MCP-1 causes leukocyte recruitment and subsequently endotoxemic ileus in rat

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Türlер, Andreas, Nicolas T. Schwarz, Esther Türlер, Jörg C. Kalff, and Anthony J. Bauer. MCP-1 causes leukocyte recruitment and subsequently endotoxemic ileus in rat. Am J Physiol Gastrointest Liver Physiol 282: G145–G155, 2002. First published September 21, 2001; 10.1152/ajpgi.00263.2001.—Endotoxemia causes an inflammatory response within the intestinal muscularis and gastrointestinal dysmotility. We hypothesize that the resident macrophage-derived chemokine monocyte chemoattractant protein-1 (MCP-1) plays a significant role in the recruitment of leukocytes into the lipopolysaccharide (LPS)-stimulated rat intestinal muscularis. MCP-1 mRNA expression was investigated by RT-PCR. Leukocyte extravasation and MCP-1 protein localization were determined by immunohistochemistry. Contractile activity was assessed by using a standard organ bath in rats that were treated with saline, recombinant MCP-1, LPS, LPS + nonspecific antibody, or LPS + MCP-1 antibody. Endotoxemia caused a significant 280-fold increase in MCP-1 mRNA expression in the muscularis, peaking at 3 h. MCP-1 protein was immunohistochemically located to muscularis macrophages. LPS application caused significant leukocyte recruitment into the muscularis and a 51% decrease in muscle contractility. MCP-1 antibody treatment significantly averted leukocyte recruitment and significantly prevented muscle dysfunction. These parameters were not significantly altered by the nonspecific antibody. Results show that resident muscularis macrophage-derived MCP-1 plays a major role in the recruitment of monocytes during endotoxemia, which then subsequently secrete kinetically active substances that cause ileus.

sepsis; intestinal muscularis; smooth muscle; macrophage

ENDOTOXEMIA FOLLOWING COMPLICATED abdominal surgery, hemorrhagic shock, trauma, and burns is still a leading cause of death because it leads to septic shock and multiorgan failure. Sepsis represents the largest cause of morbidity and mortality in the critically ill (2). Furthermore, with advances in life-support strategies, it can be predicted that endotoxemia will increasingly impact on the management of the seriously ill patient. There is increasing evidence that intestinal barrier failure and bacterial translocation may play a role in the development of sepsis. In fact, frequently the source of the contaminating bacteria can be traced to the endogenous flora of the gastrointestinal tract. Intuitively, it can be readily accepted that the gut, due to a breakdown in barrier function, is a frequent bacterial source after abdominal surgery, shock, or trauma (10, 42).

In addition to being the source of bacteria, the intestine also actively participates as one of the target organs in the phenomenon of sepsis-induced multiorgan failure. The induction of sepsis in experimental animals by the systemic administration of live bacteria or endotoxin has a detrimental effect on the metabolic and barrier functions of the small intestine and thus furthers the translocation of bacteria (13, 20, 38, 43). Along with mucosal dysfunction, endotoxemia is known to be associated with alterations in gastrointestinal motility. It has been shown that a single, sublethal dose of endotoxin temporarily disrupts gastrointestinal motility and transit (8, 9, 12, 30, 54). Furthermore, gastrointestinal dysmotility may result in luminal bacterial overgrowth, producing a further source of bacterial products that cause ileus and mucosal dysfunction. Thus the demise of the intestine appears to be the result of its own incompetence.

It has recently been reported that a dense network of resident macrophages, which appear to be inactive in their basal state, populates the intestinal muscularis (26, 36). However, there is increasing evidence that endotoxin rapidly activates these resident intestinal macrophages and that they subsequently initiate a molecular and cellular inflammatory response that causes intestinal dysmotility (12, 30, 48). Lipopolysaccharide (LPS)-activated macrophages are able to secrete a variety of biologically active substances into their local milieu. To date, more than 100 agents secreted by macrophages have been reported that mediate physiological and pathological functions of macrophages in inflammation, acute phase response, and repair mechanisms (40). Some of these substances could have a direct impact on smooth muscle activity,
such as prostanoids or nitric oxide (48). Other secreted agents act as chemokines and cytokines by initiating subsequent inflammatory reactions, which can further potentiate gastrointestinal dysmotility. One of these potential pathways is the recruitment of immunocompetent leukocytes during endotoxemia. We have previously shown that exogenous LPS causes the extravasation of leukocytes into the intestinal muscularis, which consists mainly of monocytes, polymorphonuclear neutrophils (PMNs), and mast cells (11). We have also demonstrated that a similar inflammatory response occurs in a variety of local and systemic gut injury models such as intestinal manipulation (25), hemorrhagic shock (24), small bowel transplantation (23), and ischemia-reperfusion injury (19). Our studies suggest that leukocytes infiltrating the muscularis release mediators that inhibit smooth muscle contractility. Thus we hypothesize that the activation of macrophages by LPS elicits an inflammatory cascade that is amplified through leukocyte recruitment (22).

However, the specific mechanisms that initiate leukocyte recruitment into the intestinal muscularis during endotoxemia are not well defined. Macrophages have been reported to secrete monocyte chemoattractant protein-1 (MCP-1) in response to LPS and to a wide range of cytokines such as interleukin (IL)-6, tumor necrosis factor-α, and IL-1β (4, 7). Interestingly, the production of these three cytokines is also known to be enhanced during endotoxemia (51) and may result in an intensified expression of MCP-1. MCP-1 is a potent chemoattractant capable of promoting monocyte recruitment into an inflammatory site as well as activating monocytes and macrophages (14, 29).

We developed the hypothesis that MCP-1 production by resident intestinal macrophages may play a critical role in the recruitment and activation of leukocytes into the intestinal muscularis during endotoxemia and therefore may be an important determinant for the development and progression of intestinal smooth muscle dysfunction. Therefore, our objectives were to 1) determine and delineate the time course of MCP-1 mRNA expression within the intestinal muscularis, 2) locate the site of MCP-1 production, 3) investigate the impact of exogenously applied recombinant (r) MCP-1, and 4) determine the potential ameliorative effects of MCP-1 protein neutralization during endotoxia on leukocyte recruitment and functional intestinal smooth muscle dysfunction caused by LPS.

MATERIALS AND METHODS

Animals and experimental groups. Male ACI (black agouti) rats weighing 180–220 g were obtained from Harlan (Indianapolis, IN). The experimental design was approved by the University of Pittsburgh Institutional Animal Care and Use Committee. All animals were kept in a pathogen-free facility accredited by the American Association for Accreditation of Laboratory Animal Care and complies with the requirements of humane animal care as stipulated by the U.S. Department of Agriculture and the Department of Health and Human Services. Animals were maintained on a 12:12-h light/dark cycle and provided with commercially available rat chow and tap water ad libitum.

Endotoxemia was induced by a single intraperitoneal injection of LPS at 15 mg/kg. LPS derived from Escherichia coli serotype 0111:B4 of lot 70K4108 was used. During the course of this study, LPS caused no mortality in the ACI animals, even in those surviving for 48 h or in those which had received antibody treatment. This is in contrast to a 25% mortality rate (12 h after LPS) that we have previously reported (11). We have observed that the lethal effects of LPS are rat-strain specific and that biologically active lipoproteins are present and variable in commercially available LPS. A time course to investigate the expression of MCP-1 within the isolated rat muscularis after LPS was performed by harvesting the small intestine and isolating the muscularis at various time points (1, 3, 6, 24, and 48 h) after LPS injection (n = 4 at each time). All of the results were compared with saline-injected control animals.

The effect of MCP antibody neutralization after LPS was investigated using four groups. The first experimental group of animals received only LPS. The second group was treated before LPS administration with an intravenous injection of a specific antibody to MCP-1 (250 μg/kg). To exclude the effectiveness of a nonspecific antibody treatment, a third group of animals was pretreated with a similar dose of nonspecific IgG isotype antibodies. All of the results were compared with saline-injected controls. Rats were killed 18 h after the LPS administration for functional and immunohistochemical studies (n = 6/group).

The effectiveness of MCP-1 as a chemoattractant in the muscularis externa was determined by the local direct application of exogenous MCP-1 to the small bowel through a midline incision. A total dose of 1 μg lyophilized rMCP-1, suspended in 0.5 ml sterile sodium chloride (0.9%), was continuously dropped on a distal jejunal loop located ~2 cm proximal to the ileocecal junction. This procedure took 10 min, and the midline incision was closed using a continuous running two-layer suture. Animals were killed 18 h after the MCP-1 application. The proximal jejunum of unoperated and sham-operated animals (laparotomy with saline application) served as controls (n = 4/group).

Small bowel preparation. Rats were anesthetized by isoflurane inhalation, and the abdomen was opened by midline laparotomy. The abdominal aorta was clamped above and below the superior mesenteric artery and flushed with 5 ml of cold (4°C) sodium chloride (0.9%) to remove nonadherent blood cells from the vasculature. The small bowel was then removed and placed iniced preoxygenated Krebs-Ringer buffer (KRB). For gene expression analysis, the rat jejunal muscularis was isolated from the mucosa-submucosa by slitting 5-cm-long portions of the intestine over a glass rod and stripping the muscularis from the jejunal mucosa, as described previously (22). The isolated muscularis was snap-frozen in liquid nitrogen and stored at −80°C. Functional studies described below were carried out immediately on bowel specimens taken from the middle jejunum. Three jejunal segments per animal were used for histochemical and immunohistochemical analysis.

MCP-1 mRNA expression. Total RNA extraction was performed as previously described using the guanidium-thiocyanate phenol-chloroform extraction method (11). The RNA pellets were resuspended in RNAsecure resuspension solution (Ambion, Austin, TX). Following the resuspension, a DNase treatment was carried out (DNA-free reagent; Ambion). Then, equal aliquots (5 μg) of total RNA from each sample were quantified by a spectrophotometer (at 260 nm) and processed for cDNA synthesis by using an oligo(dT) primer and Moloney murine leukemia virus reverse transcription enzyme. The time course of the MCP-1 mRNA
expression after LPS treatment was analyzed using semi-quantitative RT-PCR. Briefly, amplification of synthesized cDNA from each sample was carried out using PCR with 32P-labeled primers. GAPDH was used as the housekeeping gene. The specificity of the MCP-1 primer pair was previously confirmed by Wang et al. (55) through DNA sequencing, and the specificity of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers was confirmed using restriction site analysis as described previously (52). Sequences of the PCR primers were as follows: MCP-1 sense 5’-CAGGTCCTGTCGACGCTTCT-3’ and antisense 5’-AGTATTACTGGAGGGAATAG-3’ (product size 527 bp); GAPDH sense 5’-GCCATACAGGCCCCCTTCAT-3’ and antisense 5’-GCCTGTCTCTACCACCTTCT-3’ (product size 702 bp). MCP-1 and GAPDH amplification was carried out for 30 cycles (MCP-1: 92°C for 1 min, 62°C for 1 min, 72°C for 1 min; GAPDH: 94°C for 1 min, 57°C for 1 min, 72°C for 2 min) on a model PTC-100 thermal cycler from MJ Research (Watertown, MA). Rat peritoneal macrophages elicited with thioglycolate and RAW cells (RAW 264.7 macrophage cell line) stimulated in vitro with LPS served as positive controls; water added instead of DNA template served as a negative control. Twenty microliters of the PCR amplification product and a 123-bp DNA ladder were separated using polyacrylamide gel electrophoresis. The gel was dried on a gel dryer, and the PCR product bands were visualized by autoradiography. A phosphorImager (Molecular Dynamics, Sunnyvale, CA) was used to quantitate each gel band product. To ensure equal cDNA synthesis, data from all samples were normalized against their respective control counts. To ensure equal reaction conditions, all samples received pooled reagents and comparisons were made only between experiments performed simultaneously.

MCP-1 mRNA peak expression at 3 h after LPS was quantified by a SYBR Green two-step real-time RT-PCR. Aliquotted RNA (200 ng) was subjected to first-strand cDNA synthesis using random hexamers (PE Applied Biosystems, Foster City, CA) and SuperScript II (Life Technologies, Rockville, MD). Primers were designed according to published sequences (50) [GenBank accession Nos. M57441 (MCP-1) and NM_017008 (GAPDH)] using Primer Express software (PE Applied Biosystems). Sequences of the real-time PCR primers were as follows: MCP-1 sense 5’-CAGGCAAGATGCAGTTAATGCC-3’ and antisense 5’-AGCCGACTTATCGG GATCAT-3’ (product size 74 bp); GAPDH sense 5’-ATGGCA CAGTCAGGCTGGA-3’ and antisense 5’-GCCTCTGGGA AAGATGTTGAT-3’ (product size 70 bp). The specificity and equality of both primer efficiencies were confirmed in validation experiments. GAPDH was used as an endogenous control. Each sample was estimated in triplicate. The PCR reaction mixture was prepared using the SYBR Green PCR core reagents (PE Applied Biosystems). PCR conditions were as recommended by the manufacturer. The reaction was incubated at 50°C for 2 min to activate the uracil N'-glycosylase and then for 10 min at 95°C to activate the AmpliTaq Gold polymerase followed by 40 cycles at 95°C for 15 s and at 60°C for 1 min on an ABI PRISM 7700 sequence detection system (PE Applied Biosystems). The real-time PCR data were plotted as the ΔRn fluorescence signal versus the cycle number. An arbitrary threshold was set to the midlinear portion of the log ΔRn cycle plot (46). The threshold cycle (Ct) was defined as the cycle number at which the ΔRn crosses this threshold. Relative quantification was performed using the comparative Ct method as described previously by Schmittgen et al. (46) (see also User Bulletin #2, PE Applied Biosystems).

Histochemistry and immunohistochemistry. Histochemistry and immunohistochemistry were performed on whole mounts of the intestinal muscularis as described previously (26). Briefly, jejunal segments were cut from the bowel and immersed in KRB in a Sylgard-filled glass dish. The length and width of each jejunal segment were measured with a caliper, and then the segments were gently pinned down along the mesenteric border. The bowel was opened along the mesentery and stretched to 150% of the length and 250% of the width. The opened jejunal segments were fixed in 100% ethanol for 10 min. Each segment was washed twice in KRB, and mucosa and submucosa were stripped off under microscopic observation (Wild, Heerbrugg, Switzerland). The mucosa-free muscularis whole mounts were cut into 0.5 × 1 cm pieces and used for staining procedures. Myeloperoxidase (MPO)-positive cells were detected using a mixture of 10 mg Hanker-Yates reagent, 10 ml KRB, and 100 μl 5% hydrogen peroxide (Sigma, St. Louis, MO) for 10 min. For immunohistochemistry, whole mounts were incubated for 24 h at 4°C in the primary antibody, followed by 3 × washes in 0.05 mol/l PBS. Horseradish anti-rat-ED1 antibody (for monocyte staining), a mouse anti-rat-ED2 antibody (for macrophages), and a rabbit anti-rat-MCP-1 antibody were used as primaries. Each specimen was then incubated with suitable fluorescently labeled secondary antibodies for 4 h at 4°C: Cy3 donkey anti-mouse for ED1/ED2 and Alexa 488 goat anti-rabbit for the MCP-1 antibody. Leukocytes were counted in five randomly chosen areas in each specimen (3 specimens/animal) at a magnification of ×200. Secondary antibodies without primary antibody preincubation were used in parallel in all staining procedures to ensure specificity.

Functional studies. Mechanical activity was measured as previously described (11). In brief, a segment of mid-jejunum was pinned in a dissecting dish containing ice-cold preoxygenated KRB and opened along the mesentery. The mucosa and submucosa were stripped off, and the muscularis was cut into strips (1 × 10 mm) parallel to the circular muscle fibers and suspended in standard horizontal mechanical organ chambers. One end of the strips was pinned down in the organ bath, and the other was attached to an isometric force transducer (WPI, Saratosa, FL). Muscle strips were continuously superfused with KRB equilibrated to 95% O2-5% CO2 and maintained at 37 ± 0.5°C. After stabilization for 30 min, strips were incrementally stretched to L0 (length at which maximal spontaneous contraction occurs). The spontaneous activity and the response of the circular muscle to increasing doses of the muscarinic agonist Bethanechol (0.3–300 μmol/l) were recorded and analyzed as grans per square millimeter per second using a A/D computer hardware and software package (Acknowledge; Biopac, Santa Barbara, CA).

Data analysis. Data were compiled as means ± SE. Statistical analysis was performed using the unpaired Student’s t-test. Data were considered statistically significant at a P < 0.05 level.

Drugs and solutions. A standard KRB was used as described previously (26). KRB constituents were purchased from Sigma. Rat rMCP-1 and mouse anti-rat-ED1 and -ED2 antibodies (1:100 each) were obtained from Serotec (Ralegh, NC). The rabbit anti-rat MCP-1-antibody (1:100 for immunohistochemistry) and the nonspecific rabbit anti-rat antibody were purchased from Chemicon International (Temecula, CA). Alexa 488-conjugated goat anti-rabbit secondary antibody (1:100) was obtained from Molecular Probes (Eugene, OR). Indocarbocyanine (Cy3)-conjugated donkey anti-mouse antibody (1:500) was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). For immunohistochemistry, antibodies were diluted in 0.05 mol/l PBS contain-
ing 0.2% bovine serum albumin, 1,000 U/ml penicillin G, and 1 mg/ml streptomycin (Sigma). LPS from *E. coli* serotype 0111:B4 was obtained from Sigma.

RESULTS

Effect of LPS on muscularis MCP-1 mRNA expression. We have previously shown that LPS causes the rapid activation of resident muscularis macrophages and that this is followed by the extravasation of numerous monocytes (11). MCP-1 is known as a major monocytic chemoattractant after LPS challenge (29, 34, 37). To determine the role of MCP-1 expression in the LPS-stimulated intestinal muscularis, we analyzed MCP-1 mRNA levels by semiquantitative RT-PCR. LPS caused a significant increase in MCP-1 mRNA expression in the intestinal muscularis, peaking at 3 h and remaining elevated through 48 h (Fig. 1), GAPDH expression was unchanged. The basal expression of MCP-1 mRNA was detectable in the isolated muscularis of control animals. Anesthetized saline-injected animals also served as controls and did not differ from control animals without injection (data not shown). SYBR Green real-time PCR was performed to quantify the MCP-1 peak mRNA expression at 3 h following LPS injection. As illustrated in Fig. 2, the samples of the endotoxemic animals entered the exponential amplification phase distinctly earlier than those of the controls, which demonstrates a higher template concentration. The average MCP-1 $C_T$ value of the LPS animals (17.9 ± 0.41) differed significantly from the controls (25.7 ± 0.25). Normalization of the data with the endogenous reference gene GAPDH to MCP-1 showed that the muscularis cDNA copy number for MCP-1 was 280-fold increased following LPS at 3 h compared with untreated controls ($\Delta \Delta C_T$ -8.1 ± 0.25).

MCP-1 immunohistochemistry. LPS has proven to be a potent activator of resident intestinal macrophages and recruiter of circulating leukocytes. We have reported that the cellular recruitment begins at ~4 h and reaches a maximum at 24 h (11). Since MCP-1 expression occurred before the onset of extravasating leukocytes, we hypothesized that it must come from a cell type within the muscularis. Therefore, we performed immunohistochemistry to determine the cellular localization of MCP-1 using a specific polyclonal antibody. As indicated in Fig. 3, 18 h after LPS, MCP-1 immunoreactivity was distinctly present in stellate-shaped cells. The distribution and pattern of the MCP-1 staining was similar to the morphological appearance of resident muscularis macrophages. Immunohistochemical staining with the rat macrophage marker ED2 confirmed the identification of these cells as macrophages. No MCP-1-positive cells were detected in whole mounts from control animals.

Local effect of exogenous MCP-1 on the intestinal muscularis in vivo and in vitro. MCP-1 is highly induced in response to LPS within muscularis macrophages. We decided to determine if exogenous MCP-1 could initiate the local recruitment of immunocompetent cells into the intestinal muscularis even in the absence of LPS. We explored this by directly applying MCP-1 to the intestinal wall through an abdominal incision. Using this approach, we were able to initiate the recruitment of leukocytes into the intestinal muscularis. As demonstrated in Fig. 4, local MCP-1 ap-

![Fig. 1](image1.png)

**Fig. 1.** Time course and representative gel bands of monocyte chemoattractant protein-1 (MCP-1) mRNA expression in the intestinal muscularis during endotoxemia, determined by semiquantitative RT-PCR. The radioactive intensity for each gel band was quantified by phosphor imaging and averaged. The MCP-1 message increased significantly at 1 h after lipopolysaccharide (LPS), peaked at 3 h, and remained elevated for 48 h.

![Fig. 2](image2.png)

**Fig. 2.** Real-time 2-step RT-PCR analysis of MCP-1 mRNA levels at 3 h after LPS injection using SYBR Green. The MCP-1-specific amplification was normalized to an endogenous control [glyceraldehyde-3-phosphate dehydrogenase (GAPDH), right]. Estimations were performed in triplicate. $\Delta R_n$ values are plotted vs. cycle number. MCP-1 amplification in the endotoxemic muscularis extracts started significantly earlier [threshold cycle ($C_T$) = 17.9 ± 0.41] than the amplification in the controls ($C_T$ = 25.7 ± 0.25).
Application resulted in the significant infiltration of leukocytes into the muscularis externa. In addition to many strongly reactive MPO-positive cells, representing PMNs, we observed numerous cells with a round morphology exhibiting weak MPO reactivity (Fig. 4B). Using the monocyte/macrophage marker ED1, these dense plaques of infiltrating cells were immunohistochemically identified as monocytes (Fig. 4, C and D). Specimens from the proximal jejunum, a region that was not exposed to exogenous MCP-1, and from sham-operated animals did not show this leukocyte recruitment. However, in accordance with previously reported results (25), the whole mounts of these animals showed a slight infiltration of MPO-positive cells that was due to mild intestinal manipulation. A monocytic infiltration was not observed in these specimens.

We next sought to determine the functional long-term effect of the MCP-1-recruited leukocytes into the intestinal muscularis. As in the above experiments, MCP-1 (1 μg/0.5 ml) or saline alone was applied to a segment of the distal jejunum. Then, after 18 h of in vivo activity, muscle strips from the applied region were prepared for mechanical recordings. After preparing the muscles from a segment of bowel, the remaining muscularis was stained for MPO activity to confirm that the muscle was prepared from the appropriate region. As shown in Fig. 5, spontaneous and bethanochol-stimulated contractile activity (at 100 μM) of the MCP-1-induced infiltrated muscularis was significantly suppressed compared with the saline-pretreated muscularis (39% and 47%, respectively).

MCP-1 has been shown to stimulate production of leukotrienes in culture (32). Therefore, we investigated the direct acute exogenous effect of MCP-1 on spontaneous contractions generated in vitro from rat jejunal circular smooth muscle. In four experiments, MCP-1 superfused at the relatively high concentration of 4 μg/100 ml KRB had no immediate direct effect on spontaneous muscle contractions over a 15-min period (data not shown).

**Antibody neutralization of MCP-1.** As shown above, MCP-1 is significantly upregulated and expressed within the resident muscularis macrophages following LPS. Furthermore, we show that exogenous MCP-1 can initiate the recruitment of numerous leukocytes into the muscularis. Therefore, we hypothesize that endogenous MCP-1 plays a significant role in leukocyte recruitment during endotoxemia. We tested this hypothesis by quantifying PMN and monocyte extravasation after the in vivo pretreatment of animals with a specific neutralizing MCP-1 antibody. Few PMNs and monocytes were seen in whole mounts of control animals. As illustrated in Fig. 6, LPS caused a significant cellular inflammatory response within the intestinal muscularis. This cellular response was primarily dominated by the extravasation of monocytes, which increased 22.6-fold (22.3 cells/field at ×200). The total number of infiltrating PMNs was significant but quantitatively lower compared with monocytes (9.3 cells/field at ×200; 10.6-fold increase). The specific neutralization of MCP-1 in the third group of animals resulted in a significant reduction of the cellular infiltration into the intestinal muscularis. Monocytes increased only 5.7-fold and PMNs 2.9-fold over control. Nonspecific antibody administration caused no significant quantitative or qualitative changes in the infiltration pattern of PMNs or monocytes after LPS compared with nontreated LPS animals.
motility, in vitro circular muscle spontaneous contractions and bethanechol-stimulated dose-response curves (0.3–300 μM) were generated 18 h after LPS with and without antibody pretreatment. Using standard organ bath techniques, we found that a single intraperitoneal bolus injection of LPS caused a distinct reduction in rat spontaneous circular muscle contractility (Fig. 7, A–D). Control spontaneous circular muscle generated 0.31 ± 0.03 g·mm⁻²·s⁻¹, whereas LPS caused a significant 60% reduction in contractile area to 0.12 ± 0.03 g·mm⁻²·s⁻¹ (Fig. 7E). As illustrated in Fig. 7, A–D, the specific MCP-1 blockade significantly prevented the suppression in spontaneous contractions caused by LPS in untreated animals. Quantitatively, MCP-1 neutralization caused a significant (64%) prevention of contractile impairment observed in LPS-treated rats (0.24 ± 0.05 g·mm⁻²·s⁻¹). Pretreatment of animals with the nonspecific IgG polyclonal antibodies did not improve the circular muscle spontaneous contractility after LPS compared with untreated rats injected with LPS.

As we have demonstrated previously, LPS (15 mg/kg) causes a significant (51% at 300 μmol/l) reduction of bethanechol-stimulated circular muscle activity compared with controls (Fig. 8). Pretreatment of animals with the neutralizing MCP-1 antibody before LPS prevented circular muscle inhibition, with bethanechol-stimulated activity restored to normal levels. Control experiments using a nonspecific antibody showed a slight improvement in bethanechol-stimulated activity (reduction: 38% at 300 μmol/l) compared with LPS.

Fig. 4. Histological whole mounts of the intestinal muscularis 18 h after the selective bowel application of recombinant (r) MCP-1. A: myeloperoxidase (MPO) staining shows a significant and sharply demarcated leukocytic infiltration. B: infiltrate consisted of darkly stained polymorphonuclear neutrophils (PMNs) and lighter-stained cells with a round morphology. C and D: immunostaining with the monocyte marker ED1 identified these cells as extravasated monocytes. Magnification: A, ×40; B, ×100; C, ×100; D, ×400.

Fig. 5. Contractile histogram showing effects of exogenous MCP-1 (1 μg/0.5 ml) applied to a segment of bowel from which spontaneous and bethanechol-stimulated muscle contractions were recorded after an 18-h period of in vivo exposure. Both spontaneous and bethanechol-stimulated contractions at a concentration of 100 μM were significantly suppressed by MCP-1 pretreatment (closed bars) compared with the saline-treated group (open bars). KRB, Krebs-Ringer buffer. Data were expressed as means ± SE (n = 4/group). *Significant compared with the saline-treated group.
alone. However, this improvement did not achieve statistical significance. The differences between the MCP-1-specific and the nonspecific antibody pretreatments were significant at bethanechol doses >10 μM.

**DISCUSSION**

The extravasation of immunocompetent phagocytes into organs is an important component of the inflammatory response during sepsis and tissue trauma. To mount a sufficient host defense, leukocytes must selectively migrate toward the site of infection or injury. Chemotactic cytokines, derived from the location of the inflammatory event, act as signaling proteins to coordinate the selective recruitment of specific leukocyte populations (14). It has been suggested that MCP-1, a β-chemokine, plays an essential role in the recruitment of monocytes to sites of injury in several inflammatory models (31). Furthermore, MCP-1 produced from pulmonary macrophages has been suggested to mediate monocyte infiltration in the lung in response to endotoxemia (5). We have previously demonstrated that the quiescent network of resident macrophages is rapidly activated in response to LPS and that it is able to initiate a moderate leukocytic infiltrate within the muscularis, mostly attributed to extravasated monocytes (11). This LPS-stimulated monocytic recruitment resulted in a distinct suppression in jejunal circular smooth muscle contractility. The suppression in contractility was dose dependent and developed at a relatively high injected dose of LPS (10 mg/kg). Bacteria in an in vivo peritonitis model are known to release high concentrations of LPS into the surrounding peritoneal fluid (47). It may be appropriate to interpret our study in the context of a patient with a peritoneal infection during which locally produced endotoxin can easily reach the levels used in this study. However, this is not to imply that the mechanisms elucidated in this study do not apply to other clinical situations or lower doses of LPS. In light of our previous observations, we investigated the potential role of intestinal MCP-1 upregulation as a key causative mechanism for LPS-induced monocytic infiltrates into the intestinal muscularis and the subsequent dysfunction in circular smooth muscle contractility.
This study demonstrates that intestinal MCP-1 plays a key role in the initiation of cellular inflammatory events within the intestinal muscularis during endotoxemia. A single LPS challenge caused a pronounced increase in MCP-1 mRNA expression, which was rapidly upregulated within 1 h, peaked at 3 h, and remained elevated for at least two days. It has been shown that many cell types are able to produce MCP-1, including leukocytes, smooth muscle cells, fibroblasts, and endothelial cells (29). The release of MCP-1, potentially from both resident cells and recruited leukocytes, has led to the conclusion that MCP-1 promotes monocyte recruitment during the initiation, as well as the chronic inflammatory phase, of various diseases (27). However, in our sepsis model, the very early demonstration of MCP-1 upregulation excludes recruited leukocytes as an initial cellular source of MCP-1. This conclusion is based on our previous study, which showed that the onset of monocyte and neutrophil infiltration into the intestinal muscularis begins 3 h after LPS but did not become significant until 12 h after LPS (11). Coincidentally, the onset of recruitment coincides with the peak in MCP-1 message expression. Immunohistochemical results from the current study support the role of resident cells, and furthermore, we show that MCP-1 protein is highly localized to the resident network of muscularis macrophages. These findings indicate that the major source of MCP-1 in this endotoxemia model is the resident muscularis macrophages. However, smooth muscle cells, fibroblasts, or endothelial cells cannot be definitely excluded as further sources of MCP-1. Nevertheless, we propose that MCP-1 expressed from the dense network of resident macrophages is the primary source of the increased MCP-1 expression within the jejunal muscularis. This conclusion is further supported by previously reported data concerning macrophage activation in response to LPS, which suggest that resident macrophages play a key role in the development of subsequent inflammatory events during endotoxemia (11, 48).

In the present study, endotoxemia caused a significant leukocyte infiltration into the intestinal muscularis at 18 h, which was mostly attributed to monocytes. We hypothesized that the endogenous upregulation of MCP-1 played a seminal role in this cellular recruitment. We explored this possibility by using direct exogenous MCP-1 application to the bowel wall and by performing antibody neutralization of endogenous MCP-1 experiments. The exogenous direct application of MCP-1 to the jejunal wall in vivo resulted in the significant recruitment of monocytes and PMNs into the muscularis after 18 h. These results are consistent with findings from MCP-1 transgenic mice in which a pronounced accumulation of monocytes was seen (18). Interestingly, the induced infiltrates in our model consisted not only of monocytes but also of PMNs, though in smaller numbers. MCP-1 functions primarily as a chemoattractant for monocytes and macrophages (28). However, it has been shown that MCP-1 acts not only as a chemoattractant but also as a cytokine that enhances intercellular adhesion molecule-1 (ICAM-1) expression in an ischemia-reperfusion model (55, 56). Furthermore, ICAM-1 expression was proven to be significantly upregulated by MCP-1 in a dose-dependent manner on glomerular cells (41). It has also been demonstrated that ICAM-1 is important in the recruitment of neutrophils into injured tissues and that it is mechanistically involved in both leukocyte adhesion and transmigration (6). Thus MCP-1 could not only participate in monocyte recruitment but also in the local regulation of neutrophil recruitment via ICAM-1. A similar mechanism has been demonstrated in a cardiac inflammation model (21). In this study, we only used a single concentration of MCP-1. It is possible that a selective monocytic recruitment could be achieved at lower application doses.

The validity of using MCP-1 neutralization on monocyte recruitment through specific antibodies has been shown in several in vitro and in vivo models of acute and chronic inflammation (15, 18, 34, 44). Using a MCP-1 antibody, Grande et al. (16) were able to elicit an 87% inhibition of LPS-induced MCP-1 chemotactic activity in supernatants from cultured mesangial cells. Matsukawa et al. (34) demonstrated a 58% inhibition of LPS-induced monocyte infiltration into the joint with MCP-1 antibody pretreatment. Neutralization of MCP-1 activity in our LPS model resulted in a 74% decrease in monocyte accumulation within the intestinal muscularis. Furthermore, PMN infiltration was also significantly reduced, supporting the results obtained in the local MCP-1 application experiment, which also demonstrated muscularis PMN infiltration.

Finally, the role of MCP-1 in causing a downstream decrease in functional circular smooth muscle activity following LPS injection was also investigated. As previously shown, LPS causes a distinct suppression in spontaneous and bethanechol-stimulated circular smooth muscle contractility (11, 30, 54). Using a local gut injury model for studying mechanisms of postoper-
sive muscularis leukocytic infiltration directly mediated through the presence of an impressive muscularis leukocytic infiltrate (22). The present study confirms this correlation between leukocyte infiltration into the intestinal muscularis and smooth muscle dysfunction and, in addition, that enhanced MCP-1 expression is a causative mechanism of leukocyte extravasation and subsequently endotoxemic smooth muscle dysfunction.

However, MCP-1 appears to play a Janus-faced role in rodents. In contrast to the above-reported beneficial effects of MCP-1 blockade on ileus, previous studies have reported that anti-MCP-1 antibodies were detrimental during lethal endotoxemia and cecal-derived bacteria (32, 33, 57). It appears that when endotoxin is used in a concentration that is normally lethal, MCP-1’s ability to induce anti-inflammatory cytokines (i.e., IL-10) may be essential to limit mortality (57). Likewise, in a cecal ligation puncture model of sepsis, MCP-1 could be seen to limit bacterial spread and also induce anti-inflammatory cytokines, both of which intuitively would benefit survival (32, 33). However, the same chemotactic ability of MCP-1 that potentially limits bacterial spread also appears to play a substantial role in LPS-recruited, monocyte/macrophage-induced intestinal ileus. We have not investigated the role of anti-inflammatory cytokines generated within the intestinal muscularis externa, but we hypothesize that these cytokines would play a role in limiting the duration of ileus once it has been initiated by MCP-1-recruited monocytes.

The present results support our hypothesis that leukocyte extravasation into the intestinal muscularis directly causes a suppression in gastrointestinal motility during endotoxemia (11). Activated monocytes and PMNs are able to secrete numerous kinetically active substances such as prostaglandins and reactive oxygen and nitrogen intermediates. We and others (12, 48) have clearly shown that nitric oxide from inducible nitric oxide synthase upregulated monocyte/macrophage functions as a major suppressor of circular smooth muscle contractility during endotoxemia. Germane to this topic is the interesting observation made by Tsao et al. (49), wherein they report that nitric oxide downregulates MCP-1 expression. It is interesting to speculate that a potential interaction between nitric oxide and MCP-1 also exists within the intestinal muscularis. However, other studies have not shown a clear interaction between inducible nitric oxide synthase-derived nitric oxide and MCP-1 expression, even though other inflammatory mediators are reportedly influenced by nitric oxide in these studies (1, 39).

Furthermore, it has been reported that superoxide radicals secreted from activated PMNs have a direct impact on smooth muscle tone (17, 35). It is also known that prostaglandins have an inhibitory effect on intestinal smooth muscle cells (3, 45). Regarding this, and based on unpublished observations concerning intestinal manipulation, it appears that prostaglandins generated from recruited monocytes may also play a key role in the relationship between leukocyte infiltration and the inhibition of rat jejunal circular smooth muscle contractility.

The current study extends previous observations by showing that the regulation of leukocyte recruitment and the subsequent dysfunction of intestinal smooth muscle during LPS-induced endotoxemia is, at least in part, mediated through the paracrine activity of the potent chemokine MCP-1. Furthermore, this study demonstrates that, along with previous observations that LPS rapidly activates the dense network of resident macrophages (11, 48), the cellular source of MCP-1 is the dense network of normally resident muscularis macrophages.

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