

Increased Sensitivity to *Salmonella enterica* Serovar Typhimurium Infection in Mice Undergoing Withdrawal from Morphine Is Associated with Suppression of Interleukin-12

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Received 18 July 2005/Accepted 23 August 2005

We have shown previously that withdrawal from morphine induces immunosuppression in mice. The present study reports the effects of morphine withdrawal on infection with *Salmonella enterica* serovar Typhimurium. Mice were made dependent on morphine by the implantation of a slow-release morphine pellet for 96 h. Controls received a placebo pellet. Withdrawal was induced by pellet removal. Mice were inoculated intraperitoneally with *Salmonella* 24 h postwithdrawal. Morphine withdrawal sensitized mice to *Salmonella* infection, as evidenced by increased mortality, shortened mean survival time, and increased bacterial load in the blood, spleen, and liver. Examination of the levels of a panel of proinflammatory cytokines in sera of infected, morphine-withdrawn mice showed that morphine withdrawal inhibited the elevation of interleukin-12p70 (IL-12p70). The production of IL-12p40 in morphine withdrawal mice was also suppressed. The administration of exogenous IL-12 significantly decreased the bacterial burden in morphine-withdrawn mice. These studies show a correlation between the suppression of IL-12 production and a heightened susceptibility to *Salmonella* infection in mice undergoing withdrawal from morphine.

Opioid abuse has been clinically observed to correlate with an increased incidence of infectious diseases (21, 23, 27). Intravenous drug use is the second most frequently reported risk behavior for human immunodeficiency virus (HIV) infection. It is well documented that HIV-infected patients have a greater risk of *Salmonella* infections than the general population (3, 10, 18, 20, 24, 26, 39). A substantial body of literature has shown that opioids are immunosuppressive in laboratory animal models, and impaired immunity has been proposed as a major cause of increased infections in intravenous drug abusers (17, 31, 37). Our laboratory has shown that subacute administration of morphine can potentiate oral *Salmonella* infection (28). In laboratory models, acute, subacute, or chronic administration of morphine has been shown to sensitize individuals to several other pathogens, including *Streptococcus pneumoniae* (43), *Toxoplasma gondii* (12), *Klebsiella pneumoniae* (42), *Candida albicans* (42), and herpes simplex virus type 1 (32).

Most opioid abusers experience periods of withdrawal between “hits.” Yet, there are only a few studies of the effects of withdrawal from morphine on immune responses (4, 7, 19, 35, 36, 45), and there are, so far, only two papers of which we are aware on the effects of morphine withdrawal on infection (5, 14). The lack of investigation in this area is particularly surprising considering that opioid abuse and dependence remain major public health problems related to the acquisition of various infectious diseases. Development of opioid tolerance and an abstinence syndrome upon termination of the drug

(manifestations of withdrawal) are among the defining characteristics of opioid addiction. The phenomena of opioid tolerance, leading to a state of physical dependence, and abstinence have been examined extensively at the anatomical, pharmacological, biochemical, behavioral, physiological, transcriptional, and molecular levels (6, 13, 33, 34, 44). Whether abstinence can affect the host’s capacity to defend against infection and the inflammatory response to infectious agents still remains an unexplored area.

In the present study, we investigated the effect of withdrawal from morphine on the resistance of mice to systemic infection with *Salmonella enterica* serovar Typhimurium. The results show that withdrawal from morphine sensitizes mice to this bacterial infection. A potential mechanism was shown to be related to a deficiency in the production of interleukin-12 (IL-12) during the infection in morphine-withdrawn animals.

MATERIALS AND METHODS

Animals. Pathogen-free, female, 6-week-old C3HeB/FeJ mice were purchased from Jackson Laboratories (Bar Harbor, ME). The mice were allowed to acclimate for at least 1 week before use. Rodent chow and fresh water were available ad libitum.

Bacterial strain. *Salmonella enterica* serovar Typhimurium strain W118-2 was used for experimental infection in this study. It has been used extensively by our laboratory (2, 15, 28, 38). The intraperitoneal (i.p.) 50% lethal dose (LD₅₀) is <10 cells. A spontaneous mutant of W118-2 with decreased virulence (LD₅₀ = 500 CFU, i.p.) was also used. For culture growth, a lyophil was rehydrated with 5 ml of brain heart infusion (BHI) broth, incubated overnight, and then streaked onto a tryptic soy agar plate and grown overnight at 37°C. Typical colonies were picked and inoculated into 10 ml of BHI broth. The culture was incubated for 3.5 h at 37°C. The top 5 ml was inoculated into 50 ml of BHI broth and grown on a shaker at 150 rpm at 37°C for 1.5 h to produce log-phase organisms. Bacteria were counted in a Petroff-Hausser counter and diluted to the desired concentration in sterile, pyrogen-free saline. The actual number of organisms injected i.p. was calculated from duplicate spread plates on TSA.

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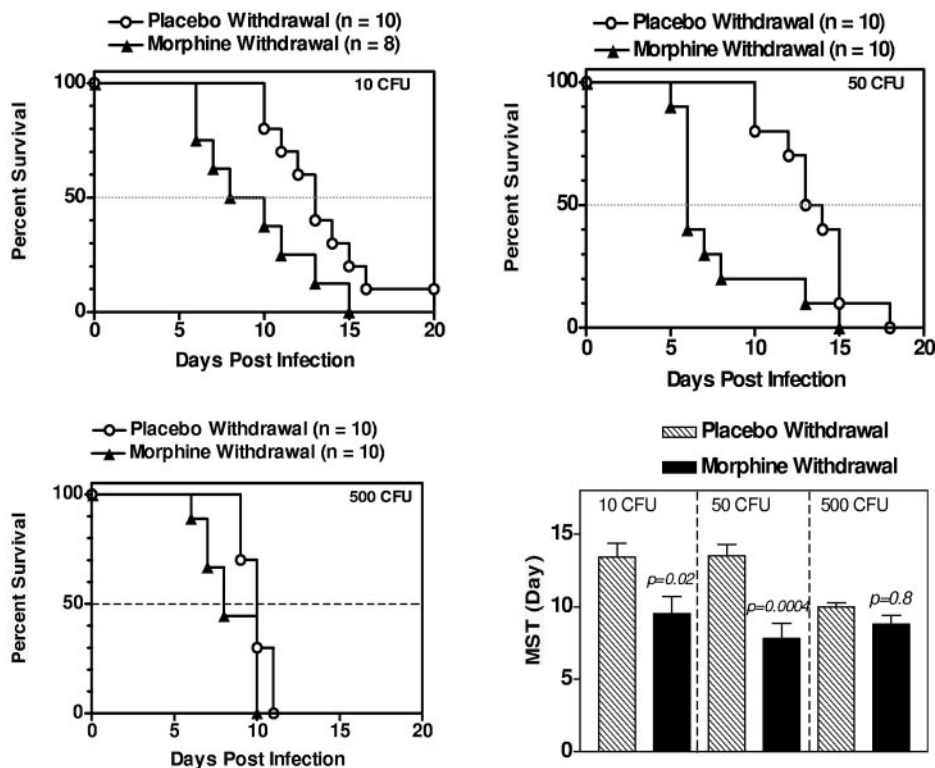


FIG. 1. Withdrawal from morphine decreases the mean survival time of mice infected with *Salmonella*. Groups of 8 to 10 mice were inoculated i.p. with the indicated doses of virulent *Salmonella* 24 h after morphine withdrawal, and mortality and mean survival time (MST) were observed for 20 days.

Experimental design and infection. Mice were anesthetized by inhaling isoflurane, and an area on the back was shaved. A 1-cm incision was made in the skin, and the mice were implanted subcutaneously with either a 75-mg slow-release morphine pellet or a cellulose placebo pellet (NIDA, Rockville, MD) sealed in a nylon mesh bag to permit easy removal. Slow-release pellets are a preferred method of opioid delivery, as the continuous presence of the drug prevents opioid withdrawal (11). The levels of morphine attained in the blood are comparable to doses that are physiological in human (9, 22). In all experiments, pellets were left in place for 96 h, at which time the animals were dependent on morphine, based on our previous work (35). Withdrawal was initiated by surgi-

cally removing the morphine pellets while the mice were under anesthesia. Placebo pellets were also removed. At 24 h postremoval of the pellets, animals were inoculated i.p. with the desired dose of log-phase *Salmonella*. Resistance to infection was assessed by survival and mean survival time over a period of 20 days. Bacterial numbers and levels of cytokines in the blood, spleen, and liver were assayed at different designed time points after inoculation. To assess the protective capacity of IL-12, in some experiments, 750 ng of mouse recombinant IL-12p70 (R&D Systems, Minneapolis, MN) in 0.2 ml of 1% bovine serum albumin (BSA) saline was injected i.p. 2 h before and 24 h after *Salmonella* inoculation. According to the supplier, the endotoxin content of the IL-12p70 preparation was less than 0.1 ng per 1 μ g.

Determination of bacterial burden. At desired time points after inoculation, the mice were bled, while under isoflurane anesthesia, from the retroorbital plexus using heparinized capillary tubes before being sacrificed by cervical dislocation. Individual spleens and livers were aseptically removed, weighed, and homogenized in 5 ml of ice-cold phosphate-buffered saline using an SDT Tissue-mixer (Tekmar Co., Cincinnati, Ohio). A 0.1-ml sample of homogenate or appropriate dilution in phosphate-buffered saline was plated on Levine eosin-methylene blue agar plates (Difco) and grown overnight, and the colonies were counted. For the determination of bacterial burden in the blood, 20 μ l of heparinized blood was plated directly onto eosin-methylene blue plates.

Collection of plasma and organ homogenates for cytokine determination. For collection of plasma used for measuring the cytokine levels in blood, the heparinized blood was centrifuged at 2,000 rpm for 10 min at 4°C and the clarified plasma was stored at -70°C until use. To prepare organ extracts used for cytokine assessment, aliquots of organ homogenates from individual mice were clarified by centrifugation at 13,000 rpm for 20 min at 4°C using a bench-top model 5415R centrifuge (Eppendorf, Germany), and the protein concentrations of samples were determined using the BCA protein assay kit (Pierce, Rockford, IL). The homogenates were stored at -70°C until used.

Cytokine measurement by CBA. Levels of multiple cytokines in plasma or organ homogenates of individual mice were assessed simultaneously by flow cytometry using the cytometric bead array (CBA) technique (BD Pharmingen, San Diego, CA). Capture beads coated with anti-murine IL-12p70, gamma interferon (IFN- γ), tumor necrosis factor alpha (TNF- α), monocyte chemoattract-

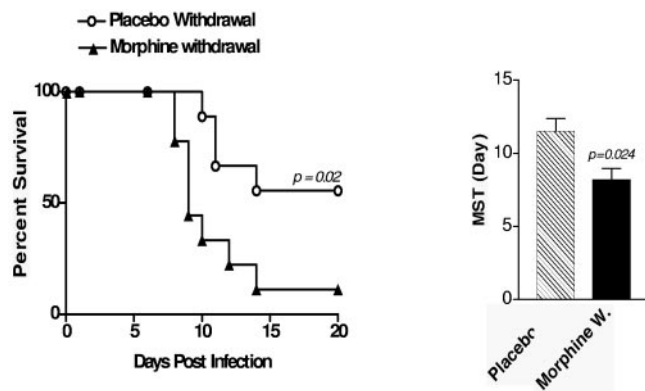


FIG. 2. Withdrawal from morphine increases the mortality of mice infected with an attenuated variant of *S. enterica* serovar Typhimurium strain W118-2. Groups of nine mice were inoculated i.p. with 500 organisms of a spontaneous mutant, W118-2, at 24 h after morphine withdrawal, and mortality and mean survival time (MST) were observed for 20 days.

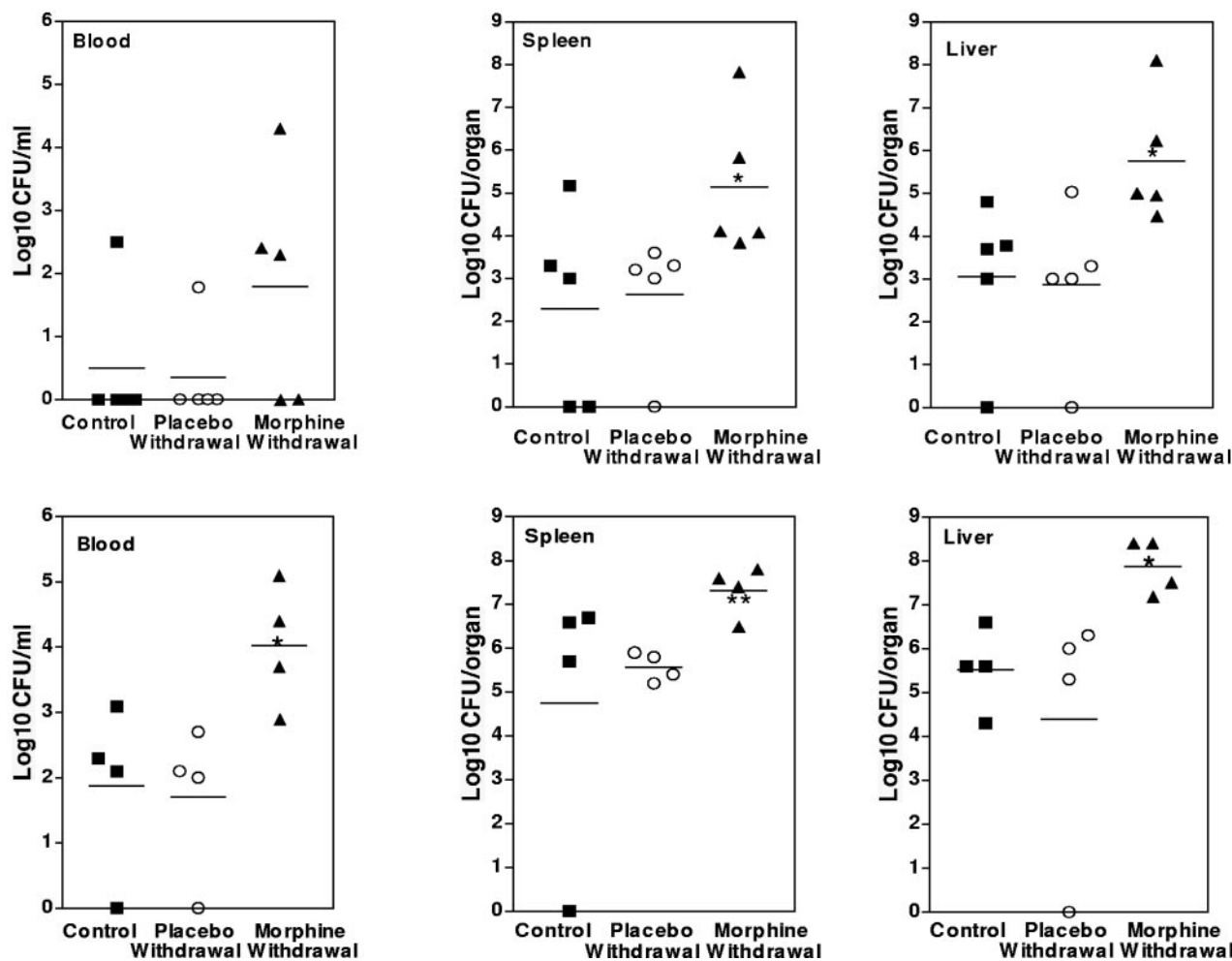


FIG. 3. Withdrawal from morphine increases the bacterial burden in infected mice. Animals were inoculated i.p. with 10 CFU of virulent W118-2 at 24 h postwithdrawal, and bacterial numbers in the blood, spleen, and liver were assayed 6 (top row) and 12 (bottom row) days later. Dashes are mean values for each group. The results are representative of two experiments. *, $P < 0.05$; **, $P < 0.001$, morphine-withdrawal group versus the placebo-withdrawal group or normal control group.

tant protein 1 (MCP-1), IL-10, or IL-6 (each with discrete fluorescence intensity characteristics) antibodies were purchased from BD Pharmingen (San Diego, CA). The assay was carried out according to the manufacturer's recommendations. Briefly, capture beads specific for the various cytokines were mixed together to form a bead suspension. Fifty-microliter aliquots of the bead suspension were then incubated with a 50- μ l sample collected from an individual mouse and 50 μ l of the anti-murine phycoerythrin detection reagent for 2 h at room temperature in the dark. One ml of wash buffer (provided by the manufacturer) was added to each assay tube and centrifuged at $200 \times g$ for 6 min. The supernatants were discarded, and 200 μ l of wash buffer was added to resuspend the bead pellets. For each sample, 3,000 events per capture bead were gated and analyzed on a BD FACScan flow cytometer (BD Pharmingen, San Diego, CA) calibrated with cytochrome setup beads that were provided by the manufacturer. Amounts of IL-12p70, IFN- γ , TNF- α , MCP-1, IL-10, and IL-6 were quantitated using standard curves generated by flow cytometric analysis of cytokine standards incubated with mixed capture beads and an anti-murine phycoerythrin detector antibody to develop the reaction.

Cytokine ELISA. Levels of IL-12p40 in plasma and homogenates of the spleen and liver were measured by capture enzyme-linked immunosorbent assay (ELISA), with antibody pairs and the IL-12 standard. Antibody pairs for cytokines were purchased from Pharmingen. The assay was carried out according to the manufacturer's recommendations. Briefly, the 96-well ELISA plates (Nunc-Immuno Maxisorp plates; Fisher Scientific, Pittsburgh, PA) were coated at 4°C overnight with 100 μ l of capture monoclonal antibody per well in 0.2 M sodium phosphate buffer (pH 6.5). After being blocked with 200 μ l of assay diluent

(Pharmingen) at room temperature for 1 h, samples were loaded in 100 μ l of the diluent and the plates were incubated at room temperature for 2 h. Serial twofold dilutions of recombinant cytokines were included as standards. A volume of 100 μ l of the horseradish peroxidase-conjugated rat anti-mouse detection monoclonal antibody was added to each well and incubated for 1 h at 37°C. Finally, a mixture of tetramethylbenzidine and hydrogen peroxide was added as a substrate for the horseradish peroxidase, and the color in each well was allowed to develop at room temperature for 30 min in the dark. The reaction was stopped by addition of 2 N H₂SO₄. The optical density value at 405 nm was read by an automated microplate reader (model 2550; Bio-Rad). Cytokine concentrations were determined by comparison with the standard curve.

Statistical analysis. Significant differences between groups were analyzed by a two-tailed Student unpaired *t* test or Fisher's exact test for survival assays by using the PRISM computer program (GraphPad, San Diego, Calif.). A $P < 0.05$ was considered statistically significant.

RESULTS

Withdrawal from morphine potentiates *Salmonella* infection. To examine the effects of morphine withdrawal on *Salmonella* infection, mice were made dependent on the drug by the implantation of a slow-release morphine pellet for 96 h. Withdrawal was induced by removing the pellet. Controls received

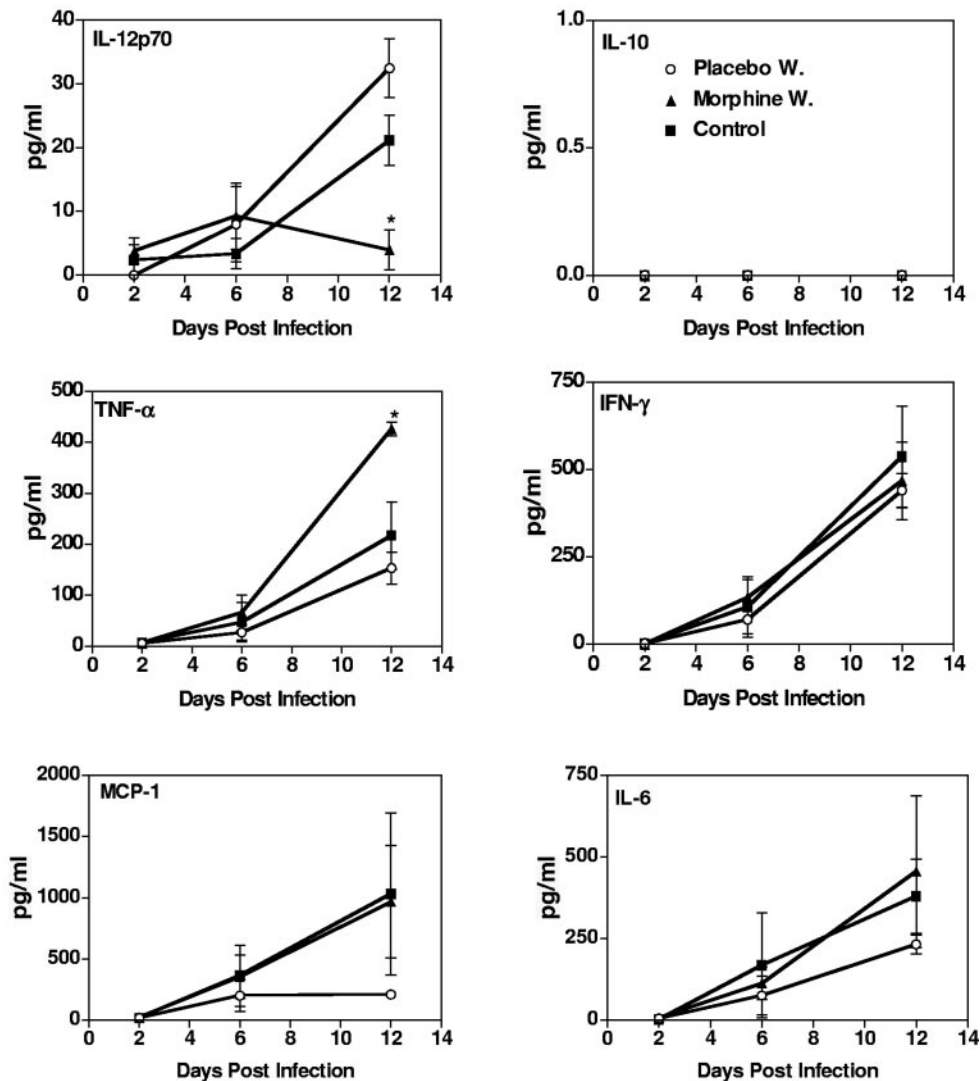


FIG. 4. Levels of plasma inflammatory cytokines in mice during the infection course. Mice were inoculated i.p. with 10 CFU of *Salmonella* at 24 h after morphine withdrawal. At 2, 6, and 12 days after inoculation, the mice were bled and plasma was harvested. The amounts of IL-12p70, TNF- α , MCP-1, IL-10, IFN- γ , and IL-6 were assessed by flow cytometry using the CBA kit. The results represent means \pm standard deviations ($n = 5$). *, $P < 0.05$, morphine-withdrawn group versus the placebo-withdrawn group. Data are representative of one of four experiments.

placebo pellets, which were also removed after 96 h. For the survival experiment, mice were infected i.p. with different doses of virulent *Salmonella* 24 h after initiation of withdrawal, and survival was scored for 20 days. As shown in Fig. 1, there was no difference in mortality between placebo and morphine withdrawal groups at any of the challenge doses. However, morphine withdrawal significantly lowered the mean survival time at challenge doses of 10 and 50 CFU. Other groups of animals were infected i.p. with an attenuated variant of *Salmonella* strain W118-2, and the mortality was observed for 20 days postinfection. As shown in Fig. 2, in the morphine withdrawal group, death started on day 7, with a survival rate of 11% and a mean survival time of 8.2 ± 1.8 days. In contrast, the survival rate of infected placebo mice was 56%, with death commencing on day 10, and a mean survival time of 11.5 ± 2.5 days ($P < 0.05$).

The more rapid mortality in morphine-withdrawal mice was supported by examining the bacterial burdens in the blood,

livers, and spleens of infected mice undergoing withdrawal from morphine. At 24 h after withdrawal from morphine, mice were inoculated i.p. with 10 CFU of the virulent strain of *Salmonella*, and bacterial numbers in the blood and organs were assayed on days 2, 6, and 12 postinfection. The results show that there was no detectable *Salmonella* growth in any of the groups on day 2 postinfection (data not shown). However, on days 6 and 12 the bacterial numbers in the blood, livers, and spleens of morphine-withdrawal mice were significantly higher than those of the placebo-withdrawal group (Fig. 3), and the differences were statistically significant.

These results clearly demonstrate that mice undergoing withdrawal from morphine are more sensitive to the lethality caused by *Salmonella* infection, as shown by a shortened survival time and increased bacterial burden after infection with the lethal strain of *Salmonella* and enhanced mortality after infection with an attenuated strain of the bacterium.

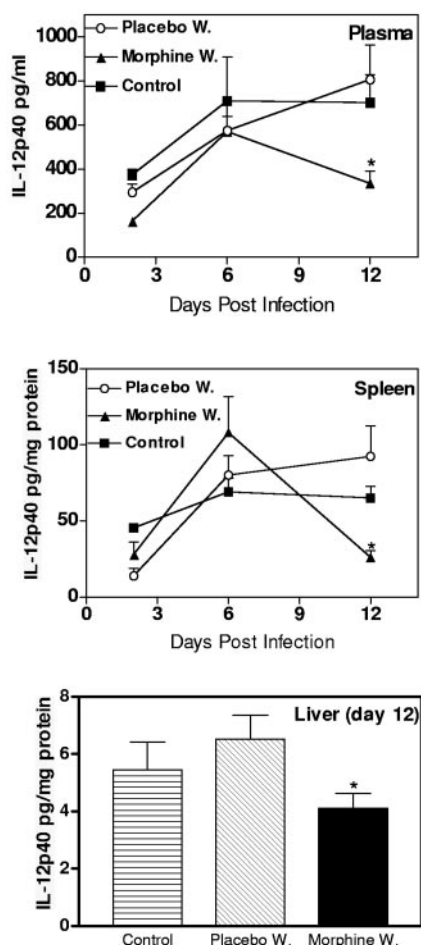


FIG. 5. IL-12p40 levels of the plasma, spleen, and liver in mice during the *Salmonella* infection course. Mice were inoculated i.p. with 10 CFU of *Salmonella* 24 h after morphine withdrawal. At 2, 6, and 12 days after inoculation, the amounts of IL-12p40 in the plasma, spleen, and liver (day 12 only) were assessed by ELISA. The results represent means \pm standard deviations ($n = 5$). *, $P < 0.05$, morphine-withdrawn group versus the placebo-withdrawn group or normal control group. Data represent 1 of 4 experiments.

Morphine withdrawal alters proinflammatory cytokine production in *Salmonella*-infected mice. To examine the mechanism underlying the increased bacterial growth and sensitivity to lethality in mice undergoing morphine withdrawal, the dynamics of production of IL-12p70, TNF- α , MCP-1, IFN- γ , IL-6, and IL-10 were assessed during the course of infection in mice undergoing withdrawal from morphine and compared to that in infected, placebo-withdrawn mice or infected normal mice (controls). Cytokine levels in the blood were assessed on days 2, 6, and 12 postinfection with 10 CFU of virulent *Salmonella*. As shown in Fig. 4, there were minimal increases in cytokine levels over the first 6 days of infection. On day 12 postinfection, the TNF- α level was significantly increased in infected morphine-withdrawn mice, compared to that in infected placebo-withdrawn or infected control mice. No significant differences were observed in the levels of MCP-1, IFN- γ , and IL-6 among these same groups at any time point. IL-10 levels were below the detectable range in all groups at all time points. In contrast, *Salmonella* infection resulted in a rise in

IL-12p70 production in both placebo-withdrawn and normal mice on day 12 postinfection, but morphine-withdrawn mice showed depressed IL-12p70 levels under the same conditions.

IL-12p40 production in *Salmonella*-infected mice undergoing withdrawal from morphine. There is evidence that IL-12 is important in mediating the host's resistance to *Salmonella* (1, 8, 25, 29, 30, 40). To further investigate IL-12 production in mice receiving *Salmonella* following morphine withdrawal, the IL-12p40 protein level in the plasma, spleens, and livers from the same mice used for Fig. 4 were measured. The results are shown in Fig. 5. The levels of IL-12p40 in the blood and spleens of all groups were significantly increased on day 6 compared to those at day 2 postinfection, with no significant differences between the infected morphine-withdrawn and infected placebo-withdrawn or infected control mice. The elevated levels of IL-12p40 in the blood and spleen were maintained through day 12 postinfection only in the placebo-withdrawn and control groups but dropped significantly in the morphine-withdrawn mice at day 12. The levels of liver IL-12p40 were also suppressed in morphine-withdrawn mice on day 12 postinfection compared to those of the placebo-withdrawn and control mice. The data clearly demonstrate that the capacity for IL-12 production in response to *Salmonella* infection in morphine-withdrawn mice was suppressed at the later stage of infection.

Exogenous IL-12 protects against *Salmonella* infection in mice undergoing withdrawal from morphine. The results above clearly demonstrate that a deficiency of IL-12 production exists in mice undergoing both withdrawal from morphine and *Salmonella* infection. Previous studies have shown that IL-12 augments the protective immune response to *Salmonella* (25). Accordingly, we tested the hypothesis that administration of IL-12 to mice undergoing morphine withdrawal would afford protection against *Salmonella* infection. Mice were inoculated i.p. with *Salmonella* 24 h postwithdrawal. At 2 h before and 24 h after the bacterial inoculation, 750 ng of IL-12p70 was administered i.p. in 0.2 ml saline to each mouse. Bacterial numbers in the blood, spleens, and livers were assayed 3 days after inoculation. Figure 6 shows that treatment of mice with mrIL-12p70 dramatically reduced bacterial burdens in the withdrawal group compared to those in animals undergoing morphine withdrawal and receiving no IL-12p70 treatment. In the spleen, the IL-12 reduced the bacterial burden significantly, to levels of *Salmonella* even below those observed in the placebo withdrawal group.

The results strongly indicate an important role for IL-12 in the pathogenesis and immune response to *Salmonella* in mice undergoing withdrawal from morphine.

DISCUSSION

The present study is the first to examine the effects of withdrawal from morphine on host defense against a bacterial infection and on cytokine production during the course of infection in mice. Morphine withdrawal sensitized mice to virulent *Salmonella* infection, as evidenced by a decreased mean survival time and increased bacterial burdens over the period 6 to 12 days postinfection. Significant differences were observed in the survival rates of mice undergoing withdrawal from morphine and challenged with an attenuated strain of *Salmonella*. There are two other reports in the literature on the effect of

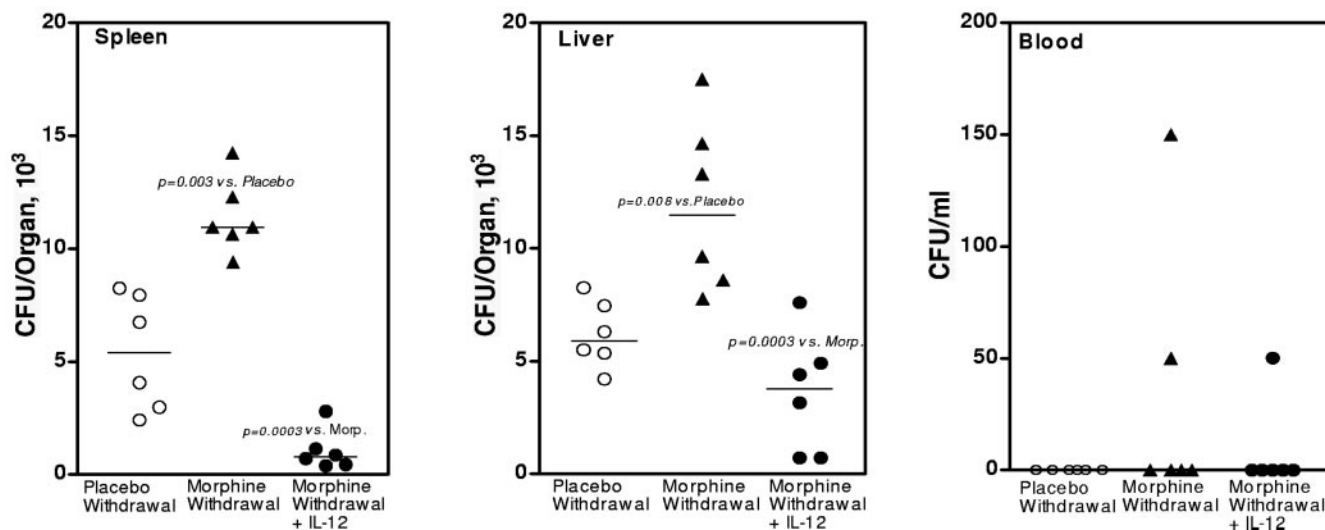


FIG. 6. Exogenous IL-12 decreases the bacterial burden in different organs of mice undergoing withdrawal from morphine and infected with *Salmonella*. Mice were inoculated i.p. with 500 organisms of the attenuated variant of *Salmonella* at 24 h postwithdrawal. Each animal received 750 ng of IL-12p70 i.p. in 0.2 ml saline containing 1% BSA 2 h before and 24 h after bacterial inoculation. Placebo-withdrawn and morphine-withdrawn groups received the same volume of 1% BSA saline. The bacterial numbers in the blood, spleens, and livers were assayed 3 days after infection. Dashes are median values for each group.

opioid withdrawal on infection, and they reached opposite conclusions. Donahoe et al. (14) found that if simian immunodeficiency virus-infected rhesus monkeys were treated chronically with morphine and withdrawn from the drug, the viral titer increased. In contrast, Barr et al. (5) found that chronic morphine treatment and withdrawal did not alter the levels of feline immunodeficiency virus infection in cats. These models are different from the one we used, as they measured the effect of withdrawal on retroviral infection. Further, the infections were present when withdrawal occurred, whereas our paradigm measured the effect of withdrawal on host defense to a subsequent infection. Our findings support the conclusion that opioid withdrawal has a potentiating effect on *Salmonella* infection.

An interesting and novel finding of our study is that at later stages of infection morphine-withdrawn mice had significantly depressed serum levels of IL-12 compared to those of infected, placebo-withdrawn mice. Administration of exogenous IL-12 before and during the early stage of *Salmonella* infection in mice undergoing withdrawal from morphine greatly decreased the bacterial burdens in spleens and livers. The essential role of IL-12 in protective immunity to *Salmonella* has been unambiguously demonstrated in murine models of *Salmonella* (8, 25, 29, 30). Evidence for the importance of this cytokine includes the observation that administration of recombinant IL-12 dramatically increased survival times of mice orally challenged with *Salmonella enterica* serovar Dublin (25). In addition, mice pretreated with anti-IL-12 antibody had an increased *Salmonella* burden and reduced mean survival time compared with those of mice receiving control antibody (25). Further, successful protective immunity to *Salmonella* was correlated with increased production of IL-12p40 mRNA in the Peyer's patches within hours after oral infection (8). These observations are supported by clinical studies showing that humans with inherited IL-12 deficiencies due to mutations in genes coding for IL-12 or the IL-12 receptor are more susceptible to *Salmonella*

enterica serovar Enteritidis and *Mycobacterium bovis* bacillus Calmette-Guérin, with the former causing disseminated infection in a child with this disorder (1, 40). The importance of IL-12 in protecting against *Salmonella* and mycobacterial infection undoubtedly relates to the role of this cytokine in polarizing immune responses to a Th1 phenotype, with subsequent macrophage activation (41).

Interestingly, in a previous study, we showed that mice in withdrawal from morphine were sensitized to a sublethal dose of bacterial lipopolysaccharide (LPS) given 24 h after the initiation of abstinence (16). Sensitization to the toxic effects of LPS correlated with elevated levels of TNF- α . However, in these mice IL-12 was also differentially suppressed, providing a second example of a paradigm in which withdrawal depressed IL-12 production (16). The mechanism by which withdrawal from opioids might modify IL-12 levels is of interest but unknown and currently under investigation. The mechanism would have to account for a selective action on IL-12 compared to those on other proinflammatory cytokines.

The possibility that other parameters of immune function besides IL-12 may be disrupted during withdrawal and may be responsible for the observed sensitivity to systemic *Salmonella* infection must also be taken into account. We have previously shown that 24 h after withdrawal from morphine, mice are immunosuppressed, as assessed by the capacity of spleen cells to mount an ex vivo antibody response, by depression of the expression of proinflammatory cytokines in response to LPS in vitro, and by the reduction in levels of B7.2 on macrophages stimulated with LPS (35, 36). Other studies reported in the literature on the effects of withdrawal from morphine on immune responses have also shown suppression of T-cell proliferation to mitogens (4, 19) and suppression of NK cell activity (7, 45), as well as alterations in T-cell subsets (19). Further studies will be needed to assess the contribution of these immune parameters to a host's defense against *Salmonella* infection during withdrawal.

The results presented in this paper show that withdrawal can

sensitize mice to a bacterial infection and modify levels of IL-12, a cytokine which is important in host defense against intracellular infections of macrophages. These findings raise the possibility that opioid addicts may experience an increased incidence of infection because of periods of immunosuppression induced during episodes of withdrawal.

ACKNOWLEDGMENTS

This work was supported by National Institute on Drug Abuse grants DA14223 and DA13429.

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