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*J Immunol* 1999; 163:6148-6154;
http://www.jimmunol.org/content/163/11/6148

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Endogenous Monocyte Chemoattractant Protein-1 (MCP-1) Protects Mice in a Model of Acute Septic Peritonitis: Cross-Talk Between MCP-1 and Leukotriene B₄

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We investigated the involvement of monocyte chemoattractant protein (MCP)-1 in a murine model of septic peritonitis induced by cecal ligation and puncture (CLP). Initial studies demonstrated that CLP induced a dramatic increase in MCP-1 production in the peritoneum, followed by an increase in the recruitment of leukocytes. MCP-1 blockade with anti-MCP-1 antiserum significantly decreased the survival rate following CLP, which was accompanied by an enhanced recovery of viable bacteria from the peritoneum. This was likely due to the reduction in the recruitment and activation of both macrophages and neutrophils. To understand the mechanisms whereby MCP-1 may influence neutrophil infiltration, levels of chemokines known to attract neutrophils were monitored, which showed that peritoneal levels of macrophage-inflammatory protein (MIP)-2, KC, and MIP-1α were not altered with anti-MCP-1 Abs. However, anti-MCP-1 Abs reduced the peritoneal levels of leukotriene B₄ (LTB₄) by 59%. The i.p. injection of MCP-1 into normal mice resulted in elevated levels of LTB₄ in the peritoneum. In vitro, MCP-1 stimulated the production of LTB₄ from peritoneal macrophages, in a dose-dependent manner. A specific LTB₄ receptor antagonist (CP-105,696) inhibited CLP-induced recruitment of both neutrophils and macrophages, which was accompanied by a reduced level of MCP-1 in the peritoneum. Finally, administration of CP-105,696 was extremely detrimental to the survival of mice following CLP. These experiments demonstrate that endogenous MCP-1 serves as an indirect mediator to attract neutrophils via the production of LTB₄, and suggest the cross-talk can occur between MCP-1 and the lipid mediator LTB₄ during septic peritonitis. The Journal of Immunology, 1999, 163: 6148–6154.

Despite the development of powerful antibiotics and advances in the management of intensive care patients, sepsis still remains a serious disorder with high rates of morbidity and mortality (1). Sepsis-related mortality is often multifactorial and can be caused by a variety of conditions, including shock, coagulopathy, and multiple organ failure. Although the overall mortality rate of sepsis is approximately 25 to 35% (1, 2), patients with septic peritonitis have a mortality rate between 60 and 80% (3). Septic peritonitis is usually characterized by a massive leukocyte infiltration into the peritoneum, as the host responds to spillage of polymicrobial flora in the peritoneal cavity (4). The recruitment and subsequent activation of leukocytes are an important effector mechanism for clearing the fecal contamination and microbial killing (5, 6). Thus, the inflammatory mediator systems and cascades that result in eliciting and activating leukocytes appear to be essential in protecting the host by restricting the polymicrobial flora to a small area in the peritoneum.

A growing body of evidence suggests that the recruitment of leukocytes is governed by cell-specific chemoattractants, called chemokines (7). Chemokines are mainly divided into two subfamilies. CXC chemokines are typically chemotactic for neutrophils whereas CC chemokines attract and activate mononuclear cells (7). IL-8, a prototype of CXC chemokines, was detected in peritoneal fluids of patients with septic peritonitis (8), while macrophage inflammatory protein-2 (MIP-2)3 a murine CXC chemokine functionally equivalent to human IL-8, was previously found in the peritoneal fluids in a murine model of septic peritonitis (9). These studies suggest a role of CXC chemokines in both clinical and experimental septic peritonitis. Monocyte chemoattractant protein (MCP)-1, a prototype of CC chemokines, is produced by a variety of cells in vitro after stimulation with TNF-α, IL-1, or endotoxin (10), which are all involved in the pathogenesis of sepsis (11). Elevated levels of MCP-1 have been detected in plasma of patients with sepsis (12), as well as after administration of endotoxin to experimental animals or human volunteers (13, 14). Collectively, these investigations have clearly demonstrated the presence of MCP-1 in association with the development of septic peritonitis; however, the mechanistic role of MCP-1 in sepsis evolving from peritonitis is unclear.

In the present study, we have assessed the function of endogenous MCP-1 in a murine model of septic peritonitis induced by cecal ligation and puncture (CLP). The pathology of this model results from a polymicrobial peritonitis due to the leakage of intestinal flora from the puncture wounds and mimics clinical sepsis with peritonitis associated with postsurgical or accidental trauma (15). Using this model, we have shown that MCP-1 can direct the host response to this pathogenic challenge via the establishment of novel in vivo inflammatory cascades. The contribution of this CC

Abbreviations used in this paper: MIP-2, macrophage-inflammatory protein-2; MCP, monocyte chemoattractant protein; CLP, cecal ligation and puncture; LTB₄, leukotriene B₄; TSA, thymic-shared Ag.

Received for publication April 29, 1999. Accepted for publication September 13, 1999.

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1 Abbreviations used in this paper: MIP-2, macrophage-inflammatory protein-2; MCP, monocyte chemoattractant protein; CLP, cecal ligation and puncture; LTB₄, leukotriene B₄; TSA, thymic-shared Ag.
chemokine was first assessed via neutralization studies, where Abs directed against murine MCP-1 significantly increased the mortality rate due to the developing peritonitis. At the mechanistic level, MCP-1 was found to play an important role in the elicitation of both macrophages and neutrophils. While monocyte recruitment is a known biological activity of MCP-1, we have found that MCP-1 can attract neutrophils via the production of leukotriene B_4 (LTB4) during the evolution of CLP. Our data also demonstrate that LTB4 can induce the production of MCP-1, suggesting that the inflammation due to CLP can be amplified by cross-talk between MCP-1 and LTB4.

Materials and Methods

Mice

Female CD-1 mice (6 to 8 wk of age) were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in the animal care facility unit (University Laboratory of Animal Medicine). The animal use committee at the University of Michigan approved all studies. The total number of mice used in this study was 330.

CLP model

CLP surgeries were performed as described previously (9, 15). In brief, the mice were anesthetized with an i.p. injection of ketamine HCl (Vetaminate; Mallinckrodt Veterinary, Mundelein, IL), followed by inhaled methoxyflurane (Metaflane; Mallinckrodt Veterinary). Under sterile conditions, the cecum was exposed through a 1- to 2-cm incision of the lower left abdomen, ligated tightly with a 3-0 silk suture without causing bowel obstruction, and then punctured through and through once with a 21-gauge needle. The cecum was replaced in the peritoneal cavity and the incision was closed with surgical staples. The mice then received 1 ml of saline s.c. for fluid resuscitation, and placed on a heating pad until they recovered from the anesthesia.

Neutralization of MCP-1

To neutralize MCP-1 activity, 0.5 ml of anti-murine MCP-1 antisera was injected i.p. 2 h prior to CLP. The volume of antiserum was considered to be sufficient, because a larger volume (1 ml) or repeated injections of the antisera (twice with 0.5 ml; −2 and 48 h after CLP) did not induce further biological effects (data not shown). Furthermore, the biological half-life of the Ab was approximately 36 h (C. M. Hogaboam, personal observations). Polyclonal anti-murine MCP-1 antiserum was raised by immunizing rabbits with murine rMCP-1 (R&D Systems, Minneapolis, MN) (16). The Abs did not cross-react with a number of other murine cytokines including CXC and CC chemokines, since MCP-1 ELISA established with the Abs did not detect any murine cytokines at a concentration of as high as 100 ng/ml. The Abs had a neutralizing activity against murine rMCP-1 in vitro chemotaxis assay. In addition, previous reports showed that administration of anti-MCP-1 antiserum effectively and specifically neutralized MCP-1 activity in vivo (16–20). As a control, preimmune rabbit serum (0.5 ml) was used.

Experimental protocol

In the first set of experiments, the mice were observed for 7 days after CLP to determine the mortality rate induced with CLP. In the second set of experiments, the CLP mice were anesthetized, bled, and euthanized at 4, 8, 24, and 48 h after CLP. The peritoneal cavities were washed with 2 ml of serum (0.5 ml) was used. Two ml of peritoneal lavage fluid was collected, centrifuged at 6000 rpm for 10 min, and then the supernatant assayed for LTB4.

Statistics

Statistical significance was evaluated by a two-tailed unpaired Student’s t test. In case of survival curve and CFU count, the data were analyzed by the log-rank test and Mann-Whitney test, respectively. A p value <0.05 was regarded as statistically significant. All data were expressed as mean ± SEM.

Results

Leukocyte infiltration in the peritoneal cavity after CLP

Investigations were first established to determine the leukocyte infiltration into the peritoneal cavity post-CLP. As shown in Fig. 1, CLP induced a rapid peritonitis composed of various leukocyte subpopulations. The number of total infiltrating leukocytes reached the peak level at 8 h, and decreased after 24 h (Fig. 1). Differential
cell analyses demonstrated that the accumulation of neutrophils peaked at 8 h, while the number of macrophages peaked at 24 h. Similar levels of macrophages still remained at 48 h. The accumulation of lymphocytes gradually increased with time (Fig. 1). These studies demonstrate that a temporal pattern of leukocyte infiltration into the peritoneum occurs after CLP that reflects an early neutrophil infiltrate followed by the elicitation of macrophages and lymphocytes.

**Generation of MCP-1 in peritoneal fluids and sera after CLP**

Previous investigations demonstrated that certain CXC chemokines, such as MIP-2, were elevated in the peritoneal fluids during the evolution of CLP-induced peritonitis (9); thus, experiments were next conducted to assess peritoneal fluid levels of the CC chemokine, MCP-1. During the evolution of CLP-induced peritonitis, levels of MCP-1 in the peritoneal fluids were detectable by 4 h, peaked at 8 h (94.6 ± 21.9 ng/cavity, n = 6), and then rapidly declined (Fig. 2A). Interestingly, the peak levels of MCP-1 in the peritoneum were extremely high, preceded the peak in the recruitment of macrophages by 16 h, and were coincident with the peak in neutrophil levels. Upon further analyses, MCP-1 levels in sera were found to peak at 8 h (1.9 ± 0.3 ng/ml, n = 6), but were much lower than those found in the peritoneal fluids (Fig. 2B), suggesting that the MCP-1 was produced in greater abundance in the peritoneum. These data indicate that MCP-1 levels were augmented during the evolution of CLP-induced peritonitis.

**Neutralization of MCP-1 increases CLP-induced lethality**

To examine the contribution of MCP-1 during the evolution of CLP-induced peritonitis leading to lethality, either anti-MCP-1 antiserum or control serum was i.p. injected 2 h prior to CLP surgery, and the survival rates were monitored. As shown in Fig. 3, neutralization of MCP-1 significantly deteriorated the survival of mice following CLP. At 24 h post-CLP, 92% of control mice (24 of 26 mice) were alive compared with 67% in anti-MCP-1 antiserum-treated mice (20 of 30 mice). The detrimental effects of neutralizing MCP-1 were most apparent at 48 h. At this time point, the mortality rate in control mice was 27% (7 of 26 mice), however, a striking 67% mortality rate (20 of 30 mice) was observed in mice that received anti-MCP-1 antiserum (Fig. 3). These findings suggest that endogenous MCP-1 may have a protective role in CLP-induced lethality, as removal of murine MCP-1 resulted in an increase in the mortality.

**Neutralization of MCP-1 decreases bacterial clearance in the peritoneal cavity**

In an attempt to identify the mechanism whereby MCP-1 may exhibit a beneficial effect during the development of CLP-induced peritonitis, we determined the bacterial load in the peritoneal fluids and sera of mice treated with either anti-MCP-1 antiserum or control serum. No bacteria were recovered from peritoneal fluid samples collected at 4 and 8 h after CLP in either the anti-MCP-1- or control serum-treated groups. However, peritoneal fluids from the 24-h time point contained a significant number of bacteria in 8 of 14 mice receiving control serum (mean CFUs 1.1 × 10^7/10 μl peritoneal fluid) (Fig. 4). At this same time point, animals treated with anti-MCP-1 antiserum and developing CLP-induced peritonitis had a 65-fold increase in bacteria recovered from the peritoneal fluids (11 of 14 mice, mean CFU = 7.1 × 10^9/10 μl, p < 0.01) when compared with controls (Fig. 4). In addition, 2 of 14 mice presented with a bacteremia at 24 h in the CLP animals treated with anti-MCP-1 antiserum. These studies support a host defense role for endogenous MCP-1 during experimental sepsis, as neutralization of MCP-1 resulted in a reduced ability to clear bacteria from the peritoneum.

**Effects of anti-MCP-1 antiserum on the leukocyte infiltration induced with CLP**

In order to assess the in vivo contribution of MCP-1 on leukocyte infiltration during the evolution of CLP-induced peritonitis, mice
were treated with neutralizing anti-MCP-1 antiserum and total leukocyte numbers were determined. As shown in Fig. 5A, neutralization of MCP-1 resulted in a decreased number of total leukocytes at 8 h after CLP. Furthermore, differential cell analyses demonstrated that the recruitment of both neutrophils and macrophages were reduced by anti-MCP-1 antiserum by 61% and 53%, respectively (Fig. 5, B and C). At the 24-h time point, no significant differences were found regarding the recruitment of total leukocytes, neutrophils, and macrophages between anti-MCP-1 antiserum or control serum-treated animals (Fig. 5, A–C). There was no significant difference in the recruitment of lymphocytes for up to 24 h (data not shown).

**Effects of anti-MCP-1 antiserum on the production of chemokines in peritoneal fluids**

To determine the effect of endogenous MCP-1 on the production of other chemokines in the peritoneum, levels of specific chemokines in the peritoneal fluids were analyzed after treatment with anti-MCP-1 antiserum or control serum. The levels of KC, MIP-1α, and MIP-2 increased in the peritoneal fluids after CLP, which peaked at 4, 8, and 24 h post-CLP, respectively. Interestingly, the peak amounts of these chemokines were much lower than the peak amount of MCP-1, which was detected at 8 h after CLP (MCP-1: 94.6 ± 21.9 ng/cavity, n = 6; KC: 7.9 ± 1.9 ng/cavity, n = 6; MIP-1α: 0.9 ± 0.2 ng/cavity, n = 6; MIP-2: 2.3 ± 0.8 ng/cavity, n = 6). The levels of these chemokines were not inhibited with anti-MCP-1 antiserum throughout the observation periods. In addition, the levels of other cytokines found in the peritoneal fluids, which included TNF-α, IFN-γ, IL-10, IL-12, and IL-13, were not affected by anti-MCP-1 antiserum (data not shown).

**MCP-1 stimulates the production of LTB₄**

The above studies indicated that the neutralization of MCP-1 resulted in a significant decrease in neutrophil numbers after CLP-induced peritonitis. In order to determine the mechanism underlying this observation, we assessed the ability of anti-MCP-1 antiserum to alter the levels of a known neutrophil chemotactic factor, LTB₄. As shown in Fig. 6A, administration of anti-MCP-1 antiserum significantly decreased the production of LTB₄ in the peritoneal fluids at 4 and 8 h by 63% and 59%, respectively, as compared with preimmune serum. Conversely, normal mice challenged i.p. with murine MCP-1 were found to have a higher level of LTB₄ in the peritoneal fluids (Fig. 6B). When peritoneal macrophages were cultured in vitro, murine MCP-1 induced the production of LTB₄ in a dose-dependent manner, and the peak levels of LTB₄ after MCP-1 stimulation were similar to that induced with LPS (Fig. 7). To elucidate, if neutrophil infiltration in CLP mice was mediated by LTB₄, a specific LTB₄ receptor antagonist was administered orally 2 h prior to CLP surgery and the numbers of infiltrating leukocytes were assessed at the 8-h time point. Treatment with the LTB₄ receptor antagonist inhibited the recruitment of both neutrophils and macrophages by 36 and 48%, respectively.
with medium alone (MCP-1: 0 ng/ml). LTb4 appears to be an important mediator involved in the recruitment of control mice was 65% (13 of 20 mice) (Fig. 9). Thus, in mice that received LTb4 receptor antagonist whereas the mortality rate was monitored. As shown in Fig. 9, LTb4 blockade dramatically decreased the survival of mice following CLP. At 72 h after CLP, a striking 100% mortality rate (20 of 20 mice) was observed in mice that received LTb4 receptor antagonist whereas the mortality rate of control mice was 65% (13 of 20 mice) (Fig. 9). Thus, LTb4 appears to be an important mediator involved in the recruitment of leukocytes and CLP-induced lethality, and the production of this lipid mediator is regulated by MCP-1.

Discussion

Previous evidence using an animal model of endotoxemia has suggested that proinflammatory cytokines, such as TNF-α and IL-1, are deleterious, while anti-inflammatory cytokines, such as IL-4, IL-10, and IL-13, are beneficial in attenuating the mortality in this model (11). In addition to these latter regulatory cytokines, we recently demonstrated that rMCP-1 protected mice in a model of lethal endotoxemia (17). However, clinical sepsis is much more complex than an experimental model of endotoxemia in which animals receive a single injection of endotoxin. For example, neutralization of TNF-α was not efficacious for the treatment of either human sepsis or experimental sepsis induced by CLP (23, 24). In additional experimental studies, the administration of IL-1 improved survival following CLP (25), which corresponded with clinical observations that the inhibition of IL-1 with IL-1R antagonist failed to improve survival in large scale trials involving human sepsis (26). In the present study, we have extended this cytokine theme and have examined the regulatory role of the chemokine MCP-1 in an experimental model of sepsis induced by CLP. Our investigations demonstrate that endogenous levels of MCP-1 are highly elevated in peritoneal fluids post-CLP. The contribution of MCP-1 was determined via in vivo neutralization studies, which demonstrated that depletion of MCP-1 resulted in enhanced lethality. These investigations indicate that endogenous MCP-1 is protective in septic peritonitis induced by CLP.

The recruitment of leukocytes into the infectious foci is an essential mechanism of our host defense system necessary to eliminate invading pathogens. One mechanism whereby endogenous MCP-1 exerts a protective role in experimental septic peritonitis appears to occur by the recruitment and subsequent activation of leukocytes. In our model of CLP-induced peritonitis, administration of anti-MCP-1 antiserum inhibited the recruitment of both macrophages and neutrophils at 8 h. However, by 24 h post-CLP, anti-MCP-1 antiserum treatment failed to reduce the accumulation of leukocytes. Interestingly, at this time point (24 h) there was an increase in viable bacteria recovered from the peritoneal cavity, suggesting that the bacteria-clearing activity of the leukocytes was impaired by this treatment. In addition to its chemotactic properties, MCP-1 can activate monocytes and cause lysozomal enzyme release (27) and H2O2 production (28), both of which are effector molecules for bacterial killing. Furthermore, earlier studies have demonstrated that an i.p. injection of MCP-1 augmented the killing effect.
activity of peritoneal macrophages without promoting an increase in the elicitation of macrophages (29). Thus, neutralization of MCP-1 can both decrease the elicitation of leukocytes and alter leukocyte activation.

During the evolution of the CLP-induced peritonitis, the recruitment of neutrophils in the peritoneum was inhibited with anti-MCP-1 antiserum, despite the fact that MCP-1 has no neutrophil chemotactic activity in vitro (30). This phenomenon is consistent with a previous report showing that neutralization of MCP-1 reduced the influx of neutrophils in a pulmonary Cryptococcus neoformans infection (18), but the mechanism in this study was not addressed. Since the peritoneal macrophages (5.4 ± 0.6 × 10^6 /cavity, n = 13) are present even in normal conditions, it is quite reasonable to speculate that MCP-1 attracts neutrophils indirectly via the activation of the resident macrophages, which are expected to be the cellular source of many inflammatory cytokines and chemokines. Once macrophages are activated, enhanced MCP-1 production can occur via stimulation with such bacterial component as muramyl dipeptide and/or LPS. In addition, cytokine cascades are likely to be established with the subsequent induction of chemokines. Therefore, we measured the production of TNF-α and the following chemokines: MIP-2, KC, and MIP-1α. Neither the levels of these mediators nor the production of putative immunoregulatory cytokines, such as IL-10 and IL-13, were changed by anti-MCP-1 antiserum treatment. Another immunoregulatory cytokine, IL-4, was not detected in the peritoneum at any time point examined. Thus, the inhibitory effects of anti-MCP-1 on the recruitment of neutrophils in CLP animals appear to be independent of a cytokine pathway.

Additional studies were performed to determine the influence of MCP-1 depletion on the levels of LTB4, a known neutrophil chemotactic and activating factor (31). In these studies, 59% of LTB4 normally expressed in the peritoneum at 8 h after CLP was inhibited by anti-MCP-1 antiserum. These data strongly suggest that anti-MCP-1 inhibited neutrophil influx via the down-regulation of LTB4 production in this model. Accordingly, the recruitment of neutrophils post-CLP was inhibited with a specific LTB4 receptor antagonist. Since LTB4 can be generated by leukocytes from arachidonic acid (31), and anti-MCP-1 antiserum inhibited the recruitment of leukocytes, it might be possible that the reduced level of LTB4 was the result of the reduced number of infiltrating leukocytes; however, the inhibition began at 4 h, a time when the recruitment of leukocytes was not inhibited. In vivo studies demonstrated that the i.p. injection of MCP-1 induced the production of LTB4, while in vitro analyses showed that MCP-1 dose-dependently stimulated the production of LTB4 from peritoneal macrophages. These studies suggest that MCP-1 induces neutrophil elicitation indirectly via the production of LTB4 in this model.

An LTB4 receptor antagonist also inhibited the recruitment of macrophages. LTB4 attracts not only neutrophils, but also monocytes (32). Interestingly, the level of MCP-1 in the peritoneum after CLP was inhibited by 51% by the LTB4 receptor antagonist, suggesting that the inhibition of the recruitment of macrophages by the LTB4 receptor antagonist occurred, at least in part, via the reduced production of MCP-1. Our results suggest that MCP-1 and LTB4 affect the production of each other, thus amplifying the local inflammatory responses. Administration of MCP-1 enhanced bacterial clearance following i.p. challenge of viable Salmonella typhimurium and Pseudomonas aeruginosa, and protected mice from the lethality (29). Exogenous LTB4 enhanced bacterial clearance in the same infectious model (33), and leukotriene-deficient mice manifested a greater degree of lethality as well as bacteremia following Klebsiella pneumoniae challenge (34). In our model of septic peritonitis, administration of LTB4 receptor antagonist was detrimental to the survival of mice post-CLP. Collectively, inflammatory responses induced and amplified by MCP-1 and LTB4
appear to be indispensable to protect mice from CLP-induced lethality.

In summary, neutralization of MCP-1 increased lethality in an experimental model of septic peritonitis, which was associated with the reduction in the recruitment and activation of both macrophages and neutrophils. While previous studies unequivocally demonstrated that MCP-1 could directly elicit macrophages, our experiments show that MCP-1 can indirectly elicit neutrophils during in vivo inflammatory responses via augmenting levels of LTβR. Thus, endogenous MCP-1 appears to possess a beneficial effect during the evolution of experimental sepsis by eliciting various leukocyte subpopulations needed to locally contain the initial traumatic insult and eliminate microbes.

References