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Calcitonin Gene-Related Peptide Partially Reverses Decreased Production of Chemokines KC and MIP-2 Following Murine Sepsis[1]

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Abstract—The secretion of calcitonin gene-related peptide (CGRP) and the chemokines KC and MIP-2 are increased in the animal models of endotoxemic and septic shock. We tested whether CGRP could modulate KC and MIP-2 secretion from different sources of macrophages after murine sepsis induced by cecal ligation and puncture (CLP). Macrophages were obtained from the peritoneal exudate and lung of female BALB/c mice 16 h after CLP and plated in culture with CGRP and/or LPS for 12 h. The results showed that peritoneal macrophage production of the chemokines (KC, MIP-2) and cytokines (TNF-α, IL-6) was markedly decreased in CLP mice. Alveolar macrophages did not display decreased cytokine/chemokines production after CLP. CGRP (0.1 nM–10 nM) partially reversed this decreased production of LPS-induced KC and MIP-2 from peritoneal macrophages. These results suggest that CGRP might be intimately involved in recruitment of neutrophils by promoting local production of the chemokines KC and MIP-2 in murine sepsis.

KEY WORDS: calcitonin; chemokines; murine sepsis

INTRODUCTION

Substantial data has been published demonstrating that neuroendocrine hormones and neuropeptides can regulate the immune response. The sensory nerve endings are in close contact with several components of the immunological system, thus immunological functions may be affected by the neurotransmitter CGRP from the sensory nerve endings (1). In addition, we have shown recently that CGRP can be synthesized and released in T lymphocytes, especially mitogen activated T lymphocytes in the rat (2). By using a $CGRP_1$ receptor antagonist, $hCGRP_{8-37}$, we have shown that although lymphocytes produce much less CGRP than dorsal root ganglia, sufficient amounts appear to be secreted to stimulate biological processes, such as suppression of proliferation and IL-2 production by T lymphocytes. Both cellular sources of CGRP may act on the same receptor of target cells, although the secretion from lymphocytes and neurons require different stimulants. It appears that CGRP performs many functions in an autocrine or paracrine fashion within the immune system. Specific receptors for CGRP are present on various cells of the immune system (3, 4) and CGRP has been shown previously to modulate a number of immune functions (5).

It has been reported that CGRP can inhibit IFN-γ-induced peroxide production, suppress antigen presentation by macrophages and increase intracellular cAMP (4, 6). The data have shown that CGRP can inhibit LPS-induced TNF and IL-12 production in cultured mouse

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peritoneal macrophages through the activation of cAMP pathway in murine macrophages (7, 8).

Also, it has been reported that CGRP promotes the rapid onset and prolonged activation of leukocyte adhesion to vaso-endothelium (9). In synergy with inflammatory mediators such as bacterial peptide, FMLP or complement fragment C5a, CGRP was reported to induce edema formation and neutrophil accumulation (10), however, the mechanism of CGRP's effects is not known, although modulation of chemokines may be involved. The chemotactic cytokines (chemokines) are potent and specific chemoattractants for inflammatory cells (11). The C-X-C chemokines primarily serve to recruit neutrophils to local sites of inflammation (12). The exact murine homolog of human IL-8, a major C-X-C chemokine in humans, has not been identified, but the two most probable candidates are KC (13) and macrophage-inflammatory protein-2 (MIP-2) (14). Therefore, we hypothesized that CGRP might stimulate chemotaxis via modulating production of the C-X-C chemokines KC and MIP-2.

Previous publications have demonstrated that the systemic levels of CGRP, KC, and MIP-2 are increased in endotoxemia or sepsis in animals and humans (15–18). Therefore, we hypothesized that CGRP may modulate chemokine secretion from the macrophages in the murine model of sepsis induced by CLP. The response of two sources of macrophages to CGRP in the mouse CLP model was investigated in the present study and the date indicated that the production of C-X-C chemokines, KC and MIP-2, and cytokines, TNF-α and IL-6, in response to LPS by peritoneal, but not alveolar macrophages was significantly reduced following CLP. Due to this suppression, CGRP reversed the decreased production LPS induced chemokines, KC and MIP-2, from peritoneal macrophages.

MATERIALS AND METHODS

Animals

BALB/c mice, 18–22 g, were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN) and maintained under standard laboratory condition. In addition to a resting period at the institutional vivarium, mice were acclimated to the laboratory environment for at least 24 h before surgery. After CLP, mice were housed in a temperature-controlled room with food and water *ad libitum*, and were kept under a 12-h light/12-h dark diurnal cycle. The experiments were performed in accordance with the National Institute of Health guidelines and approval was obtained from the University of Michigan Animal Use Committee.

CLP

CLP was performed as previously described (19). Female BALB/c mice were anesthetized with ketamine/rompun, intraperitoneally, 87/13 mg/kg of body weight (Ketamine, Fort Dodge Laboratories, Inc., Fort Dodge, IA; Rompun, Bayer Corporation, Shawnee Mission, KS). The peritoneum was opened sterilely and the cecum tied with 4.0 silk surgical thread 1 cm from the distal end, care was taken not to obstruct the illeocecal valve. Two punctures were made by passing a 21 gauge needle through the cecum distal to the point of ligation. The needle was removed and a small amount of the stool was extruded from the punctures to ensure patency. The abdominal cavity was closed with running sutures using sterile 4.0 silk sutures, and the skin closed with 9 mm stainless steel removable would clips. After the surgery, the mice were injected subcutaneously with 1 ml of prewarmed normal saline before being placed into their cage. Two h and then 12 h after surgery, each mouse received subcutaneous injections of imipenem, 0.5 mg/mouse (Primaxin, Merck and Co., Inc., West Point, PA), reconstituted in 1 ml of 5% dextrose for fluid resuscitation. We have previously reported that imipenem treatment reduces mortality and morbidity in the CLP model and was superior to the use of triple antibiotics (17). Mice were sacrificed at 16 h and samples collected for later analyses.

Peripheral Blood Analyses

At the time of sacrifice, mice were lightly anesthetized with ketamine/xylazine and 20 μl of EDTA anticoagulated blood was collected from the tail. A Hemavet Mascot Multispecies Hematology System Counter 1500R (CDC Technologies Inc., Oxford, CT) was then used to perform a complete blood count, this automated system operates through light scattering technology. Forward angle light scatter detects cells based on size (similar to flow cytometry) while the side angle (or 90°C) scatter determines cell-type-based cellular granularity.

Preparation of Macrophage Populations

After blood collection, mice were euthanized by cervical dislocation, then macrophages were obtained

from the lavage of peritoneal and lung exudate as previously described by our laboratory (18). The total cell count was carried out using a Coulter counter (model ZF, Coulter Electronics Inc., Hialeah, FL). Red blood cells were lysed using Zap-Oglobin II (Coulter) to ensure that only WBCs would be counted. The cells from two sources were then prepared for differential counts using a cytospin (final cell concentration of 1×10^5 in 200 μl of cytospin fluid), centrifuged at 700× g for 5 min; cytospin fluid consisted of RPMI 1640 and 1% FCS. The cytospins were then stained with Diff-Quick (Baxter, Detroit, MI) and WBC types were quantified under oil immersion lens after tallying 300 cells. The total for each cell type was calculated by dividing each cell type by 300 and multiplying by the total WBC obtained from the Coulter counter.

The cells were plated on culture dishes at a density of 5×10^4 cells per well for alveolar macrophages, and 2×10^5 cells per well for peritoneal macrophages in 1 ml RPMI-1640 containing 10% FCS and antibiotics (1% pen/strep) at 37°C in a humidified atmosphere at 5% CO_2 and 95% room air. Macrophages were allowed to adhere for 2 h and washed with Hank's balance salt solution 3×. This method provided adherent cells that exhibited typical macrophage morphology. The production of cytokines in the peritoneum was normalized to the total number of macrophages that were placed in culture. This was done by determining the number of macrophages in the lavage fluid and expressing the cytokine measurements as ng or pg/million plated macrophages. This was done since a million total peritoneal cells from the CLP group would contain substantially fewer macrophages than the sham group due to the recruitment of neutrophils. The percentage of macrophages in 300 cells of peritoneal exudate was decreased from 90% in the sham to 48% in the CLP due to the influx of neutrophils. No such change was observed in the alveolar cells.

Stimulation and Measurement of Cytokine Production

The macrophages were incubated with rCGRP (1 pM–10 nM, rat sequence, CGRP type 1, CGRP-α) (Peninsular Laboratory, CA) or/and 0.01 μg/ml lipopolysaccharide (LPS, *E. coli* O111:B4) (Sigma, St. Louis, MO) for 12 h in culture, then the cell supernatants were collected and frozen. All samples for cytokine production analysis were measured simultaneously to reduce errors due to assay variation. TNF-α levels were quantified by cytolytic activity directed against the WEHI 164 subclone 13 fibrosarcoma cell line as previously described (20). Briefly, samples were serially diluted across a flat bottom 96-well plate in 100 μl RPMI 1640 culture media supplemented with 1% FCS and 1 mM L-glutamine. WEHI cells in RPMI 1640 (100 μl) containing 10% FCS, 1 mM L-glutamine, 30 μg gentamicin and 0.5 μg/ml actinomycin D were added on top of the samples. The plates were allowed to incubate overnight at 37°C in a humidified chamber with 5% CO_2 followed by addition of MTT-Tetrazolium (Sigma, St. Louis, MO). Plates were returned to the incubator for an additional 4 h followed by the addition of acidified isopropanol and kept overnight at room temperature in a darkened chamber. Readings were performed at 550 nm with background subtraction at 630 nm. TNF-α levels were then extrapolated from a standard curve of human recombinant TNF-α (Cetus Corp., Emeryville, CA) that was used in the same assay, which was sensitive to 1–2 pg/ml of TNF.

IL-6 levels were determined by a bioassay using the B-9 cell, that proliferate in the presence of IL-6 (21). Serial dilutions of the samples were made in 96-well plates and B-9 cells suspended in Isocove's media containing 2 mM glutamine, 25 mM HEPES, 1% pen/strep, β-mercaptoethanol (50 μM), and 10% FCS. The cells were incubated for 3 days at 37°C in humidified chamber. MTT-tetrazolium was then added and incubated for 6 h followed by the addition of acidified isopropanol overnight. Samples were then read and the results calculated based on a standard curve of the human recombinant IL-6 (PepcoTech, Rocky Hill, NJ) run in the same assay, which reliably detected samples as low as 1–2 pg/ml.

KC and MIP-2 levels were quantitated by ELISA as described in our previously published methods (22). Briefly, rabbit polyclonal antibodies were raised against murine recombinant KC or MIP-2-α, purified, and used as capture antibodies. The plates were coated with the antibody overnight, blocked for 1 h on the nutator at room temperature before the addition of samples and standards, which were also placed on the nutator for 1 h. Biotinylated antibody was then added for 1 h followed by streptavidin-HRP (Jackson ImmunoRes. Labs, Inc., PA). Peroxidase substrate was then added and plates were allowed to develop for 20 min in the dark before the reaction was stopped with 3 M H_2SO_4. Plates were read at dual wavelengths of 490 nm and 630 nm. Sample concentrations were determined by comparison to a standard curve, which was prepared using the recombinant KC, which did not cross-react with MIP-2, and MIP-2-α.

The lower limits of detection for KC and MIP-2-α were 100 pg/ml and 25 pg/ml, respectively.

Statistical Analysis

The results were expressed as the mean ±SEM. The number of experimental animals used for each group was presented as '*n*' value in the figure legends. The data were analyzed using one-way ANOVA and further analyzed using the Student-Newman-Keuls' test for multiple comparisons between treatment groups and using unpaired student *t*-test for comparison between two groups. A *p*- value of <0.05 was considered significant for differences between treatment group means.

RESULTS

Alterations in Inflammatory Cells

In agreement with our previous report (18), the peripheral blood WBC was reduced due to a decrease in neutrophils, lymphocytes, and monocytes 16 h after CLP (Table 1). However, as we reported before (23), there was a significant elevation in the total number of cells obtained from the peritoneum 16 h after CLP while there was no increase in the recovery of cells from the lung (data not shown). The increase in peritoneal cell number was due to a substantial neutrophil influx.

Cytokine and Chemokine Changes in Plasma and Peritoneum

Similar to our previous report (23), there were significant increases in the murine cytokine IL-6 and the C-X-C chemokines, KC and MIP-2, at the site of inflammation, the peritoneum 16 h after CLP with a 21 gauge needle (Table 2). In this model, plasma IL-6, KC, and MIP-2 levels were also increased after CLP (Table 2).

Cytokine/Chemokine Production Following CLP

We evaluated the ability of cells from the two different cellular compartments to respond to an *in vitro* challenge with LPS. The results are normalized to the total number of macrophages present in the cell suspension for the peritoneal cells, as described in the methods section. Therefore, the results are all expressed as pg of cytokine/chemokine per million macrophages.

Table 1. Peripheral Blood Leukocytes 16 Hours after CLP or Sham Surgery

Group	n	WBC	PMNs	Lymphs	Monocytes
Sham	14	23.8 ± 1.1*	8.8 ± 0.4*	13.3 ± 0.7*	0.98 ± 0.05*
CLP	14	8.3 ± 1.1	2.3 ± 0.2	5.3 ± 0.7	0.4 ± 0.03

Values are cells $\times 10^{-6}$/ml. Each value is the mean ± SEM.
*n = $p < 0.001$ sham compared to CLP.

Table 2. Plasma and Peritoneal Levels of Cytokines and Chemokines 16 Hours After CLP or Sham Surgery

Group	N	IL-6	KC	MIP-2
		Plasma		
Sham	12	0.17 ± 0.05**	14.9 ± 1.8*	1.7 ± 0.4*
CLP	12	81.1 ± 0.3	1544 ± 603	22.1 ± 3.8
		Peritoneum		
Sham	12	0.20 ± 0.06**	0.09 ± 0.02*	0.15 ± 0.03**
CLP	12	407 ± 106	4.3 ± 1.4	7.6 ± 1.9

*Values are ng/ml and each value is the mean ± SEM.
* = $p < 0.02$.
**$n = p < 0.001$ sham compared to CLP.

Figure 1 shows the production of the cytokines, TNF-α, IL-6 and C-X-C chemokines, KC and MIP-2, from the peritoneal and alveolar macrophages in response to an LPS stimulus. Cells harvested from the peritoneum of mice who had undergone CLP 16 h previously had a significantly reduced capacity to produce TNF-α, IL-6, KC, and MIP-2 in response to the LPS challenge. In contrast, there was no reduction in the response of the alveolar macrophages to produce these cytokines and chemokines (Fig. 1A, B, C, and D). These data demonstrate for the first time that C-X-C chemokines, KC and MIP-2, production in response to LPS by peritoneal, but not alveolar macrophages was significantly reduced after a septic challenge due to CLP.

Effect of CGRP on Chemokine Production

We specifically sought to determine if modulation of chemokine production might occur by attempting to augment chemokine production by CGRP since CGRP has been reported to stimulate chemotaxis (9, 10). Peritoneal macrophages harvested 16 h after CLP, at a time of decreased chemokine production were stimulated with LPS (0.01 μg/ml). CGRP augmented chemokine production by the peritoneal macrophages in a dose-dependent manner, both KC (Fig. 2) and MIP-2 (Fig. 3) production was increased. This augmentation of chemokine production was observed at very low concentrations of CGRP (0.01– 10 nM), this effect was not observed in peritoneal

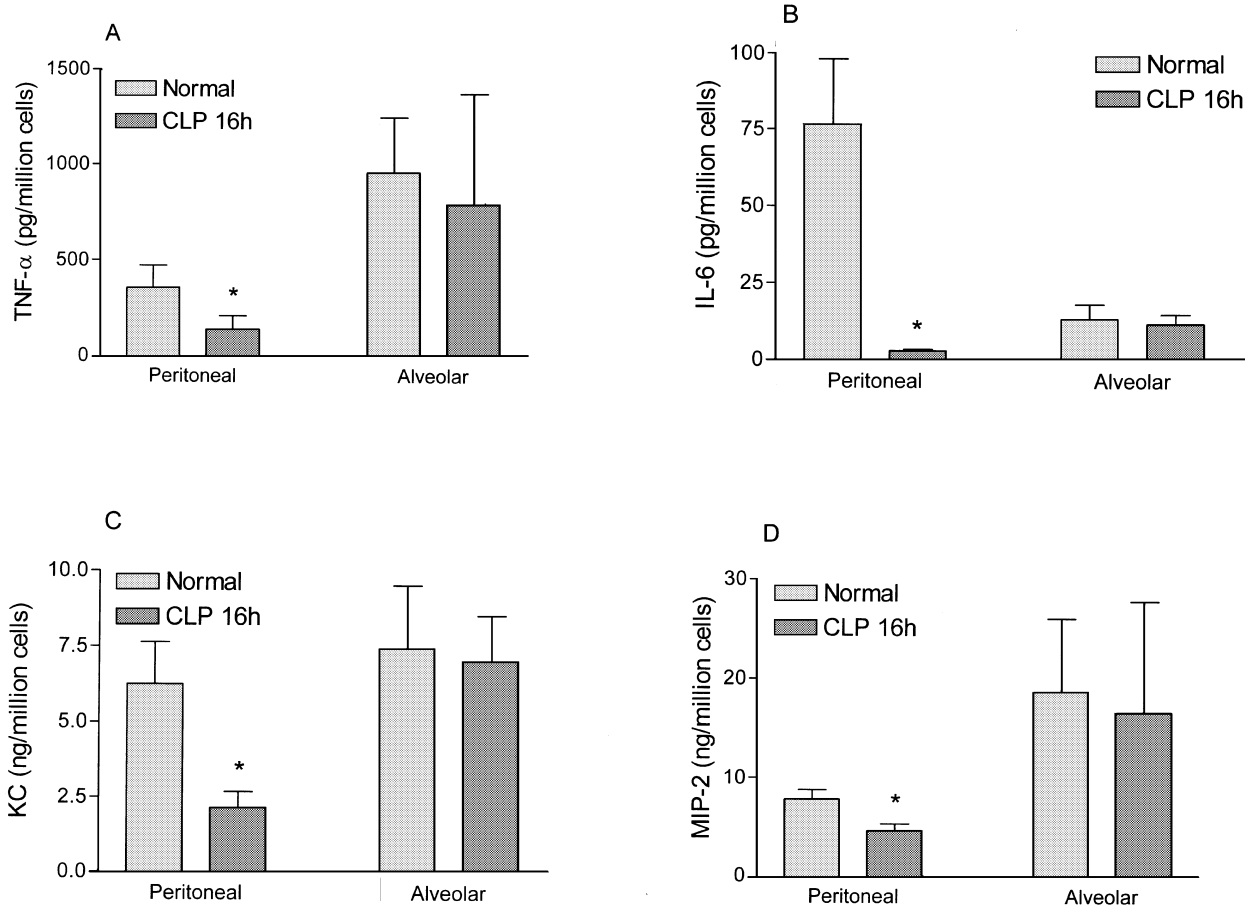

Fig. 1. Decreased TNF-α, IL-6, KC, and MIP-2 production after *in vitro* LPS stimulation of cells obtained from CLP mice. Peritoneal or alveolar macrophages were collected from the peritoneal exudates and lungs 16 h after sham surgery or CLP. Adherent macrophages were obtained and then stimulated with LPS (0.01 μg/ml) for 12 h. There was a significant decrease in TNF-α (A), IL-6 (B), KC (C), and MIP-2 (D) production from murine peritoneal macrophages, but not alveolar macrophages in the mice after CLP compared to the sham mice. The data are expressed as mean ±SEM for 6 mice. * = $P < 0.05$ CLP compared to sham.

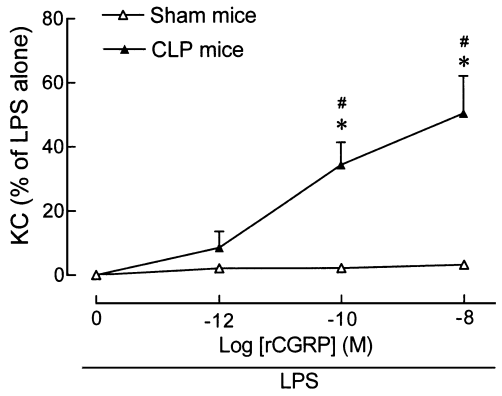

Fig. 2 Comparison of the effect of CGRP on LPS-stimulated *in vitro* levels of KC by peritoneal macrophages from sham surgery or CLP mice. Adherent macrophages were obtained from peritoneal cells after 16 h of either sham surgery or CLP. These cells were stimulated with LPS (0.01 μg/ml) and increasing concentrations of CGRP (1 pM–10 nM). CGRP augmented LPS induced KC production by macrophages obtained from mice subjected to CLP but not in the sham mice. The data are expressed as the percent of the LPS alone and each point is the mean ±SEM for 6 mice. * $P < 0.05$ *vs* LPS alone, # $P < 0.05$ CLP compared to sham surgery.

cells obtained from sham surgery mice, as can be observed in Figs. 2 and 3. Additionally, CGRP did not increase chemokine production from alveolar macrophages taken from either sham or CLP mice (data not shown).

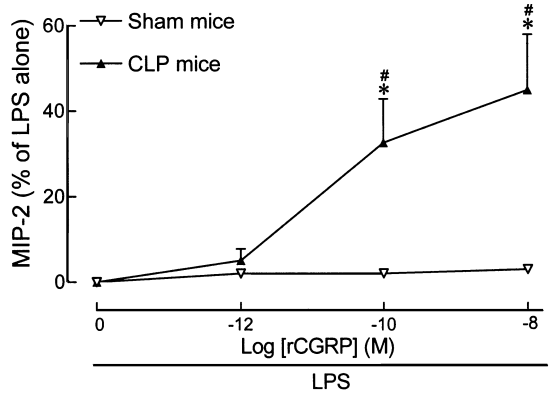

Fig. 3 Comparison of the effect of CGRP on LPS-stimulated *in vitro* levels of MIP-2 by peritoneal macrophages from sham surgery or CLP mice. Adherent macrophages were obtained from peritoneal cells after 16 h of either sham surgery or CLP. All cells were stimulated with LPS (0.01 μg/ml) and increasing concentrations of CGRP (1 pM–10 nM). CGRP augmented LPS induced MIP-2 production by macrophages obtained from mice subjected to CLP but not in the sham mice. The data are expressed as the percent of the LPS alone and each point is the mean ±SEM for mice. * $P < 0.05$ *vs* LPS alone, # $P < 0.05$ CLP compared to sham surgery.

When the two sources of macrophages were stimulated with CGRP alone, the levels of chemokine examined in the culture medium were very low. CGRP had no direct effect on KC or MIP-2 production from the macrophages taken from either the sham or the CLP mice.

DISCUSSION

This study shows, for the first time, that C-X-C chemokine (KC and MIP-2) production in response to LPS by peritoneal, but not alveolar macrophages, was significantly reduced after a septic challenge due to CLP. This reduction of LPS induced chemokine production from peritoneal macrophages following CLP was partially reversed by the polypeptide CGRP.

It has been reported that tissue-specific macrophages have a differential response to hemorrhage, trauma, endotoxin, or sepsis (24–26). Ayala and Chaudry (27–29) have reported that region-specific macrophages functions are altered after hemorrhage and resuscitation. Cecal ligation and puncture-induced sepsis differentially affects splenic, peritoneal, and pulmonary macrophages (24, 30). The differences in the local release of inflammatory mediators by adjacent cell populations represents one potential mechanism (25). Another possibility is that various macrophage subpopulations constitutively express different intracellular kinases involved in the regulation of monokine production (31). The reduced level of cytokine and chemokine release in response to LPS by peritoneal macrophages as compared to alveolar macrophages is most likely due to the prior activation *in vitro* by stimulants, i.e., endotoxin or cytokines. In CLP model, the peritoneal macrophages are more proximal to the intraabdominal septic insult, while the alveolar macrophages are more distal.

During sepsis, there are marked elevations in the number of cells and neutrophils obtained from the peritoneum following CLP (23). Since neutrophils play a key role in the elimination of invading microorganism, an important mechanism of host defense is neutrophil influx into the peritoneum which represents the local site of inflammation in this model. An improvement in the recruitment and function of neutrophils could lead to a reduced mortality due to sepsis. There are reports of significant IL-8 elevation in multiple organ failure patients (32). Previous reports have shown that an increase in two murine IL-8 analogs, KC, and MIP-2 (17, 18) occurs in murine sepsis. We have showed in the present study

there were significant increases both in the numbers of neutrophils recruited into the peritoneum and the levels of murine C-X-C chemokines, KC and MIP-2, in peritoneum and plasma after CLP with a 21 gauge needle. The murine C-X-C chemokines, KC and MIP-2, are known as neutrophil chemotactic mediators (33).

In certain pathological conditions, an abnormally high local level of CGRP may be associated with altered immune function. For example, plasma levels of CGRP were found to be elevated more then seven-fold above the basal level in patients in the late stage of septic shock. Additionally, it was elevated by 30 min in a rat endotoxin shock model, and continued to increase up to 3 h (15, 16). The CGRP release during septic shock may reach sufficiently high concentrations locally to modulate some immunocyte activities via paracrine mechanisms, including the elevation of LPS-induced KC and MIP-2 production as reported in the present study. It has been postulated that cytokines and chemokines contribute significantly to the pathophysiology of sepsis and septic shock (33). An early release of KC and MIP-2 into the local tissue milieu may initiate and achieve many of the beneficial responses aimed at improving antimicrobial function and reducing tissue damage. We anticipate that CGRP may exert neuroimmunomodulatory effects by augmenting KC and MIP-2 release from mono/macrophages at the early stage of sepsis or other inflammatory responses. This would exert a beneficial effect by augmenting recruitment of neutrophils to the local site of inflammation to clear the invading bacteria. However, the release of CGRP was thought to be associated with the hypotension, tachycarida, and immune suppression in late stage of endotoxin shock (34, 35). Further studies are needed to elucidate the role of CGRP in the sepsis *in vivo*.

In conclusion, the polypeptide CGRP can modulate a number of immune functions. Hypo-responsiveness to subsequent LPS challenge was selectively found by diminished peritoneal macrophage production of not only cytokines, TNF-α and IL-6, but also the chemokines, KC and MIP-2, in the murine CLP model. CGRP was able to partially reverse the decreased production of KC and MIP-2. These results suggest the peptide CGRP might be intimately involved in recruitment of neutrophils by promoting local production of the chemokines, KC and MIP-2, in murine sepsis and might serve to provide ongoing recruitment of neutrophils to the local site of inflammation.

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