shown are representative results from one of three independent experiments.

added to the cells. The cells were incubated for an additional 4 h in the presence of *S. pneumoniae* membrane fractions or vehicle (control). Galectin-3 released into the medium and galectin-3 synthesis in resident lung cells were analyzed by Western blotting. As shown in Fig. 8*C*, the release of galectin-3 was significantly augmented when lung resident cells were incubated with the *S. pneumoniae* membrane fraction and was markedly suppressed by morphine treatment. In contrast, the synthesis of galectin-3 in lung resident cells was not significantly altered by stimulation with the membrane fraction with either *S. pneumoniae* or morphine treatment (data not shown). These results suggest that inhibition of galectin-3 release may be one of the mechanisms by which morphine suppresses neutrophil migration.

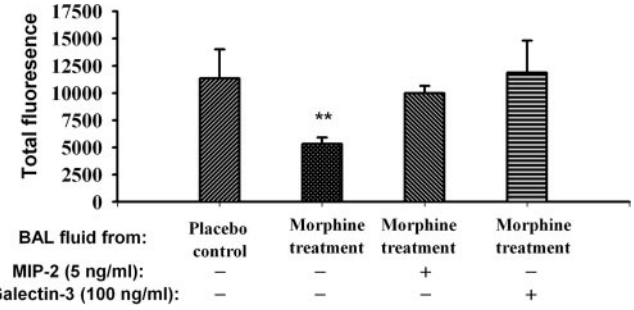

FIGURE 9. Effect of chronic morphine treatment on BAL fluid chemotactic activity. Chemoattractant activity of BAL fluid was analyzed using a neutrophil chemotaxis assay. BAL fluid was collected from morphine- and placebo-treated mice 4 h after infection. Morphine-treated BAL was supplemented with MIP-2 or galectin-3 as indicated. The values represent the mean ± SEM. Mean values from three experiments are shown. The data were normalized to the uninfected control values. **, $p < 0.01$ (compared with the placebo control group; $n = 6$).

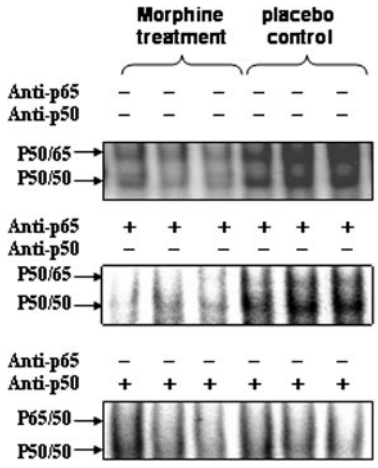

FIGURE 10. Effect of chronic morphine treatment on *S. pneumoniae*-induced NF-κB activation. The morphine-treated and placebo control mice were killed, and nuclear protein was extracted from lung tissue 2 h after *S. pneumonia* infection. NF-κB binding was measured in nuclear extracts of lung resident cells by EMSA. The results are representative of three independent experiments.

Effect of morphine on neutrophil chemotactic activity

To determine whether impaired chemokine and galectin-3 production in the chronic morphine-treated mice was sufficient to functionally decrease neutrophil chemotaxis, we measured the in vitro chemotactic response of normal neutrophils toward BAL fluid collected from morphine-treated and control groups of animals. Fluorescent-labeled normal neutrophils were placed in the upper chamber of a ChemTx chamber, and BAL fluid collected 4 h postinfection from either placebo- or morphine-treated animals was placed in the lower chamber. Neutrophil migration was determined using a fluorescence plate reader. Our results show that neutrophil migration toward BAL fluid from morphine-treated animals was significantly lower (46.5% inhibition) compared with the mean chemotactic response of neutrophils from placebo control mice challenged with *S. pneumoniae* (Fig. 9).

Next we investigated whether MIP-2 or galectin-3 can reverse the deficiency in neutrophil migration seen in morphine-treated mice. In the in vitro neutrophil migration assay system, MIP-2 (0.5 ng/ml) or galectin-3 (100 ng/ml) was added to BAL harvested from the morphine-treated group 4 h after infection. The results showed that MIP-2 and galectin-3 were able to rescue morphine's inhibitory effect, suggesting that decreased MIP-2 and galectin-3 may be responsible for the delay in neutrophil migration into infected lung seen in morphine-treated animals (Fig. 9).

Effect of morphine on NF-κB transcription activity

The coordinated expression of adhesion molecules and chemokines required for neutrophil recruitment may be mediated in part by transcription factors that bind to promoter elements common to their genes. MIP-2 and many other neutrophil ligands contain functional NF-κB sites in their upstream untranslated regions, suggesting that these genes may be regulated by the NF-κB family of

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transcription factors. Our previous studies have shown that morphine negatively regulates the NF-κB transcriptional activity in lymphocytes and macrophages (25, 27). To determine whether NF-κB may be an upstream signal of MIP-2 in our pneumonia infection model, the NF-κB nuclear protein levels of lung resident cells were measured 2 h after pneumonia infection by EMSA. As shown in Fig. 10, lung resident cells stimulated with infection resulted in a shift in the NF-κB p50/50 homodimer as wells as the inducible p50/65 heterodimer. Chronic morphine treatment significantly decreased the extent of NF-κB DNA binding to both the p50/65 heterodimer and p50/50 homodimer in pneumonia-stimulated lung resident cells.

Discussion

Drug use and abuse is associated with an increased frequency and severity of bacterial infections (29). Studies have shown that chronic morphine impairs a number of innate immune functions, including the expression of chemokine and proinflammatory cytokines, phagocytosis, and neutrophil migration (30–33). However, little is known about the molecular mechanism by which morphine modulates the innate arm of the immune system or whether modulation of innate immunity by morphine increases susceptibility to bacterial infection. In this study we examined the mediators and the molecular events involved in the recruitment of neutrophils and key components involved in the fundamental process of innate immunity in a pulmonary pneumonia infection model.

Our experiments show that pneumococcal pneumonia infection induced a rapid, time-dependent accumulation of neutrophils into the lungs of placebo-treated control animals. Neutrophil recruitment was observed as early as 6 h after infection and was sustained up to 72 h postinfection. Chronic morphine treatment resulted in a significant delay in neutrophil influx into the alveolar compartment after *S. pneumoniae* infection. This delay in neutrophil influx was associated with an increase in bacterial dissemination, which may be responsible for the 80% mortality seen in the morphine-treated group. Before neutrophil recruitment, a time-dependent increase in MIP-2 and KC protein concentration was observed in the lung tissue and BAL fluid of placebo-treated animals. MIP-2 and KC concentrations peaked at 4 h after infection, suggesting that the C-X-C chemokine MIP-2 and KC, might be involved in neutrophil recruitment after *S. pneumoniae* infection in our model system. When morphine-treated animals were investigated for MIP-2 and KC accumulation in BAL and lung tissue, our data showed that chronic morphine treatment resulted in suppression of the early accumulation of MIP-2 and KC in BAL and lung tissue after intrapulmonary infection with *S. pneumoniae*. It has been shown that MIP-2 and KC play important roles in directing neutrophils to the site of infection (34–37). Neutralization of these chemokines impairs neutrophil migration into the lung after infection (34, 35). Because chronic morphine treatment resulted in a significant suppression of MIP-2 and KC during the early phase of infection, this reduction may play a contributing role in the reduced neutrophil recruitment observed after morphine treatment.

Murine CXCR2 binds all ELR^+ CXC chemokines, including MIP-2 and KC (38). These receptors, like all chemokine receptors identified to date, are seven-transmembrane spanning, G protein-coupled receptors. Neutrophils from CXCR2 knockout mice fail to migrate in response to MIP-2 and KC in vitro (39), confirming the exclusive use by CXC chemokines of this receptor on neutrophils. To determine whether the observed differences in neutrophil recruitment that occurred in infected mice chronically treated with morphine were associated with a suppression of CXCR2 expression, CXCR2 mRNA expression was measured in leukocytes from BAL fluid after morphine treatment. Chronic morphine treatment decreased CXC receptor mRNA expression in the early phase of infection, suggesting that the effects of chronic morphine treatment on neutrophil recruitment may be due to both alterations in the expression of CXCR2 on the neutrophil surface and synthesis of CXC chemokines in BAL.

Effective pulmonary host defense against respiratory pathogens is believed to be mainly mediated via phagocytosis by alveolar macrophages and recruited neutrophils (40). Such defenses are orchestrated by a rapid inflammatory response after infection. Cytokines have been shown to be important soluble mediators responsible for coordinating this response (41). Many cytokines are known to be involved in antibacterial defenses within the lungs. TNF-α is capable of recruiting inflammatory cells to the site of infection, both directly and via up-regulation of adhesion molecules (42). TNF-α has also been shown to stimulate the release of chemokines that are directly responsible for the chemotaxis of inflammatory cells. After recruitment of phagocytic cells, TNF can also promote antimicrobial activity by activating respiratory burst activity (43) and increase degranulation of macrophages (44). These effects have been shown to be required for effective in vivo host defense against a range of microorganisms, including *Klebsiella pneumoniae* and *Pneumocystis carinii* (45). IL-1β shares several of TNF's activities, including the promotion of cell recruitment and the activation of macrophages at the site of infection (46). In some situations the two cytokines act synergistically to exert their effects (47). IL-1 may mediate similar functions as TNF-α and may facilitate the emigration of neutrophils in the lungs in the absence of TNF-α signaling. IL-6 has been ascribed both pro- and anti-inflammatory effects. IL-6 can activate monocytes (48), and it can synergize with TNF-α to increase the respiratory burst of neutrophils (49). Our results indicate that chronic morphine treatment delays neutrophil influx into the alveolar compartment after *S. pneumoniae* infection, and this delay in neutrophil influx is associated with decreased TNF-α, IL-1, and IL-6 levels in BAL fluid during the early phase of infection. Our data suggest that the susceptibility of chronic morphine-treated mice to pneumococcal pneumonia may be due in part to the reduced expression of TNF-α, IL-1, and IL-6 in lung airways during early infection. Alternatively, reduced TNF-α, IL-1, and IL-6 could result in decreased MIP-2 and KC expression and reduced neutrophil influx, facilitating bacterial proliferation in the lung tissue and dissemination into the blood and spleen in morphine-treated mice.

The NF-κB family of transcription factors is believed to be an important regulator of innate immunity (50). The activation and nuclear translocation of NF-κB have been associated with increased transcription of chemokines (IL-8 and MIP-2), adhesion molecules, and cytokines (IL-1, IL-2, TNF-α, and IL-12). Transcriptional activation of the MIP-2 promoter in the macrophage cell line RAW 264.7 by LPS has been demonstrated to be mediated through a sequence containing one copy of NF-κB binding, located between −450 and −54 of the MIP-2 promoter. The MIP-2 promoter has been shown to be activated by ectopical expression of either NF-κB p65 or c-Jun transcription factors (51). In our experimental model, *S. pneumoniae* challenge resulted in a significant increase in NF-κB migration into the nuclear compartment 2 h after infection. Chronic morphine treatment significantly impaired translocation of both NF-κB p65 and p50 transcription factors into the nuclear compartment of lung resident cells. These results are in agreement with our previous findings that both inhibition of IFN-γ promoter activity in T cells (25) and LPS-induced TNF-α activation in macrophages (27) after chronic morphine treatment were mediated through a NF-κB-dependent pathway. Thus, we speculate that a decrease in NF-κB activation after morphine treatment

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may be a conceivable molecular mechanism by which morphine increases susceptibility to *S. pneumoniae* lung infection.

The recruitment, attachment, and extravasation of leukocytes are dependent on cell adhesion molecules. Galectin-3 has been implicated in the adhesion and extravasation of neutrophils to the endothelium during streptococcal pneumonia in a β_2 integrin-independent manner. Galectin-3 has been shown to be synthesized and accumulated in the cytosol of phagocytic leukocytes. Because galectin-3 does not contain the sequence necessary for sorting through the secretory pathway, extracellular release of galectin-3 is proposed to be mediated by either passive release through cell damage or active release through a leaderless secretory pathway that may be dependent on macrophage phagocytosis (52). Our data showed that in placebo-treated animals, the release and accumulation of galectin-3 in BAL fluids occurred 4 h postinfection, peaked ~12 h postinfection, and then reached a plateau for the next 48 h. The accumulation of galectin-3 in BAL fluid (Fig. 9) of infected animals correlated closely to the kinetics of neutrophil extravasation into alveolar spaces and lung tissue (Fig. 4). Chronic morphine treatment, however, significantly reduced the release and accumulation of soluble galectin-3 in BAL fluid during the early phase of infection. This correlated with a decrease and delay in neutrophil recruitment in the early phase of infection. During the late phase of infection, an increase in galectin-3 accumulation is seen in the morphine-treated animals. We speculate that the decrease in galectin-3 release in the early phase after morphine treatment may be due to a decrease in the phagocytic activity of resident macrophages (53). In the later phase of infection, increased tissue damage resulted in a greater accumulation of galectin-3 in BAL fluid from the morphine-treated animals.

In conclusion, our data suggest that in an *S. pneumoniae* infection model, chronic morphine treatment suppresses NF-κB gene transcription in resident cells of lung tissue, which, in turn, modulates the transcriptional regulation of MIP-2 and TNF-α. In addition, morphine treatment also results in a decrease in galectin-3 release into the BAL in the early phase of infection. A combination of a decrease in MIP-2 and inflammatory cytokine synthesis and galectin-3 release in the early phase of infection leads to a reduction in neutrophil recruitment, resulting in an increase in pneumococcal bacterial burden within the lung tissue and the initiation of systemic disease. Our research provides a clearer understanding of the cellular and molecular targets of morphine action within the innate immune system during *S. pneumoniae* lung infection. These studies implicate that patients treated with morphine as an analgesic and heroin abusers are at an increased risk for *S. pneumoniae* and other bacteria infection. Our results also suggest that interventions aimed at increasing neutrophil recruitment at the site of infection during the early phases of infection may have therapeutic value.

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