

Elevated Levels of Macrophage Inflammatory Protein 2 in Severe Murine Peritonitis Increase Neutrophil Recruitment and Mortality

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We hypothesized that chemokines may play important roles in a cecal ligation and puncture (CLP) model of septic peritonitis in CD-1 mice. Concentrations of C-X-C (macrophage inflammatory protein 2 [MIP-2] and ENA-78) and C-C (MIP-1 α and JE) chemokines were measured (by enzyme-linked immunosorbent assay) in serum, peritoneal lavage fluid, lung, and liver at 4, 8, 24, 48, and 96 h after CLP. Significant elevations in all measured chemokines occurred in peritoneal fluid after CLP ($P < 0.05$). MIP-2, in particular, increased dramatically (>400-fold, $P < 0.001$) in peritoneal fluid, serum, and to a lesser extent lung and liver ($P < 0.05$). Increased MIP-2 was correlated with severity of sepsis ($P < 0.001$). To determine the significance of this finding, mice were passively immunized prior to CLP with polyclonal antibody to MIP-2, which decreased mortality from 85 to 38% at 96 h ($P < 0.01$). To further understand the mechanism of the effect of MIP-2, additional measurements demonstrated that anti-MIP-2 prior to CLP decreased the percent neutrophils in peritoneal fluid ($55\% \pm 12\%$, compared with $82\% \pm 10\%$ in controls), but no significant changes in tumor necrosis factor alpha, interleukin-6, or interleukin-10 occurred. MIP-2 contributes to the inflammatory response and overall mortality in this model of severe septic peritonitis, possibly by increasing recruitment of neutrophils, which clear bacteria but may also injure the host.

The physiologic consequences of sepsis, including organ dysfunction and death (3), depend on the nature of the associated inflammatory response, involving a complex interaction of multiple inflammatory mediators and inflammatory cells (25). Therefore, cytokines specifically chemotactic for inflammatory cells, called chemokines (23), may play important roles in coordinating the septic inflammatory response (22, 26). However, little is known about the pattern of expression of chemokines or about the importance of chemokine expression in the response to severe septic peritonitis (1). The two best-understood classes of chemokines are the C-X-C and C-C families (23), named for the distinctive cysteine residue motifs observed in these molecules. With regard to leukocyte elicitation, the C-X-C chemokines are typically chemotactic for granulocytes whereas the C-C chemokines are more important in mononuclear leukocyte chemotaxis. Interleukin-8 (IL-8) appears to be a key C-X-C chemokine (14, 24) involved in neutrophil recruitment in a number of human inflammatory conditions (8, 12, 15). Neutrophils are the most prominent leukocyte population involved in the inflammatory response in acute severe bacterial infections. Murine macrophage inflammatory protein 2 (MIP-2) is a murine C-X-C chemokine that has been considered functionally analogous to human IL-8 (18, 29).

Based on these observations, we hypothesized that chemokine expression may be significantly increased during sepsis. We further hypothesized that of the various C-X-C and C-C chemokines expressed, MIP-2 may be particularly important in the local and systemic inflammatory responses in septic peri-

tonitis. To test these hypotheses, we first measured expression of several C-X-C (MIP-2 and ENA-78) and C-C (MIP-1 α and JE) chemokines in a murine cecal ligation and puncture (CLP) model of septic peritonitis. To determine the significance of MIP-2 expression on mortality in this model of sepsis, mice were passively immunized with a polyclonal antibody to murine MIP-2 prior to CLP. To further understand the mechanism of the effect of MIP-2, expression of pro- and anti-inflammatory cytokines and peritoneal lavage leukocyte differential counts were measured in control and anti-MIP-2-treated mice at 24 h after CLP. These studies demonstrate that chemokines may have a profound effect on the outcome of systemic inflammation associated with sepsis.

MATERIALS AND METHODS

Animal model. Pathogen-free CD-1 mice were used for CLP as previously described (2). Briefly, under sterile conditions, a 1- to 2-cm midline incision was made on the anterior abdomen, and the cecum was exposed. With a 3-0 silk suture, the cecum was tightly ligated at its base without causing bowel obstruction. The cecum was then punctured through-and-through once with a 21-gauge needle. Sham-operated animals underwent identical laparotomy but did not undergo CLP and served as controls. The abdominal incision was closed, and 1 ml of saline was administered subcutaneously for fluid resuscitation.

In the first set of experiments, survival curves were determined following CLP or following sham laparotomy. In the second set of experiments, CLP animals were euthanized at 4, 8, 24, 48, 72, and 96 h, and serum, lung, and liver samples were collected. The peritoneal cavity was lavaged with 1 ml of sterile saline, and this peritoneal lavage fluid was collected. A minimum of five surviving animals at each time point were used. In the third set of experiments, mice were pretreated 2 h before and again 24 h after CLP with 0.5 ml of intraperitoneal control rabbit serum or polyclonal rabbit antiserum to either murine MIP-2, MIP-1 α , or JE (murine analog of MCP-1), and survival curves for the four groups of animals were determined. In the fourth set of experiments, tumor necrosis factor alpha (TNF- α), IL-6, and IL-10 concentrations and peritoneal lavage fluid leukocyte differential counts were measured in anti-MIP-2- and control serum-treated animals 24 h after CLP.

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Serum and peritoneal lavage fluid were frozen at -20°C immediately after collection from the mice, and all assays were performed upon the first thaw. The right lung and the left lobe of the liver were rapidly excised and immediately frozen in liquid nitrogen and then stored at -70°C . Just prior to assay, these tissue samples were homogenized in 1 ml of 0.1% Triton X-100 in phosphate-buffered saline (PBS). After brief centrifugation, the supernatant was removed and used immediately for further assays.

Production of antibodies. Rabbit anti-murine MIP-2, anti-MIP-1 α , and anti-JE antibodies were prepared by multiple-site immunization of New Zealand White rabbits with recombinant murine MIP-2, MIP-1 α , and JE, respectively (R&D Systems, Minneapolis, Minn.) in complete Freund's adjuvant. Specificity of these polyclonal antibodies was verified by the failure to cross-react with the other two cytokines of this set and with murine TNF, IL-2, IL-4, KC, and RANTES (10). A 1:1,000 dilution of the rabbit serum containing anti-MIP-2 completely neutralized neutrophil chemotaxis to 30 ng of murine MIP-2 per ml. This serum was used for passive immunization. In addition, the immunoglobulin G portion of the serum was purified by using a protein A column. These polyclonal antibodies were subsequently used in sandwich enzyme-linked immunosorbent assays (ELISAs).

ELISAs. Antigenic murine MIP-2, ENA-78, MIP-1 α , JE, and IL-10 concentrations in serum, peritoneal lavage fluid, and homogenized aqueous lung and liver extracts were measured by using a double-ligand ELISA. All ELISAs used polyclonal antibodies raised in rabbits, as described above, using immunization with recombinant murine MIP-2, MIP-1 α , JE, and IL-10 (R&D Systems). The ELISA for ENA-78 uses a rabbit anti-human ENA-78 antibody which cross-reacts with murine ENA-78. Briefly, each well of flat-bottom 96-well microtiter plates (Nunc Immuno-Plate I 96-F; Nunc, Roskilde, Denmark) was coated with 50 μl of rabbit antibody specific to one of the above-mentioned cytokines diluted to 3.2 $\mu\text{g}/\text{ml}$ in borate-buffered saline (pH 8.6), and the plates were incubated overnight at 4°C . Plates were washed three times with PBS (pH 7.5) containing Tween 20. Nonspecific binding was blocked with 2% bovine serum albumin in PBS, and plates were incubated for 90 min. Plates were washed three times, diluted cell-free supernatants (1:2 and 1:10) in duplicate were added, and the plates were incubated for 1 h at 37°C . Plates were washed three times, biotinylated rabbit antibody was added, and the plates were incubated at 37°C for 45 min. Plates were again washed three times, streptavidin-peroxidase conjugate (Bio-Rad Laboratories, Richmond, Calif.) was added, and the plates were incubated for 30 min at 37°C . Plates were washed three times, and chromogen substrate (*o*-phenylenediamine; Bio-Rad) was added. Plates were incubated at room temperature to the desired extinction, and the reaction was terminated with 50 μl of 3 M H_2SO_4 per well. The plates were read at 490 nm in an ELISA reader. These ELISAs consistently detected cytokine and chemokine concentrations above 25 to 50 pg/ml and did not cross-react with murine TNF, IL-2, IL-4, KC, RANTES, and the other chemokines in this set.

Bioassays. TNF- α concentrations were measured by using the WEHI bioassay (9), and IL-6 concentrations were measured by using the B9 cell proliferation bioassay (16). In the TNF- α bioassay, 5×10^5 WEHI 164 subclone 13 cells in 100 μl were added to 100- μl volumes of serial dilutions of test samples and were incubated overnight. In the IL-6 bioassay, 5×10^3 IL-6-dependent murine hybridoma cell line B13.29 clone B9 cells in 100 μl were added to 100- μl volumes of serial dilutions of test samples and were incubated for 72 h in a humidified incubator at 37°C and 5% CO_2 . In both cases, cell viability was measured with a colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Co.). During the final 6 h of the incubations, 20 μl of MTT (5 mg/ml in PBS) was added to each sample. Supernatant (150 μl) was aspirated from each well, and 100 μl of acidified isopropanol was added. Absorbance was measured at 550 nm. TNF- α and IL-6 concentrations in experimental samples were calculated from a standard curve generated by serial dilutions of recombinant murine TNF- α and IL-6, respectively. These bioassays consistently detected TNF- α concentration above 1 pg/ml and IL-6 concentrations above 0.1 pg/ml.

Leukocyte differential counts. In the fourth set of experiments, the peritoneal cavity was lavaged with 1 ml of PBS; the lavage fluid was immediately centrifuged at $400 \times g$ for 10 min and washed once in 1 ml of cold PBS. Cell differential counts were determined by Wright-Giemsa staining of cytopins and α -naphthyl acetate esterase staining (Sigma).

Data analysis. A two-way analysis of variance was used to test for differences in cytokine levels between control and CLP groups in the second set of experiments and between control serum- and antiserum-treated CLP groups in the fourth set of experiments. When a difference was found, specific differences were identified by using a sequentially rejective Bonferroni test procedure (11), choosing $P < 0.05$ as significant. A log-rank test was used to test for differences in survival curves. Data are reported as means \pm standard errors in the text, table, and figures.

RESULTS

Twenty-one-gauge CLP resulted in severe peritonitis, with more than half of the animals dying by 48 h and three-quarters of the animals dying by 6 days after CLP (Fig. 1). CLP resulted in approximately 10-fold increases ($P < 0.05$) in peak serum

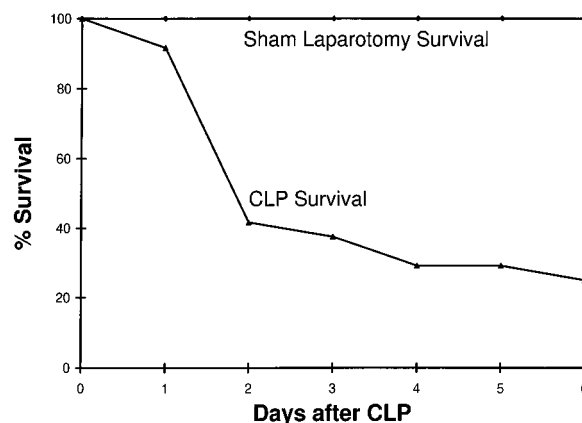


FIG. 1. Percent survival of mice over time after CLP using a 21-gauge needle ($n = 24$) is significantly less than after sham laparotomy ($n = 10$) ($P < 0.001$).

concentrations of the C-X-C chemokine ENA-78 from a baseline value of 39 ± 7 pg/ml (Fig. 2). MIP-2 serum levels increased approximately 400-fold at 8 and 24 h from a baseline value of 72 ± 3 pg/ml (Fig. 2). Peritoneal lavage measurements of the C-X-C chemokines were similar to serum measurements, with a marked increase in MIP-2 from a baseline value of 98 ± 9 pg/ml and a smaller increase in ENA-78 from a baseline value of 96 ± 20 pg/ml (Fig. 2). C-C chemokines also increased in serum and peritoneal lavage fluid after CLP (Fig. 3). MIP-1 α increased markedly at 24 h in serum from a base-

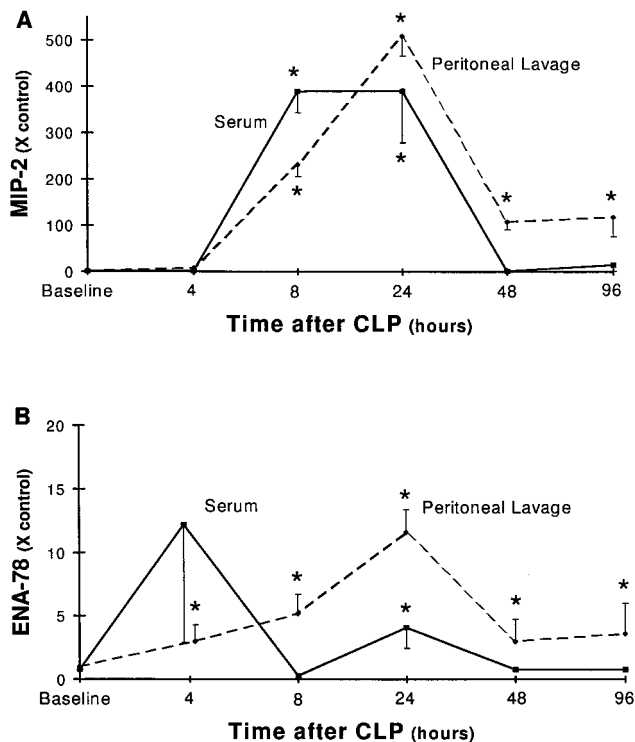


FIG. 2. ELISA-measured increases, relative to sham laparotomy time-matched controls, of the C-X-C chemokines MIP-2 (A) and ENA-78 (B) in serum and in peritoneal fluid over time after CLP ($n = 5$ to 8 animals per data point). While ENA-78 increases significantly at peak ($P < 0.05$), the peak increase of MIP-2 is much greater ($P < 0.05$). Significant differences from baseline are indicated by asterisks ($P < 0.05$).

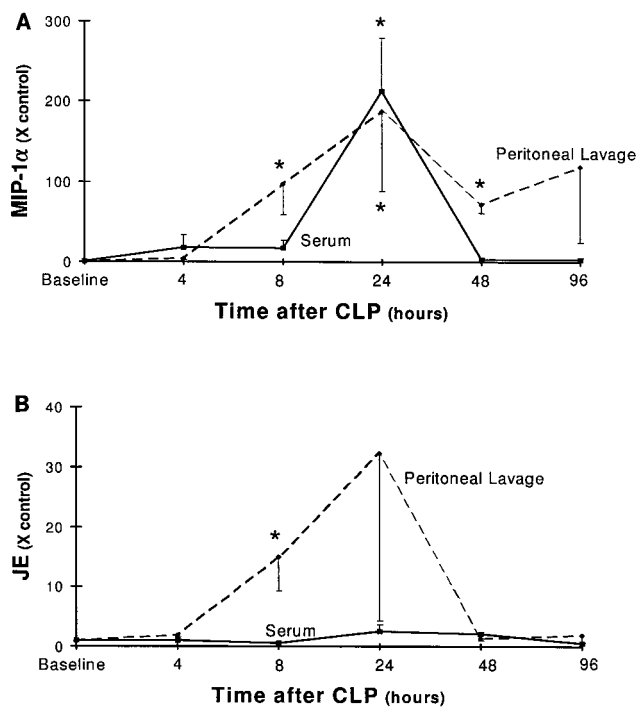


FIG. 3. ELISA-measured increases, relative to sham laparotomy time-matched controls, of the C-C chemokines MIP-1 α (A) and JE (B) in serum and in peritoneal fluid over time after CLP. Increases of MIP-1 α are much greater than increases of JE in serum and peritoneal lavage fluid ($P < 0.05$). Significant differences from baseline are indicated by asterisks ($P < 0.05$).

line value of 33 ± 13 pg/ml and in peritoneal lavage fluid from a baseline value of 39 ± 13 pg/ml. The increases in MIP-1 α were much more prominent than those in JE (Fig. 3). JE did not change significantly from a baseline value of 109 ± 6 pg/ml in serum but increased significantly from a baseline value of 503 ± 130 pg/ml in peritoneal lavage fluid, peaking at 24 h after CLP.

The greatest increase in MIP-2 expression following CLP occurred in serum and peritoneal lavage fluid. However, significant increases in expression also occurred in lung and liver (Fig. 4). Of all of the C-X-C and C-C chemokines measured, only MIP-2 increased significantly in the liver (Fig. 4) from a baseline value of 294 ± 56 pg/ml. In the lung, MIP-2 increased (Fig. 4) from a baseline value of 674 ± 57 pg/ml. MIP-1 α also

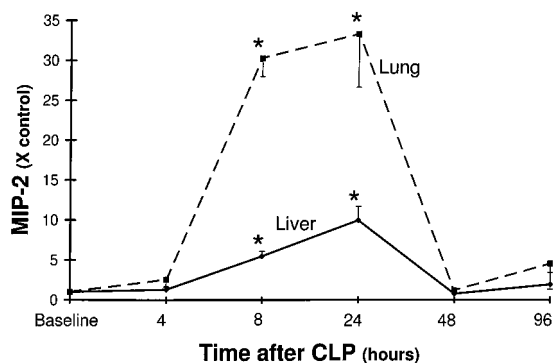


FIG. 4. Expression of MIP-2 in lung and liver, relative to sham laparotomy time-matched controls, over time after CLP. Significant differences from baseline are indicated by asterisks ($P < 0.05$).

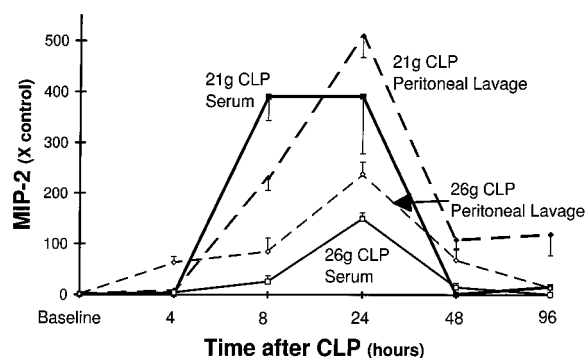


FIG. 5. Serum and peritoneal lavage fluid MIP-2 expression for 21-gauge cecal puncture (severe sepsis) and for 26-gauge cecal puncture (less severe sepsis). The increases in MIP-2 concentrations are related to severity of sepsis ($P < 0.05$).

increased in the lung at 24 h after CLP by 6.9 ± 1.2 times ($P < 0.05$) the baseline value of 3.01 ± 0.59 ng/ml.

To determine whether MIP-2 expression was correlated with mortality, an additional set of experiments was performed by using small-diameter (26-gauge) cecal puncture to produce a milder peritonitis. Mortality at 48 h after CLP was less after small-gauge CLP (20%, $n = 10$) than after 21-gauge CLP (40%, $n = 24$, $P < 0.05$). The expression of MIP-2 was substantially less in serum and peritoneal lavage fluid after 26-gauge CLP than after 21-gauge CLP (Fig. 5), demonstrating that increased severity of sepsis was associated with increased MIP-2 concentrations.

To determine the significance of MIP-2 for mortality in this model of sepsis, mice were passively immunized with polyclonal anti-MIP-2 prior to CLP. Anti-MIP-2 resulted in a substantial improvement in survival curves ($P < 0.01$) (Fig. 6), with survival increasing from 15% at 96 h in control CLP mice to 62% at 96 h in anti-MIP-2-treated CLP mice. Anti-MIP-2 did not appreciably alter the early mortality rate in the first 24 h after CLP. Thereafter, anti-MIP-2 had a substantial effect.

To better understand the mechanism of the effect of MIP-2 in this model of severe septic peritonitis, we measured expression of the proinflammatory cytokines TNF- α and IL-6, as well as the anti-inflammatory cytokine IL-10, 24 h after CLP. There were no significant differences in cytokine levels between mice pretreated with anti-MIP-2 and mice pretreated with control

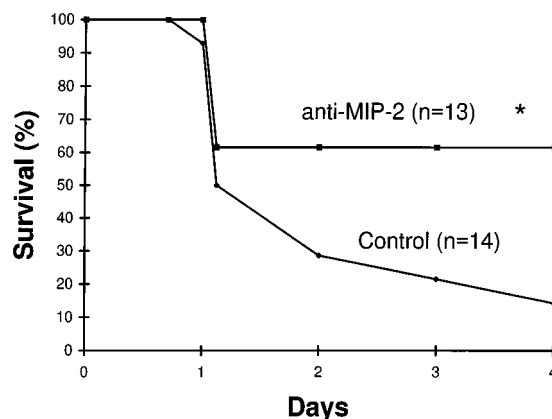


FIG. 6. Survival following CLP after pretreatment with anti-MIP-2 or control serum. Anti-MIP-2 increases survival (*, $P < 0.01$ by log-rank test).

TABLE 1. Cytokine expression 24 h after CLP in six control and eight anti-MIP-2-treated mice

Group	Mean concn (ng/ml) \pm SE ^a					
	TNF- α		IL-6		IL-10	
	Serum	Peritoneal fluid	Serum	Peritoneal fluid	Serum	Peritoneal fluid
Control	0.43 \pm 0.12	0.93 \pm 0.23	9.9 \pm 1.6	118 \pm 32	1.3 \pm 0.3	10.3 \pm 2.1
Anti-MIP-2 treated	0.47 \pm 0.14	1.13 \pm 0.30	14.4 \pm 4.8	215 \pm 83	2.3 \pm 1.1	26.1 \pm 9.4

^a In all cases, there was no significant difference between groups by unpaired *t* test.

serum (Table 1). There was a trend toward increased IL-10 in the anti-MIP-2-treated group in serum and peritoneal fluid; however, this increase was not statistically significant. Differential cell counts on cytopins from peritoneal lavage fluid are illustrated in Fig. 7. Anti-MIP-2 decreased the percentage of neutrophils recruited to the peritoneal cavity after CLP ($P < 0.05$).

DISCUSSION

Several chemokines were expressed in increased concentrations in this murine CLP model of severe sepsis. Of the chemokines measured, MIP-2 expression increased more than that of the other chemokines in serum and peritoneal lavage fluid. MIP-2 also increased significantly in liver and lung homogenates. The increase in MIP-2 expression was correlated with severity of sepsis. Furthermore, anti-MIP-2 pretreatment substantially improved survival. The mechanistic effect of MIP-2 did not appear to be related to alterations in pro- and anti-inflammatory cytokine levels in this setting. Instead, MIP-2 was important in neutrophil recruitment to the peritoneal cavity. We speculate that while recruited and subsequently activated neutrophils are important for bacterial clearance (19), neutrophils also contribute to the intense inflammatory response that leads to mortality (28) in this model of severe septic peritonitis.

The role of chemokines in the septic inflammatory response is incompletely understood. C-X-C chemokines are 8- to 10-kDa peptides which have significant neutrophil chemotactic and stimulatory activity (17). Included in this family are IL-8 and its functional murine analog MIP-2, as well as ENA-78, GRO- α (murine KC), NAP-2, and IP-10. The C-C chemokines act preferentially as chemotactic and stimulatory molecules for the monocyte/macrophage cell line (23). The C-C chemokine

family includes MCP-1 and its murine analog JE, as well as RANTES, MIP-1 α , and MIP-1 β . Despite the general patterns of activity of C-X-C and C-C chemokines, specificity for particular leukocyte subsets varies widely. MIP-1 α appears to have neutrophil chemotactic and stimulatory functions (31) in addition to its effects on macrophages (7, 20). During intraperitoneal endotoxin administration in mice, MIP-1 α is important for neutrophil recruitment into the lungs (22). MIP-1 α may mediate part of this effect by upregulation of ICAM-1 expression (22). Similarly, the C-C chemokine RANTES appears to be important in recruiting macrophages into the lungs during endotoxemia (26). Both MIP-1 α expression and RANTES expression appear to be dependent on endogenous TNF- α production (22, 26). In a similar endotoxemia model in mice, IL-10 appears to exert a protective effect by down regulating MIP-2 production (21), suggesting an important role for MIP-2 in the septic inflammatory response.

In this model of severe septic peritonitis, MIP-2 increased dramatically in serum and peritoneal lavage fluid. Passive immunization with antibody to MIP-2 significantly improved survival. In separate preliminary experiments, survival at 4 days after CLP was no more different from that of controls (25%, $n = 8$) than after passive immunization with antiserum to MIP-1 α (29%, $n = 7$) or after treatment with antiserum to JE (29%, $n = 7$). Thus, MIP-2 may be particularly important in severe sepsis. MIP-2 has been considered to be a functional analog of human IL-8 (18, 29). IL-8 has been shown to be an important chemokine associated with neutrophil recruitment to the lungs in patients with adult respiratory stress syndrome (8), idiopathic pulmonary fibrosis (15), cystic fibrosis (23), and other inflammatory diseases of the lung (23). In addition, IL-8 is also associated with neutrophil recruitment to nonpulmonary sites of inflammation in patients with rheumatoid arthritis (12, 13) and hepatic ischemia-reperfusion injury (5). Thus, our new finding that MIP-2 plays a pivotal role in severe murine peritonitis is consistent with these previous data for other inflammatory states.

The increases in MIP-2 concentrations in this model of severe septic peritonitis were related to severity of injury, being less with a small-gauge cecal puncture and greater with a large-gauge cecal puncture. Indeed, in both small-gauge and large-gauge CLP, variations in mortality rates over time were correlated with the time course of MIP-2 concentrations ($r^2 = 0.80$), suggesting that MIP-2, or factors that directly covary with MIP-2, is important in the inflammatory cascade leading to death. Decreased mortality after passive immunization with anti-MIP-2 demonstrated that MIP-2 played a causal role in this association.

Since MIP-2 may interact with other cytokines and their production (21, 27), one potential mechanism of MIP-2's effect was via increased proinflammatory mediator expression (4, 6, 27). Our measurements at 24 h after CLP were chosen to occur at approximately the peak in proinflammatory mediator levels in peritoneal fluid and at the peak of MIP-2 expression. Lack

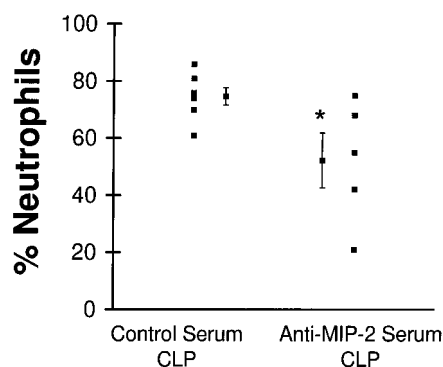


FIG. 7. Means (solid squares with standard errors) and individual animal cytopsin differential counts from peritoneal lavage fluid 24 h after CLP in mice pretreated with control serum and anti-MIP-2 serum. Anti-MIP-2 reduces the percent neutrophils recruited to the peritoneal cavity (*, $P < 0.03$, unpaired *t* test).

of a difference between control and anti-MIP-2 groups (Table 1) suggests that this is not the main mechanism of MIP-2's effect in this setting. Instead, passive immunization with anti-MIP-2 significantly decreased the percentage of neutrophils recruited to the peritoneal cavity. MIP-2 is also important in recruiting neutrophils into the lung and in preventing systemic dissemination of bacteria in a model of murine bacterial pneumonia (10). In the present study, we measured leukocyte differential counts in peritoneal fluid rather than total counts because total cell counts would depend on complete recovery of peritoneal fluid, which was not possible. The increase in the fraction of neutrophils suggests that MIP-2 is also important in recruiting neutrophils into the peritoneal cavity in severe septic peritonitis. These activated neutrophils are crucial in clearing bacteria. However, in addition to bacterial killing, neutrophils may damage tissue and propagate the inflammatory response by release of neutrophil-generated oxygen free radicals and proteases (28). We speculate that neutrophils, recruited in part by MIP-2, contribute to the host defense against infection (19) but may, in severe sepsis, also contribute to mortality (28). Our results do not rule out other explanations, because deaths may involve mechanisms other than excessive recruitment of neutrophils. For example, bowel obstruction, due to edema and the intense inflammatory response surrounding the cecal puncture site, could conceivably contribute to later deaths. Nevertheless, antibody to MIP-2 decreased mortality. The main currently known function of MIP-2 is neutrophil chemotaxis and activation. Therefore, it is reasonable to hypothesize that this is the main role of MIP-2 in this model of severe septic peritonitis.

In summary, these results indicate that chemokines, and in particular MIP-2, are expressed in severe sepsis and play important roles in regulating the character of the inflammatory response, in part by their leukocyte chemotactic activities.

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