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Lysozyme-Modified Probiotic Components Protect Rats against Polymicrobial Sepsis: Role of Macrophages and Cathelicidin-Related Innate Immunity

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Severe sepsis is associated with dysfunction of the macrophage/monocyte, an important cellular effector of the innate immune system. Previous investigations suggested that probiotic components effectively enhance effector cell functions of the immune system in vivo. In this study, we produced bacteria-free, lysozyme-modified probiotic components (LzMPC) by treating the probiotic bacteria, Lactobacillus sp., with lysozyme. We showed that oral delivery of LzMPC effectively protected rats against lethality from polymicrobial sepsis induced by cecal ligation and puncture. We found that orally administrated LzMPC was engulfed by cells such as macrophages in the liver after crossing the intestinal barrier. Moreover, LzMPC-induced protection was associated with an increase in bacterial clearance in the liver. In vitro, LzMPC up-regulated the expression of cathelicidin-related antimicrobial peptide (CRAMP) in macrophages and enhanced bactericidal activity of these cells. Furthermore, we demonstrated that surgical stress or cecal ligation and puncture caused a decrease in CRAMP expression in the liver, whereas enteral administration of LzMPC restored CRAMP gene expression in these animals. Using a neutralizing Ab, we showed that protection against sepsis by LzMPC treatment required endogenous CRAMP. In addition, macrophages from LzMPC-treated rats had an enhanced capacity of cytokine production in response to LPS or LzMPC stimulation. Together, our data suggest that the protective effect of LzMPC in sepsis is related to an enhanced cathelicidin-related innate immunity in macrophages. Therefore, LzMPC, a novel probiotic product, is a potent immunomodulator for macrophages and may be beneficial for the treatment of sepsis. The Journal of Immunology, 2006, 177: 8767–8776.

Severe sepsis is a serious and extremely costly medical problem in the U.S. The disease develops in >750,000 people annually, and ~30% of them die (1, 2). It is often caused by infection of commensal bacteria derived from mucosal or skin surfaces. In past decades, extensive efforts have been made to understand how microbes trigger the innate immune response to infections and the pathophysiology of sepsis (3, 4). We have learned that the innate immune system (including humoral and cellular components) is in a dynamic state during sepsis (reviewed in Refs. 3, 5, and 6). The sepsis syndrome is associated with an initially overwhelming innate immune response, characterized by unabated activation and release of proinflammatory mediators, i.e., humoral effectors. Subsequently, the exaggerated systemic inflammatory response is counterbalanced by a sustained expression of potent anti-inflammatory mediators, which often results in the desensitization of effector cells (such as phagocytes) and the development of immunosuppression. Both the excessive inflammation and the profound immunosuppression are major determinants to an adverse clinical outcome in sepsis. Furthermore, physiological functions of cellular effectors of the innate immune system such as macrophages/monocytes and neutrophils are altered in sepsis. Recently, several investigations suggested that modulation of immune cell function might be a novel therapeutic strategy for attenuation of sepsis (7–12).

Cathelicidin is a protein stored in granules as inactive propeptide precursors in polymorphonuclear leukocytes (PMN) and monocytes/macrophages (13–15). Upon stimulation, cathelicidin is released from phagocytes. After release, the C-terminal end of cathelicidin is processed into active peptides. It has been demonstrated that human and rodent phagocytes express a single cathelicidin peptide, namely, hCAP-18/LL-37 in humans and cathelicidin-related antimicrobial peptide (CRAMP) in rodents (16, 17). hCAP-18/LL-37 and CRAMP are effective killers of a variety of bacteria, including Escherichia coli, Pseudomonas aeruginosa, and Staphylococcus aureus (14, 16–19). CRAMP has been shown to impair intracellular replication of pathogens (20). Apart from its antimicrobial properties, hCAP-18/LL-37 and CRAMP are effective killers of a variety of bacteria.
play an important role in the maintenance of protective innate immunity.

Probiotics are nonpathogenic microorganisms, which confer benefits to the host when administered in sufficient amounts (23). Previous studies have shown that oral administration of probiotics modulates intestinal immunity, improves the balance of the gut microflora, enhances the recovery of the disturbed gut mucosal barrier, and prevents microbial translocation (23, 24). Experiment-
al and clinical studies have provided evidence for the possible use of probiotics in several inflammatory diseases, including inflammatory bowel disease, enteritis, diarrhea, and pancreatitis (24–27). In addition, studies have shown that the protective effect of probiotics is not limited to the gut (24, 28, 29). However, enteral delivery of intact probiotic bacteria has not been shown to prevent sepsis. Although probiotics have been suggested to enhance natural and acquired immunity, it is unclear whether their effects are associated with cathelicidin-related innate immunity. There have been a few reports indicating that heat-killed probiotics or their cell wall fractions effectively enhance effector cell functions of the immune system in vivo (30–32). Despite these promising results, the effect of probiotic components in the prevention and treatment of sepsis has not been investigated. In this study, we sought to use probiotic bacteria treated in vitro with lysozyme, an enzyme break-
ging down bacterial cell walls and releasing bacterial cell wall com-
ponents. Then we tested the hypothesis that oral delivery of ly-
sozyme-modified probiotic components (LzMPC) modulates the
innate immune system and protects against sepsis-induced lethality in
rats. We also sought to determine the tissue uptake of LzMPC and study whether LzMPC targets macrophages. Furthermore, we in-
vestigated the mechanism of LzMPC action by examining the reg-
ulatory effect of LzMPC on the cathelicidin-related innate immune
capacity of macrophages and studied whether CRAMP mediates the protective effect of LzMPC on sepsis.

Materials and Methods

Materials

All cell culture medium was obtained from Invitrogen Life Technologies. Lactobacillus sp. (ATCC 53103) and E. coli (ATCC 25922) strains were obtained from American Type Culture Collection. Chicken egg white ly-
sozyme (Sigma-Aldrich; L-6876), chemicals, and molecular biology re-
agents were purchased from Sigma-Aldrich. Limulus amebocyte lysate as-
say for measuring endotoxin contents was obtained fromCambrex.
BacLight Green Stain was provided by Molecular Probes. Rat TNF-α QuantiKine Colormetric Sandwich ELISA Kit was purchased from R&D Systems. RNA extraction kit and real-time PCR reagents were supplied by Qiagen. PCR primers were synthesized from Integrated DNA Technolo-
gies. All tissue culture plasticware were supplied by Costar. Goat poly-
clonal Ab (pAb) against rat CRAMP (catalog SC-34170) was purchased from Santa Cruz Biotechnology. The Ab used for in vivo neutralization of CRAMP did not contain sodium azide and gelatin.

Preparation of LzMPC and lysozyme-modified E. coli components (LzMEcC)

Lactobacillus sp. was used for preparation of LzMPC. Lactobacillus is a bacteria genus, which is the most often used probiotic (33). The safety and the risk-to-benefit ratio of Lactobacillus have been carefully studied and assessed (34, 35). Previous studies have shown that enteral administration of cell wall components of Lactobacillus provides enhancement of immu-
nity in animals (28, 31). Thus, this commercially available strain was used in this study. The bacteria were cultured in Lactobacillus MRS broth (Difco 0881) at 37°C with 5% CO2. A commercial E. coli strain was used for LzMEcC. The E. coli bacteria were cultured in Luria-Bertani medium at 37°C.

Preparation of prep for in vivo experiments. Fresh cultured bacteria (1011 CFU) were washed with PBS (pH 7.0) containing lysozyme (2 mg/ml), and incubated at 37°C for 60 min. After the digestion, cell suspension was centrifuged at 7000 × g for 30 min at 4°C. Supernatant was collected and cell pellets were dis-
carded. To inactivate lysozyme, supernatant was heated at 100°C for 10 min. Then it was cooled down to room temperature and centrifuged at 10,000 × g for 30 min at 4°C. The supernatant (i.e. LzMPC or LzMEcC) was used in various in vivo experiments in the present study. In prep used for in vivo experiments, 1 ml of LzMPC contains probiotic components derived from 107 bacteria. The LzMPC prep was gavaged to rats at a dose of 10 ml/kg.

Preparation of prep for in vitro experiments. Fresh cultured bacteria (1 g) were washed with PBS and digested with lysozyme (2 mg/ml) in PBS, as described above. After the digestion, cells were processed for centrifugation at 10,000 × g for 30 min at 4°C. The supernatant was collected, and cell pellets were discarded. To inactivate lysozyme, the supernatant was boiled for 30 min. Then it was cooled down to room temperature and centrifuged at 10,000 × g for 30 min at 4°C. The supernatant was collected, processed for column chromatography on a Detoxi-gel to remove endotoxin from the prep, and then filtrated through a 0.2-μm filter before applying to in vitro experiments. An aliquot of prep was routinely pre-
tested for Limulus amebocyte lysate assay to ensure no contamination with endotoxin. Our preliminary data showed that LzMPC prep activates macrophages at a dose of 10 μl/ml.

Polymicrobial sepsis induced by cecal ligation and puncture (CLP)

The CLP procedure was modified from previously described protocols (36, 37). All surgical instruments were steam autoclaved. Male Sprague Dawley rats (120–150 g) were purchased from Harlan Sprague-Dawley. They were anesthetized by i.p. injection of Nembutal (65 mg/kg) and placed under a heating lamp. A 2-cm midline incision was made through the abdominal wall. The cecum was identified and removed from the abdominal cavity. The distal portion (30%) of the cecum was ligated with 5-0 silk suture. The ligated cecum was then punctured twice with an 18-gauge needle and slightly compressed with an applicator until a small amount of stool ap-
peared. On sham-operated animals, the cecum was manipulated, but with-
out ligation and puncture. Thereafter, the cecum was replaced in the peri-
toneum. The incision was closed using a two-layer procedure: 5-0 silk suture on the muscle layer and surgical staples (9 mm) on the skin. Rats were monitored and weighed on a daily basis until the end of the experi-
ments. The protocol was approved by the Institutional Animal Care and Use Committee.

Bacterial load of liver tissues

Rats were sacrificed 3 days after CLP. Liver tissues were collected, weighed, extensively washed with sterile PBS four times, and homoge-
nized in sterile PBS (1 ml/g). Serial dilutions of liver homogenates in PBS were placed on Luria-Bertani agar plates. Bacteria colonies were counted after incubation overnight at 37°C. Bacterial counts were expressed as CFU/g tissue.

Fluorescent labeling of LzMPC prep with BacLight Green Stain

The BacLight Green Stain is a nonfluorescent acid fluorescent labeling reagent. It has a high affinity to the bacterial cell wall. The reagent is nonfluorescent when not associated with bacteria or cell wall components. The protocol suggested by the manufacturer was followed. Briefly, 1 μl of working dye solution was added into 1 ml of LzMPC prep, followed by incubation of samples at room temperature for 15 min. The intensity of fluorescence in the labeled LzMPC was determined with LS55 Luminescence Spectrom-
eter (PerkinElmer) with excitation at 480 nm and emission at 516 nm to ensure labeling efficiency.

Preparation and examination of tissue sections for determining tissue uptake of LzMPC labeled with BacLight Green Stain

The labeled LzMPC (450,000 fluorescent U/kg) was gavaged to rats. At the end of the experiments, the rats were sacrificed. Tissues were extensively washed with cold saline, embedded into the OCT compound, and snap frozen in liquid nitrogen. Cryosections were cut at 10 μm, postfixed in 10% neutral buffer formalin for 10 min, washed with PBS, and mounted in VECTASHIELD Mounting Medium with 4’,6’-diamidino-2-phenylindole (DAPI; Vector Laboratories). Slides were visualized under an upright fluo-
rescence microscope (model MD R; Leica Microsystems). The filter set for fluoroscein was used for detecting LzMPC labeled with BacLight Green Stain in cells, and the filter set for DAPI was used for visualizing nuclei. Immunoreactivities were acquired with a digital camera (Hamamatsu). They were transferred to a G4 Macintosh computer (Apple Computer), ana-
yzed by the image-analysis software OpenLab, and assembled with software Adobe Photoshop 8.0.
Isolation of peritoneal resident macrophages

Rats were sacrificed with inhalation of CO2 and used for isolation of peritoneal resident macrophages. The protocol described by Davies and Gordon (38) was followed. Briefly, the peritoneum was lavaged with cold serum-free RPMI 1640 several times. The exudate cells were washed and plated at 1 × 10^5 cells/ml in cell culture plates. After incubation for 2 h at 37°C in a humidified air atmosphere containing 5% CO2, nonadherent cells were removed by washing with HBSS buffered with 10 mM HEPES. Cells were then cultured with RPMI 1640 medium with 10% FBS overnight and used for various experiments.

Rat PMN isolation

A standard method in our laboratory was used (39). Briefly, rats were injected i.p. with 10 ml of 10% casein (in sterile water) under anesthesia. After 4 h, the peritoneal cavity was lavaged with 10 ml of sterile Hank’s salt solution, centrifuged at 500 × g. Contaminated RBC were removed by hypotonic shock. Then the cells were resuspended in DMEM with 10% FBS, counted, and used. PMNs were >95% pure, as assessed by May-Giemsa staining.

Macrophage bacterial assay

Intracellular bacterial killing was determined with a method described by Hamrick et al. (40). Briefly, macrophages were exposed to E. coli at 37°C for 15 min with a multiplicity of infection rate at 10 bacteria per macrophage. Extracellular bacteria were removed by washing twice with PBS and further killed by culturing macrophages with RPMI 1640 containing 5 µg/ml gentamicin for 30 min. Then wells were randomly named as T0 (for determining initial count Tinitial) and T90 (for determining final count Tfinal). Respective bacterial counts were determined by interpreting from the appropriate standard curve.

Determination of TNF

The protocol provided by the manufacturer was followed. Two microliters of the resulting cDNA was then amplified with the specific primers rat 18S housekeeping gene primers are as follows: forward, GCGCTCACTGTCACTGCTAT and reverse, 5’-TTGATTAGTTCCCTGCCC TTG-3’. These primers were used to perform the PCR. Rat 18S housekeeping gene primers are as follows: forward, 5’-GAGCCCCAAGGGGATGAGGA-3’ and reverse, 5’-CATGTGGGCCATGAGGTCCACCAC-3’. The rat CRAMP target gene primers are as follows: forward, 5’-TTGATTAGTTCCCTGCCC TTG-3’ and reverse, 5’-TGAAGGTCGGAGTCAACGGATT-3’. The rat CRAMP target gene primers are as follows: forward, 5’-GAGCCCCAAGGGGATGAGGA-3’ and reverse, 5’-CATGTGGGCCATGAGGTCCACCAC-3’.

Isolation of RNA, reverse transcription, and conventional and real-time RT-PCR

Total RNA from rat liver or peritoneal macrophages was extracted with an RNAeasy Mini Kit (Qiagen). The protocol provided by the manufacturer was followed. Exactly 1 µg of total RNA from each tissue was annealed at 42°C with random hexamers for 30 min in the iScript reaction mix (Bio-Rad) containing dNTP and 1U of Moloney murine leukemia virus-derived iScript reverse transcriptase (Bio-Rad), which was preblended with RNase inhibitor. The resulting cDNA was then amplified with the specific primers for annealing and extension. Data were analyzed with 7500 SDS software.

ELISA for quantitation of TNF in conditioned medium

Conditional medium from macrophage culture was collected, and TNF was detected using rat TNF-α Quantikine colorimetric sandwich ELISA kit, as described previously (41). The protocol provided by the manufacturer was followed. Standard curves were generated for TNF using the standard provided in the kit, and the concentration of TNF in the cell supernatant was determined by interpreting from the appropriate standard curve.

Western blotting (41)

Total cellular protein was isolated from macrophages, resolved on 4–20% SDS-PAGE gel, and transferred onto a nitrocellulose membrane, as described previously (42). The membranes containing sample proteins were used for immunodetection of CRAMP protein. Briefly, blots preincubated with PBS containing 5% nonfat dry milk (Bio-Rad; 170-6404) were reacted with primary Ab against CRAMP (1:100) for 1 h at room temperature. After incubation, the blot was washed four times with PBS containing 0.05% Tween 20 (PBS-T), and then incubated with PBS-T containing 1/2,000 diluted HRP-conjugated donkey anti-goat IgG for 1 h at room temperature. After additional washing with PBS-T, immune complexes on the blot were visualized by the ECL system. Blots were stripped and reprobed with mAb against β-actin (1/10,000) following a standard procedure (43).

RESULTS

Preparation and characterization of LzMPC

LzMPC were prepared from Lactobacillus sp. (ATCC 53103), a probiotic strain isolated from human feces, with a protocol described in Materials and Methods. LzMPC prep contains heat-stable molecules and is free of bacteria. LzMPC used for in vitro studies was further subjected to column chromatography on a Detoxi gel to remove endotoxin from the prep. To ensure removal of endotoxin from the prep, LzMPC was processed for the Limulus amebocyte lysate assay. LzMPC used for cell culture experiments contained <0.4 EU/ml endotoxin. As a control for LzMPC, we also prepared components named LzMeC using a commensal bacteria strain (ATCC 25922) utilizing the same procedure.

LzMPC specifically protects against sepsis-induced lethality in rats

To investigate the role of LzMPC in sepsis in vivo, a polymicrobial sepsis model of CLP was used (44). The CLP procedure involves surgical stress and triggers disseminated infection. It leads to development of peritonitis, bacteremia, and polymicrobial sepsis. In many respects, this model mimics the clinical course of sepsis in humans (45, 46). Thus, this model was used in the present study. To test the hypothesis that oral delivery of LzMPC provides protection against sepsis-induced lethality, we randomly assigned rats to either an experimental or several control groups, including the following: 1) LzMP + CLP (i.e., experimental group, enteral administration of LzMPC and operation with CLP; n = 18); 2) vehicle + CLP (enteral administration of vehicle and operation with CLP; n = 18); 3) viable Lactobacillus + CLP (enteral administration of Lactobacillus (10⁹ CFU/gavage) and operation with CLP; n = 10); 4) LzMeC + CLP (enteral administration of LzMeC and operation with CLP; n = 10); and 5) sham operated (enteral administration of vehicle and sham surgery; n = 6). The enteral administration of vehicle or bacterial components and CLP were conducted using a protocol described in Fig. 1A. Survival was monitored for 9 days after CLP or sham surgery. In vehicle + CLP and LzMeC + CLP groups, as shown in Fig. 1B, survival was 83 and 80%, respectively, 24 h after CLP. This diminished progressively each day until day 6, at which time only 33 and 40% were alive, respectively, in these groups. LzMPC markedly improved the survival. In the LzMPC + CLP group, ~15% of rats died by day 1 and 75% of animals survived by day 9 after the CLP challenge. In contrast, 60% of animals in alive Lactobacillus + CLP group died within 9 days after CLP.

Statistics

Data are expressed as means ± SEM. ANOVA and one-way ANOVA, followed by Fisher’s least significant difference post hoc test were used to assess the significant of differences; p < 0.05 was considered significant. Survival was analyzed and plotted by the Kaplan-Meier method using GraphPad Prism version 4.0 for Macintosh (GraphPad).

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FIGURE 1. LzMPC protects rats against polymicrobial sepsis-induced lethality. A, Protocol for delivery of LzMPC and induction of sepsis by CLP. Rats were gavaged with vehicle, LzMPC, or other bacteria components, and subjected to CLP at time points indicated in the figure. B, Survival rate of animals after CLP. Rats were randomly assigned to four groups, including the following: 1) LzMPC + CLP, n = 18; 2) vehicle + CLP, n = 18; 3) alive Lactobacillus (LB) + CLP, n = 10; and 4) LzMEcC + CLP, n = 10. The protocol shown in A was used for enteral administration of vehicle or bacterial components and conducting CLP. An 18-gauge needle was used for puncturing the cecum in CLP. Survival was monitored for 9 days after CLP. *p < 0.05 compared with the vehicle + CLP group. C and D, Enteral delivery of LzMPC does not preserve rat intestinal mucosa in polymicrobial sepsis. Rats were subjected to vehicle + CLP or LzMPC + CLP treatment, as described in A. Tissues of the small intestine were taken from animals on day 3 after CLP for histological examination. Typical sections are shown in vehicle + CLP (C), and LzMPC + CLP (D). H&E staining, original magnification ×100.

In addition, small intestinal tissue samples were taken from LzMPC + CLP and vehicle + CLP groups on day 3 after CLP for histological examination. We found extensive small intestinal injury in animals from both groups (Fig. 1, C and D).

Taken together, the data indicated that oral administration of LzMPC protected rats against CLP-induced death, whereas oral delivery of LzMEcC or live probiotics was ineffective. However, enteral delivery of LzMPC did not ameliorate intestinal injury in sepsis or preserve mucosal barrier function.

Oral administration of LzMPC enhances bacterial clearance in the liver during post-CLP period

Invasion by enteral commensal bacteria contributes to the development of sepsis in the CLP model. The level of the bacterial count in tissues is known to be associated with the severity of CLP-induced inflammatory response (45–47). Because the liver is a major organ responsible for bacterial clearance in abdominal infection, we examined whether the survival benefit afforded by LzMPC was related to bacterial elimination function in the liver. Briefly, rats were subjected to vehicle + CLP or LzMPC + CLP treatment, as described above. Livers were harvested 72 h after CLP and processed for measurement of bacterial load. As shown in Fig. 2, livers isolated from animals in vehicle + CLP group (n = 10) contained a substantial amount of bacteria. In contrast, the bacterial load in the liver of LzMPC + CLP group (n = 9) was markedly reduced (p < 0.01). The data indicated that LzMPC treatment reduced bacterial load in sepsis.

Orally administrated LzMPC is engulfed by cells in the liver after crossing the intestinal barrier

Because oral administration of LzMPC results in the enhancement of bactericidal activity in the liver, we investigated whether LzMPC is translocated into the liver. To this end, we gavaged rats with BacLight Green-labeled LzMPC. Then we examined cryo-sections of the liver from rats 16 h after enteral feeding with the labeled LzMPC. As illustrated in Fig. 3, particles with green fluorescence were found in the liver sinusoids and cells in the liver sections from rats fed with LzMPC labeled with BacLight Green Stain (Fig. 3A). Some of these cells displayed a distinct morphology of Kupffer cells (Fig. 3B), the residual macrophages in the liver. In contrast, the green fluorescent signal was barely found in the liver sections from normal control animals (Fig. 3C). The data indicated the uptake of LzMPC into the liver and macrophages.

Furthermore, we examined cryosections of the small intestine from rats 90 min after enteral feeding with the labeled LzMPC. This effort enabled us to identify labeled LzMPC at the early stages after it crossed the intact mucosal barrier. When comparing sections from rats fed with labeled LzMPC with sections from controls, cells throughout the lamina propria contained particles with
a strong degree of green fluorescence (Fig. 3D), whereas they exhibited only a weak autofluorescence in the controls (Fig. 3E). The data suggested translocation of LzMPC from the gut lumen into intestinal lamina propria.

**LzMPC activates macrophage’s bactericidal activity**

Macrophages play an essential role in the innate immune response against bacterial invasion. They eliminate bacteria from tissues during sepsis. Because LzMPC enhances bacterial clearance, we further examined whether LzMPC directly targeted the innate immune activity of macrophages. First, freshly isolated rat residential peritoneal macrophages were treated with LzMPC (10 μl/ml) or vehicle (control) for 6 h and examined under a microscope. As illustrated in Fig. 4A, LzMPC profoundly induced pseudopod formation in rat macrophages, as compared with the control group (Fig. 4B).

Furthermore, rat residential peritoneal macrophages were pre-treated with LzMPC (10 μl/ml) or vehicle (control) overnight, then processed for a bacterial killing assay. As shown in Fig. 4C, macrophages from the control group killed ~50% of ingested bacteria within 90 min. Pretreatment with LzMPC led to a significant increase in intracellular killing of bacteria by macrophages (p < 0.05). Together, the data suggested that LzMPC activated bactericidal activity of macrophages in vitro.

**LzMPC induces expression of CRAMP in macrophages**

To understand the mechanism whereby LzMPC stimulates protective innate immunity of macrophages against invasion of bacteria induced by CLP, we determined whether LzMPC modulated the expression of CRAMP gene, which encodes an important antimicrobial peptide in rat phagocytes (17). Residential peritoneal macrophages were stimulated with LzMPC (10 μl/ml) for 2 h. In control groups, cells were treated with LPS (1 μg/ml) or medium instead. Total cellular RNA was then isolated, and CRAMP gene expression was determined with semiquantitative conventional RT-PCR. As shown in Fig. 5A, CRAMP gene was constitutively expressed in rat macrophages. LzMPC, but not LPS, enhanced CRAMP gene expression.

Next, we stimulated macrophages with LPS (1 μg/ml) or LzMPC (10 μl/ml) for 6 h, and determined CRAMP gene expression quantitatively with real-time RT-PCR. As shown in Fig. 5B,
LPS had no effect on CRAMP expression in macrophages. In contrast, the gene expression was increased >14-fold within 6 h in response to LzMPC stimulation ($p < 0.01$ compared with control).

To confirm that both LPS and LzMPC activated macrophages, the culture supernatants were assayed for TNF using ELISA. As shown in Fig. 5C, TNF production was increased by $\sim$50-fold in LPS-stimulated cells and 10-fold in LzMPC-treated cells, indicating that cells were activated by both stimulations, although only LzMPC induced CRAMP gene expression. Together, these observations suggested that LzMPC specifically up-regulated CRAMP-associated innate immune capacity of macrophages.

**Surgical stress or CLP causes decrease in CRAMP expression in the rat liver, whereas LzMPC restores CRAMP gene expression in the liver of septic rats**

To assess the in vivo contribution of LzMPC treatment on CRAMP expression during the development of sepsis triggered by CLP, rats were divided into groups of the following: 1) normal control, 2) sham surgery, 3) CLP, and 4) LzMPC + CLP. The enteral feeding of LzMPC was conducted using a protocol described in Fig. 1A. Animals were sacrificed 72 h after surgery. Total cellular RNA was isolated from the liver, and CRAMP gene expression was quantitatively determined with real-time RT-PCR. Results are the means $\pm$ SEM; **, $p < 0.01$ compared with the control group.

**Oral administration of LzMPC enhances the expression of CRAMP mRNA and protein in macrophages, but not in neutrophils**

To examine whether LzMPC modulates CRAMP gene expression in macrophages in vivo, rats were gavaged with LzMPC for 5 days. Peritoneal macrophages were then isolated, and total cellular RNA and protein were extracted, respectively. Then CRAMP mRNA was measured with real-time RT-PCR and CRAMP protein was analyzed with Western blotting. As demonstrated in Fig. 7A, CRAMP mRNA was constitutively expressed in macrophages. Treatment with LzMPC in vivo resulted in a marked up-regulation of CRAMP mRNA in the peritoneal resident macrophages. Furthermore, we found that LzMPC up-regulated the expression of CRAMP protein in macrophages (Fig. 7B).

Neutrophils/PMNs also express CRAMP and play a critical role in killing bacteria (17). Thus, we further examined whether LzMPC modulates CRAMP gene expression in PMNs in vivo. As demonstrated in Fig. 7C, LzMPC does not modulate CRAMP gene expression in PMNs.
Ab against CRAMP blocks the protective effect of LzMPC on sepsis

Because the survival benefit of administrating LzMPC is associated with induction of CRAMP, we next examined the role of cathelicidin-related innate immunity in LzMPC-induced protection against sepsis by using an Ab against CRAMP. To accomplish this, we divided rats into groups of the following: 1) LzMPC + CLP (n = 10); 2) LzMPC + CLP + control IgG (n = 13); and 3) LzMPC + CLP + anti-CRAMP pAb (n = 17). Animals were subjected to treatment with LzMPC and challenged with lethal CLP using a protocol described in Fig. 8A. As stated above, LzMPC treatment resulted in a marked decrease in mortality 2 days after CLP challenge (Fig. 1B). Animals who survived 2 days after CLP were then treated with goat anti-CRAMP Ab (0.75 mg/kg, i.p.). The normal goat IgG was used as a control. The animals were continuously treated with LzMPC and monitored for an additional 8 days after administration of Abs. As illustrated in Fig. 8B, survival in LzMPC + CLP + control IgG group was similar to that in LzMPC + CLP group, indicating that administration of control goat IgG (i.e., IgG prepared from nonimmunized animals) did not result in any change in survival rate. In contrast, rats in LzMPC + CLP + anti-CRAMP pAb group had worse survival after CLP. The data suggest that endogenous CRAMP is a mediator for LzMPC action.

Effect of repeated enteral delivery of LzMPC on 1) cytokine production by peritoneal macrophages and 2) enteral flora in cecum

Pretreatment with LPS (a bacterial component) could result in the development of tolerance, which may lead to protection against septic peritonitis (48). Because LzMPC is also derived from bacteria, we investigated whether treatment with LzMPC would alter cytokine production by phagocytes. Briefly, rats were subjected to LzMPC or vehicle treatment for 5 days. Peritoneal macrophages were then isolated and stimulated with LzMPC (10\(^{-10}\) pg/ml) for 6 h, and TNF secretion was determined by ELISA. As shown in Fig. 9, macrophages spontaneously secreted TNF in vitro. Oral administration of LzMPC had no effect on the basal level of TNF production in macrophages. However, TNF secretion by macrophages was increased by LzMPC stimulation in vitro. Macrophages from LzMPC-treated animals had an increased capacity of TNF production in response to LzMPC stimulation in vitro. In addition, cells were also stimulated with LPS (1 \(\mu\)g/ml) for 6 h, and TNF secretion was determined. We found that LPS induced TNF production at 954 \(\pm 26.35\) pg/10\(^6\) cells in macrophages of vehicle group and 1033 \(\pm 16.95\) pg/10\(^6\) cells in LzMPC group (p < 0.05; vehicle vs LzMPC). Together, the data indicated that enteral treatment with LzMPC caused an increase in the capacity of cytokine production.
by macrophages rather than tolerance against LzMPC or cross-tolerance against bacterial component stimulation.

Enteral delivery of probiotics may influence the status of the gut flora. To examine the effect of LzMPC on bacterial growth in the cecum, we subjected rats to LzMPC or vehicle treatment for 5 days, collected luminal contents from the cecum by needle-puncture, and processed samples for measurement of bacterial count. As shown in Fig. 10, cecal bacterial colony count in the LzMPC group was similar to the count in the control, indicating that LzMPC did not modulate bacterial growth in the gut. This observation suggested that LzMPC-induced protective effect in sepsis was derived from mechanisms other than reduction of the amount of commensal bacteria in the intestine.

Effect of LzMPC treatment on serum TNF level during CLP-induced sepsis

As stated above, LzMPC induces the capacity of TNF production by macrophages (Fig. 9). Therefore, we further analyzed the effect of LzMPC treatment on systemic TNF level during the development of sepsis. Rats were divided into the following groups: 1) CLP alone (n = 3); 2) LzMPC + CLP (i.e., enteral feeding LzMPC for 5 days, followed by CLP; n = 5); and 3) LzMPC + CLP + LzMPC (i.e., enteral feeding LzMPC for 5 days, challenging with CLP, and enteral feeding LzMPC 2 h after CLP; n = 6).

All rats were sacrificed 6 h after CLP. ELISA analysis of serum TNF level of rats in each group demonstrated a similar degree of TNF production 6 h after CLP stimulation (Fig. 11). The data suggested that LzMPC treatment did not lead to the alteration of systemic TNF level in response to acute polymicrobial sepsis.

Discussion

Treatment of severe sepsis is still a major challenge in clinical practice (1, 3, 6, 49). In the past decade, many therapeutic approaches have focused on targeting humoral effectors (such as pro- or anti-inflammatory cytokines) of the innate immune system, which govern the development of pathophysiological disorders (such as hyper- or hypoinflammation) in sepsis (50, 51). However, attempts to neutralize proinflammatory cytokines such as TNF or IL-1β have failed to significantly improve the outcome of sepsis (52–54). In contrast, targeting anti-inflammatory cytokines such as IL-10 has been shown to be a complicated issue (55–60). Thus, it is a great practical and intellectual challenge to use immunoregulation as a means to prevent and treat sepsis. In the present study, we found that oral delivery of LzMPC protected rats against CLP-induced death. LzMPC enhanced bacterial killing and proinflammatory cytokine production by macrophages. The protective effect of LzMPC in sepsis was associated with augmentation of innate immunity in macrophages rather than induction of tolerance to bacterial products in macrophages or modulation of enteral flora. Previously, Iwata et al. (7) demonstrated that overexpression of Bcl-2 in phagocytes enhanced survival of the cells, thereby providing protection in experimental sepsis. Administration of molecules targeting phagocytes has been shown to be effective in the prevention and treatment of sepsis (8–11). Recently, Chung et al. (12) showed that preserving functional capacity of macrophages resulted in improvement of survival to sepsis. Taken together, our findings in this study in conjunction with these previous studies suggest that the immunomodulatory strategy to modulate macrophage/monocyte function should be pursued as a treatment for sepsis.

A growing body of evidence has suggested the potential usage of probiotic bacteria for therapy and immunomodulation (reviewed in Refs. 23 and 29). Because severe sepsis is associated with dysfunction of the innate immune system, theoretically, using probiotic bacteria should be an ideal immunomodulatory strategy to prevent or fight the disease. However, recent clinical studies using live probiotics yielded discouraging results: enteral delivery of live probiotics to patients at high risk of sepsis could cause secondary
nosocomial infections in these individuals, because the gut mucosal barrier function is impaired during sepsis (61–63). Thus, we prepared a novel, bacteria-free probiotic product, namely LzMPC, by digesting the probiotic bacteria with lysozyme. We demonstrated for the first time that oral delivery of LzMPC resulted in improvement in the survival of animals challenged with lethal CLP. The beneficial effect of oral administration of LzMPC was associated with enhancement of bacterial clearance in tissues. In contrast, enteral delivery of live probiotic bacteria increased death rate after CLP. These observations suggested a potential clinical use of LzMPC to protect against polymicrobial sepsis, or as a supplement for patients with immature mucosal barrier who are at high risk for sepsis.

For decades, probiotics have been defined as a live microbial food ingredient that is beneficial to health (reviewed in Ref. 33). This concept guides investigators to study the effects of probiotics in vivo. Recently, Salminen and colleagues (23, 64, 65) proposed that components of probiotic bacteria should also be included in the probiotic family. This updated definition for probiotics is supported by several studies. For example, Jiron et al. (66) reported that DNA from killed probiotics inhibits the production of IL-8 and IFN-γ by intestinal epithelial cells in response to commensal bacterial DNA. Several investigators demonstrated that functions of immune cells are modulated by oral administration of components derived from probiotic bacteria (31, 32). In addition, enteral delivery of killed probiotics results in enhancement of the host systemic innate immunity (28, 30). LzMPC is also a bacteria-free product derived from a probiotic bacteria strain. Our data suggested that ingesting LzMPC resulted in not only an improvement of innate immune capacity, but also an increased resistance to severe systemic inflammation. Thus, we believe that the study of probiotic elements is at least as important as the study of probiotics themselves.

Another important issue concerns the tissue uptake of LzMPC and its active ingredients. In the present study, we showed that LzMPC crossed the mucosal barrier and was then distributed in the liver. However, mechanisms of the gastrointestinal uptake of LzMPC remain undetermined. Previous investigations have demonstrated a normal physiological event called persorption, the paracellular uptake of substances from the digestive tract into the body (reviewed in Ref. 67). It is possible that LzMPC crosses the gut barrier via the mechanism of persorption. In addition, LzMPC is a complex preparation. It contains heat-stable components derived from probiotics. Previous investigations demonstrated that oral feeding heat-killed probiotics resulted in modulation of host immune function (28, 30). Hydrolysis of the bacterial cell wall with lysozyme yields several components such as N-acetylgalactosamine-N-acetylmuramic acid peptides and polysaccharides, which are resistant to heat. Further investigations will be required to examine whether these components play a role in the modulation of sepsis.

The mechanisms of action of probiotics are multiple (reviewed in Ref. 24). Lactobacillus is the most commonly used probiotic strain. Perdigon et al. (68) have shown that oral administration of lactobacilli activated macrophages in mice. However, it has not been elucidated whether any host factors in the intestinal lumen play a role in mediating the beneficial effect of probiotics. Lysozyme is a peptidoglycan (an essential cell wall component of bacteria) recognition protein (69, 70). It is an important bacterial-recognition molecule secreted by phagocytes and Paneth cells in the small intestine (69, 71). In the intestinal lumen, host lysozyme interacts with ingested probiotic bacteria and releases components from bacterial cells. However, little is known about whether components derived from lysozyme-modified probiotics play a role in vivo. In the present study, we mimicked this event by modifying Lactobacillus bacteria with lysozyme in vitro and administering them into the intestinal lumen. We showed that these components (i.e., LzMPC) had potent immune-enhancing properties and increased resistance against sepsis. In contrast, LzMEC did not protect against sepsis. Our observation suggests that lysozyme is an important host factor, which may mediate probiotic function in vivo.

CRAMP is a unique antimicrobial peptide expressed in phagocytes and epithelial cells (17). Recently, Nizet et al. (21) demonstrated that CRAMP-deficient mice lack innate immunity against bacterial infection, suggesting that CRAMP plays an essential role in the host defense. In addition, CRAMP-related peptides have also been demonstrated to neutralize LPS, protect mice from LPS lethality, and attenuate sepsis triggered by bacterial infection (14, 18, 22, 72). In the present study, we found that: 1) LzMPC upregulated the expression of CRAMP in macrophages; 2) LzMPC preserved CRAMP expression in tissues, which was correlated to the protective effect of LzMPC on sepsis; and 3) anti-CRAMP Ab attenuated LzMPC protection against CLP-induced death in rats. Collectively, these findings suggest that the protective effect of LzMPC in sepsis is related to increased cathelicidin-associated innate immunity of macrophages and is mediated by CRAMP. It is likely that LzMPC targets cellular effectors such as macrophages, thereby enhancing CRAMP-related protective innate immune capacity of macrophages and protecting against sepsis.

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References


