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Effect of Garlic Oil on Neutrophil Infiltration in the Small Intestine of Endotoxin-Injected Rats and Its Association with Levels of Soluble and Cellular Adhesion Molecules

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ABSTRACT: Garlic (*Allium sativum*) possesses anti-inflammatory effects. This study investigated the effects of garlic oil on endotoxin-induced neutrophil infiltration in the small intestine. Wistar rats received by gavage 10, 50, or 100 mg/kg body wt garlic oil (GO) or the vehicle (corn oil; 2 mL/kg body wt) every other day for 2 weeks before being injected with endotoxin (ip, 5 mg/kg body wt). Control rats were administered corn oil and injected with sterile saline. Blood samples for the measurement of soluble adhesion molecules were collected at various time points after injection, and all other samples were collected 18 h after injection. The 10 and 50 mg/kg doses suppressed endotoxin-induced neutrophilia, serum levels of sL-selectin and sICAM-1, cellular CD11b on neutrophils, intestinal ICAM-1 content, and neutrophil infiltration ($P < 0.05$). The 100 mg/kg dose significantly lowered local ICAM-1 and cellular CD11b on neutrophils ($P < 0.05$) but did not have a beneficial effect on neutrophil infiltration. In addition, 100 mg/kg of GO worsened the elevation of the local TNF-α level and neutrophilia. Appropriate doses of garlic oil have a preventive effect on endotoxin-induced neutrophil infiltration and damage to the small intestine.

KEYWORDS: endotoxin, intestinal mucosa, garlic oil, organosulfur compounds, rats, *Allium sativum*

INTRODUCTION

Endotoxins commonly cause systemic insults in critically ill patients. Damage to the function of the intestinal barrier by endotoxins has been demonstrated in healthy humans and experimental animals and has clinical implications for nosocomial infection in critically ill patients.[1−5] Because endogenous proinflammatory cytokines, including tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), are considered to be among the most important factors for the pathogenesis of endotoxin-induced organ failure, the main prophylactic and therapeutic strategies currently under development target these molecules with the aim of inhibiting their expression and activity.[6] On the other hand, during an inflammatory response, the number of neutrophils in peripheral blood increases. These cells transmigrate through the blood vessel wall via the interaction between adhesion molecules expressed on neutrophils and on vascular endothelial cells that are recruited to the inflammatory site by a gradient of soluble chemoattractants such as IL-8 in humans and cytokine-induced neutrophil chemoattractant-1 (CINC-1) in mice.[7] The activation of migration and phagocytosis also activates the destructive mechanisms of neutrophils and thus also represents the pathophysiology of acute and chronic inflammation. Consequently, inhibiting the interaction between neutrophils and endothelial cells has been suggested as an anti-inflammation strategy.[8,9]

Intercellular adhesion molecule-1 (ICAM-1) on epithelial cells and the β2 integrin CD11b/CD18 on neutrophils are two important adhesion molecules for stable interaction between these two cells.[10,11] Such interaction may play an important role in inflammatory injuries in various epithelial systems, including the gastrointestinal system[12−14] and the respiratory system.[15] Recently, it was shown that the amelioration of endotoxin-induced lung injury by compounds such as genistein or sevoflurane can be attributed to the inhibited expression of local ICAM-1 and lowered neutrophil infiltration.[16,17] Because ICAM-1 can be shed from cells and become soluble in serum, the serum levels of soluble ICAM-1 (sICAM-1) in various inflammatory diseases may reflect the activation of endothelial cells and thus the severity of certain inflammatory conditions.[18] Furthermore, the constitutively expressed adhesion molecule L-selectin can also be shed in activated neutrophils, and thus the serum level of the soluble form of L-selectin (sL-selectin) is a useful indicator of neutrophil activation.[19] However, the association of serum levels of these soluble adhesion molecules with the severity of endotoxin-induced tissue injury is not clear.

Garlic (*Allium sativum*), which belongs to the family Liliaceae, has a long history of medicinal use for various health problems. The anti-inflammatory activity of garlic preparations is mainly attributed to its antioxidant effect, its inhibitory effects on enzymes involved in the generation of inflammatory prostaglandins and thromboxanes, and its effect at suppressing the activation of NF-κB and the subsequent expression of proinflammatory cytokines and inducible nitric oxide synthase.[20,21] Current in vitro evidence suggests that garlic components can suppress IL-1α- and

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TNF-α-induced expression of ICAM-1 in endothelial cells,[22,23] thus suggesting a novel mechanism for the anti-inflammatory effects of garlic. We previously prepared stable garlic oil preparations and investigated some of their immune modulatory activities.[24−27] The aim of the present study was to investigate how the intake of garlic oil affects endotoxin-induced neutrophil infiltration in the intestine and its association with serum levels of sICAM-1 and sL-selectin and the expression of CD11b/CD18 on peripheral neutrophils. We were also interested in studying how the intake of garlic oil affects endotoxin-induced alterations of local cytokines and ICAM-1 in the intestine and its association with neutrophil infiltration into this tissue.

MATERIALS AND METHODS

Garlic Oil Preparation. Garlic oil was prepared from the same batch used in a previous study with steam distillation[28] and was composed mainly of 40.83% diallyl disulfide, 38.93% diallyl trisulfide, 7.17% methyl allyl trisulfide, 3.77% diallyl sulfide, 2.75% methyl allyl disulfide, and minor amounts of many other volatile compounds.

Animals and Experimental Procedure. Four-week-old weanling male Wistar rats were purchased from the National Animal Breeding and Research Center (Taipei, Taiwan). The animals were kept under a 12 h light−dark cycle at an ambient temperature of 23 °C and were given free access to water and standard rat feed (Rodent Diet 5001; Purina Mills, Richmond, IN) and were allowed to adapt to the environment for 1 week after their arrival before the experiment started. Animals were randomly assigned to five groups and received by gavage garlic oil (10, 50, or 100 mg/kg body wt) or the vehicle (corn oil; 2 mL/kg body wt) every other day for 2 weeks. The doses of garlic oil used in the present study were according to the findings of our previous study.[26] In that study, garlic oil was prepared in the same way and was found to significantly improve the integrity of the intestinal mucosa in endotoxin-injected rats at a dosage of 50 mg/kg body wt three times per week for 2 weeks.

During the 2 weeks of treatment, the animals were housed in metabolic cages and were given free access to water and a powdered diet (Rat Diet 5012; Purina Mills). Endotoxin was injected 15 days after the first administration of the garlic compounds or vehicle. The rats' food supply was withdrawn, followed by intraperitoneal injection of endotoxin from *Salmonella typhimurium* (5 mg/kg body wt; Sigma Chemical Co., St. Louis, MO). The control rats, which had received corn oil for 2 weeks, were injected (ip) with the same volume of sterile saline. Immediately before and at various time points after the injection, blood samples were withdrawn from the lateral tail vein for measurement of soluble adhesion molecules. The rats were killed by carbon dioxide euthanasia at 18 h after the injection. Blood was collected, and the intestine was removed immediately. Serum samples were prepared freshly from all blood samples by centrifugation at 500*g* at 4 °C for 10 min and stored at −80 °C until use within 1 week. Organs including liver, spleen, and kidney were then removed and weighed. Housing conditions and experimental procedures were performed according to the *NIH Guide for the Care and Use of Laboratory Animals*, and all protocols were approved by the ethical committee for animal experimentation of Chung Shan Medical University, Taichung, Taiwan.

Neutrophil Isolation and Flow Cytometric Analysis of CD11b and CD18 Expression. Peripheral neutrophils were isolated with a standard density gradient separation method by using commercially available separation media. Briefly, heparinized blood was mixed with 4.5% dextran/saline (w/v) at a ratio of 4:1 (v/v) to separate erythrocytes from leukocytes. After the leukocytes had been washed with Hanks' balanced salt solution (HBSS), the cells were resuspended in HBSS and layered over Histopaque-1077 density gradient (Pharmacia Biotech) medium in a centrifuge tube for the isolation of neutrophils from mononuclear cells. The residual erythrocytes were then removed with the RBC lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA, pH 7.2). The neutrophils were then washed and resuspended in phosphate-buffered saline (PBS) at a final density of $1 \times 10^6/100\ \mu L$ for flow cytometry analysis of CD11b/CD18 expression. Viability determined by trypan blue dye exclusion and morphological investigation showed sample yields of >95% neutrophils with >95% viability.

Flow cytometry on a FACScan Calibur system (Becton Dickinson) was carried out on neutrophils stained with a monoclonal antibody (mAb) to CD11b or CD18. Flow cytometric analysis of CD11b and CD18 expression was performed on rat neutrophils by using FITC-conjugated mouse anti-rat CD11b mAb (AbD Serotec) and RPE-conjugated mouse anti-rat CD18 (AbD Serotec), respectively, in accordance with the instructions of the manufacturer. Cells from the control group that did not stain with antibody were used as negative control. Ten thousand cells were analyzed in each sample. Data were analyzed with commercially available software (WinMDI2.8) and are expressed as mean fluorescence intensity (MFI).

Preparation of Intestinal Tissue Samples. Immediately after the intestine was removed, the ileum segment (defined as the intestinal segment 20 cm proximal to the cecum) was irrigated with cold PBS (pH 7.2) containing 1 mM phenylmethanesulfonyl fluoride to remove the intestinal contents and was separated into three segments. The segment 1−11 cm proximal to the cecum was used to prepare mucosa as described previously[26] and was stored at −80 °C for the analysis of TNF-α, IL-1β, CINC-1, and ICAM-1 within 1 week. The segment 12−13 cm proximal to the cecum was used for the analysis of myeloperoxidase (MPO) activity. The segment 13−14 cm proximal to the cecum was fixed in 10% neutral buffered formalin for histologic analysis. The segment 14−15 cm proximal to the cecum was embedded in 22-oxacalcitriol (OCT; Sakura Finetek, Torrance, CA) and was used to prepare cross sections (8 μm) with a cryostat (CM3050S, Leica Microsystems, Germany). The cryostat section of the ileum was mounted on poly(L-lysine)-coated glass microscope slides for MPO staining.

Biochemical Analysis of Blood Samples and Intestinal Tissue. Levels of serum soluble intercellular adhesion molecule (sICAM)-1 and mucosal ICAM-1 were determined by using a rat ICAM-1 ELISA kit (R&D Systems Inc., Minneapolis, MN). Levels of serum soluble L-selection (sL-selectin) and mucosal CINC-1 were analyzed by rat L-selectin and CINC-1 ELISA kits (R&D Systems Inc.), respectively. Levels of mucosal TNF-α and IL-1β were analyzed by rat IL-1β and TNF-α ELISA kits (Biosource International Inc., Camarillo, CA), respectively. The assay procedure was in accordance with the manufacturer's instructions, and the results were analyzed with a microplate reader (VersaMax; Molecular Devices Ltd., Sunnyvale, CA). Protein assays were performed by using Bio-Rad protein assay kits (Bio-Rad Laboratories, Richmond, CA).

Histochemical Analysis and Enzymatic Assay of Myeloperoxidase. The cryostat section mounted on poly(L-lysine)-coated glass microscope slides was stained with an MPO staining kit (Sigma-Aldrich) according to the manufacturer's method. After the counterstaining with hematoxylin, the sections were rinsed with ice-cold PBS twice followed by serial dehydration with ethanol and xylene and were examined by light microscopy at a magnification of 200×. Positive reactions were visible as distinct gray-black. Recruited neutrophils in intestinal mucosa were also evaluated by measuring MPO activity as described by Bradley et al.[29] with some modifications. The intestinal mucosa collected was homogenized in lysis buffer [0.5% (w/v) hexadecyltrimethylammonium bromide in 50 mM potassium phosphate buffer, pH 6.0] at 1:20 (w/v). Homogenized samples were frozen and thawed three times followed by centrifugation at 20000*g* for 15 min at 4 °C. MPO activity in the supernatants was analyzed

Table 1. Effect of Garlic Oil (GO) on Body Weight Gain, Food Intake, Organ Weights, and Neutrophil Count of Rats[a]

	control	endotoxin	endotoxin + GO10	endotoxin + GO50	endotoxin + GO100
body wt gain[b] (g)	112.2 ± 12.9	112.2 ± 12.9	108.4 ± 20.8	110.5 ± 11.1	110.1 ± 25.2
food intake[b] (g/24 h)	23.3 ± 2.9	23.3 ± 2.9	22.1 ± 1.7	23.3 ± 2.6	23.1 ± 3.1
liver wt/body wt[c] (%)	4.173 ± 0.171	4.453 ± 0.187b#	4.454 ± 0.176b	4.550 ± 0.284ab	4.739 ± 0.358a
spleen wt/body wt[c] (%)	0.339 ± 0.023	0.411 ± 0.044b#	0.410 ± 0.041b	0.461 ± 0.086b	0.532 ± 0.084a
kidney wt/body wt[c] (%)	0.927 ± 0.018	1.057 ± 0.049b#	1.034 ± 0.027b	1.037 ± 0.044b	1.143 ± 0.143a
neutrophil count[c] (cells/mL blood × 10^5)	15.8 ± 2.3	26.9 ± 7.3b#	13.5 ± 1.7c	23.0 ± 5.9bc	50.8 ± 15.6a

[a] Values are the mean ± SD for six rats per group. Endotoxin-injected groups not sharing the same letter (a−c) are significantly different ($P < 0.05$). #, significantly different from the control group ($P < 0.05$). [b] Determined before the administration of endotoxin. Both control and endotoxin groups were treated with vehicle, and thus the data were combined . [c] Determined at 18 h after the injection of endotoxin or saline.

Table 2. Concentrations of the Adhesion Molecule sL-Selectin in Peripheral Blood of Control Rats or Endotoxin-Injected Rats That Did or Did Not Receive Garlic Oil (GO)[a]

	sL-selectin (ng/mL)				
time after injection (h)	control	endotoxin	endotoxin + GO10	endotoxin + GO50	endotoxin + GO100
0	2.57 ± 0.45	2.80 ± 0.82	1.98 ± 0.93	1. 63 ± 0.93	2.67 ± 0.79
1	1.74 ± 1.32	2.91 ± 0.76a	2.76 ± 1.17a	1.75 ± 0.78a	5.73 ± 1.97b
2	1.58 ± 1.05	3.01 ± 1.59a	3.00 ± 1.67a	1.56 ± 0.93a	6.77 ± 1.52b
4	1.47 ± 0.97	3.33 ± 1.16a#	3.44 ± 1.45a	1.45 ± 0.31b	1.91 ± 0.29b
18	1.69 ± 0.62	3.12 ± 0.84a#	1.36 ± 0.62b	1.59 ± 0.82b	2.08 ± 0.46ab

[a] Values are the mean ± SD for six rats per group and were determined during 0−18 h after the injection of endotoxin or saline. Endotoxin-injected groups not sharing the same letter (a, b) are significantly different at the indicated time point ($P < 0.05$). *, significantly different from the control group at the indicated time point ($P < 0.05$).

spectrophotometrically at a wavelength of 460 nm with an ultraviolet−visible spectrophotometer (U-3000, Hitachi, Japan) with *o*-dianisidine as a substrate. MPO activity was expressed as units per gram of tissue.

Histologic Analysis of Intestinal Integrity. The distal ileum fixed in 10% neutral buffered formalin was embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin to evaluate the destruction of the villus architecture of the mucosa.

Statistical Analysis. The data are expressed as the mean ± SD and were analyzed by one-way analysis of variance. Student's *t* test was used to detect differences in means between the control group and the endotoxin-injected rats. Duncan's multiple-comparison test was used to detect differences among the means of the endotoxin-injected groups. *P* values of <0.05 were considered to be significant. All statistical analyses were performed with commercially available software (SPSS 12 for Windows; SPSS Inc., Chicago, IL).

RESULTS

Animal Characteristics. The administration of garlic oil did not significantly affect either body weight gain or food intake in the rats before the injection of endotoxin, which suggested that the doses of garlic oil used did not affect the rats' normal growth (Table 1). In rats that did not receive garlic oil, the injection of endotoxin significantly elevated ratios of organ weight to body weight compared with those of the saline-injected controls. Garlic oil at doses of 10 and 50 mg/kg did not affect endotoxin-induced organ hypertrophy; however, rats pretreated with 100 mg/kg garlic oil showed slightly but significantly worsened organ hypertrophy induced by endotoxin (Table 1). Compared with the saline-injected control group, rats injected with endotoxin showed elevated peripheral neutrophil counts ($P < 0.05$). The elevated blood neutrophil count induced by endotoxin was significantly reversed in rats pretreated with garlic oil at a dose of 10 mg/kg ($P < 0.05$); however, garlic oil at a dose of 100 mg/kg significantly elevated the endotoxin-induced increase in the neutrophil count ($P < 0.05$) (Table 1).

Serum Concentrations of sICAM-1 and sL-Selectin. Compared with the saline-injected control group, the injection of endotoxin significantly elevated the level of serum sL-selectin at 4 h after the insult, and this effect lasted up to 18 h after the insult (Table 2). Rats pretreated with 10 mg/kg garlic oil showed a significantly lowered sL-selectin level at 18 h after endotoxin injection compared with those treated with vehicle. Rats that received 50 mg/kg garlic oil showed a similar effect, but it occurred much earlier. Even though rats that received 100 mg/kg garlic oil showed a significantly worse endotoxin-induced elevation of the serum sL-selectin level at 1 and 2 h after the insult, these levels appeared to be lowered at a much earlier time point than in rats that received 10 mg/kg garlic oil (Table 2).

The serum concentration of sICAM-1 achieved a plateau state at 4 h after the injection of endotoxin (Table 3) and remained at levels significantly higher than those of the saline-injected control rats up to 18 h after the insult. Pretreatment with 10 or 50 mg/kg garlic oil significantly ameliorated the endotoxin-induced elevation of sICAM-1, and this effect was significant at 4 and 18 h for 10 mg/kg and at 4, 12, and 18 h for 50 mg/kg. In contrast, pretreatment with 100 mg/kg garlic oil did not significantly affect endotoxin-induced elevation of the serum sICAM-1 level. It is worth noting that pretreatment with garlic oil at 100 mg/kg significantly elevated the serum sICAM-1 level compared with the other groups even before the injection of endotoxin (Table 3).

Cellular CD11b/CD18 level on Neutrophils. Compared with the saline-injected control rats, endotoxin significantly elevated the expression of CD11b on these cells (Figure 1A,C).

Table 3. Concentrations of the Adhesion Molecule sICAM-1 in Peripheral Blood of Control Rats or Endotoxin-Injected Rats That Did or Did Not Receive Garlic Oil (GO)[a]

	sICAM-1 (ng/mL)				
time after injection (h)	control	endotoxin	endotoxin + GO10	endotoxin + GO50	endotoxin + GO100
0	7.26 ± 1.24	7.70 ± 1.45a	7.77 ± 0.81a	7.12 ± 2.40a	10.71 ± 2.14b
4	7.77 ± 0.90	27.73 ± 3.82a#	23.41 ± 2.77b	18.79 ± 2.65c	24.56 ± 4.56ab
8	8.10 ± 2.78	27.75 ± 5.35#	28.52 ± 6.62	24.56 ± 4.18	27.09 ± 2.56
12	6.34 ± 1.57	29.78 ± 5.17a#	26.60 ± 5.57ab	20.07 ± 4.30b	25.82 ± 5.30ab
18	6.99 ± 1.06	30.26 ± 2.66a#	22.28 ± 1.68b	18.13 ± 2.69c	26.54 ± 4.19ab

[a] Values are the mean ± SD for six rats per group and were determined during 0−18 h after the injection of endotoxin or saline. Endotoxin-injected groups not sharing the same letter (a−c) are significantly different at the indicated time point ($P < 0.05$). #, significantly different from the control group at the indicated time point ($P < 0.05$).

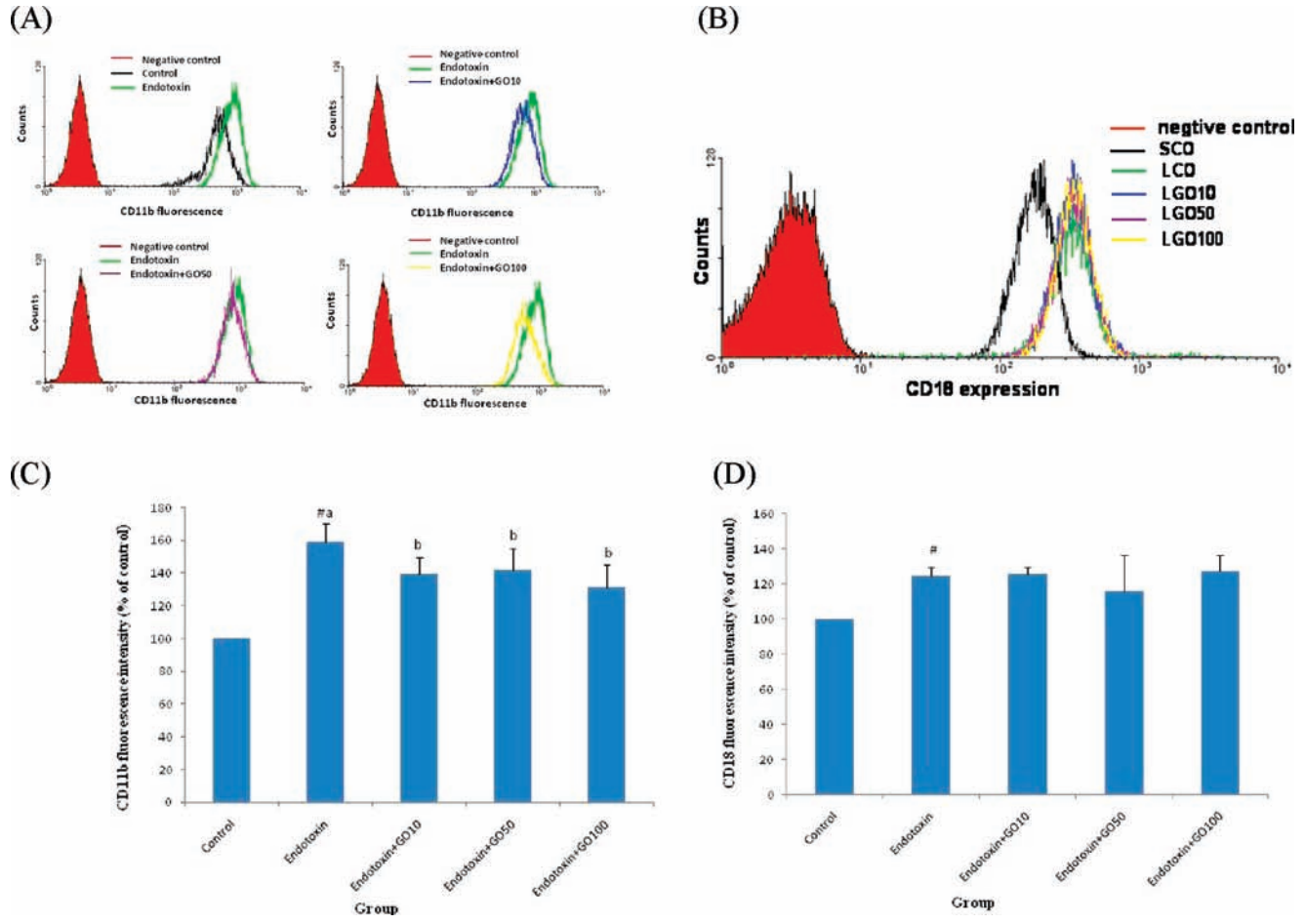

Figure 1. Effect of garlic oil on cellular levels of CD11b or CD18 on neutrophils isolated from rats injected with endotoxin. (A) Fluorescence intensity of peripheral neutrophils stained with FITC-conjugated anti-rat CD11b mAb and determined by flow cytometry. (B) Fluorescence intensity of peripheral neutrophils stained with RPE-conjugated anti-rat CD18 mAb and determined by flow cytometry. (C) Calculated mean fluorescence intensity expressed as a percentage of the control group. (D) Calculated mean fluorescence intensity expressed as a percentage of the control group. Rats received by gavage 10 mg/kg garlic oil (GO10), 50 mg/kg garlic oil (GO50), 100 mg/kg garlic oil (GO100), or the vehicle (corn oil) for 2 weeks and were then injected with endotoxin from *S. typhimurium* (ip, 5 mg/kg body weight). Control rats were pretreated with vehicle followed by injection with saline. Blood was collected, and neutrophils were isolated at 18 h after injection. Data are the mean ± SD for six rats per group. #, significantly different from the control group ($P < 0.05$). Endotoxin-injected groups not sharing the same letter (a, b) are significantly different ($P < 0.05$).

Pretreatment with garlic oil at all tested doses slightly but significantly suppressed the cellular level of CD11b on neutrophils ($P < 0.05$). On the other hand, although the cellular level of CD18 on neutrophils was significantly elevated by endotoxin ($P < 0.05$), pretreatment with garlic oil did not affect the CD18 level (Figure 1B,D).

Contents of CINC-1, ICAM-1, TNF-α, and IL-1β in the Intestinal Mucosa. Compared with the levels in saline-injected

Table 4. Contents of CINC-1, ICAM-1, IL-1β, and TNF-α in the Ileum of Control Rats or Endotoxin-Injected Rats That Did or Did Not Receive Garlic Oil (GO)[a]

	pg/mg protein				
	control	endotoxin	endotoxin + GO10	endotoxin + GO50	endotoxin + GO100
CINC-1	ND	41.17 ± 9.22#	23.09 ± 8.21	36.79 ± 20.80	33.26 ± 9.16
ICAM-1	2389 ± 1123	5569 ± 1729a#	4374 ± 1897ab	2942 ± 767b	2614 ± 779b
IL-1β	40.65 ± 20.79	245.8 ± 103.9#	275.9 ± 136.7	205.3 ± 83.1	255.5 ± 104.6
TNF-α	12.06 ± 1.70	18.55 ± 1.77b#	23.79 ± 4.94ab	15.36 ± 1.72b	29.83 ± 8.60a

[a] Values are the mean ± SD for six rats per group and were determined at 18 h after the injection of endotoxin or saline. Endotoxin-injected groups not sharing the same letter (a, b) are significantly different ($P < 0.05$). #, significantly different from the control group ($P < 0.05$). ND, not detectable.

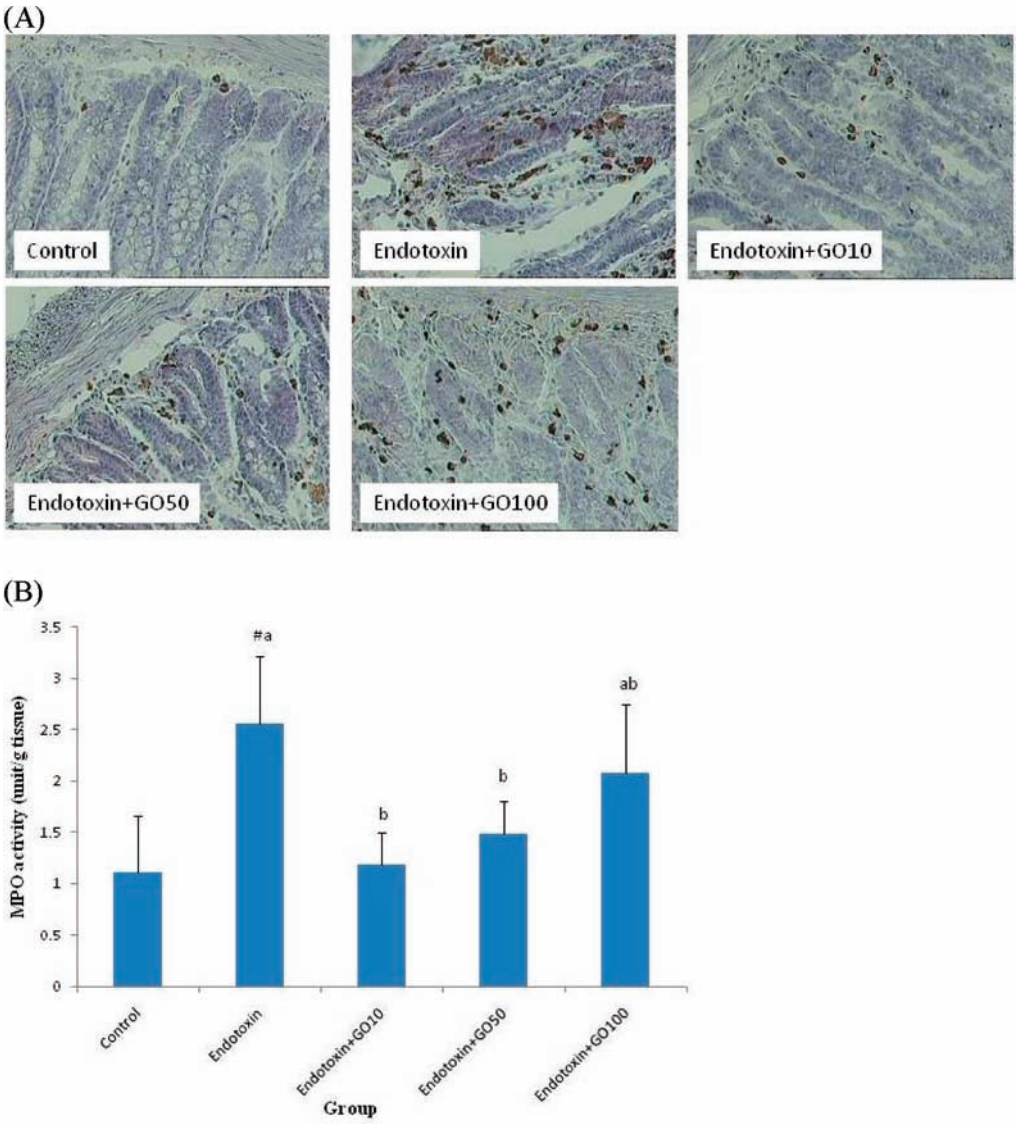

Figure 2. Effect of garlic oil on neutrophil infiltration in the ileum prepared from rats injected with endotoxin. (A) Cryostat sections of ileum were assessed for neutrophil infiltration with histochemical MPO staining and counterstained with hematoxylin (original magnification ×200). (B) MPO activity in ileum. Rats received by gavage 10 mg/kg garlic oil (GO10), 50 mg/kg garlic oil (GO50), 100 mg/kg garlic oil (GO100), or the vehicle (corn oil) for 2 weeks and were then injected with endotoxin from *S. typhimurium* (ip, 5 mg/kg body weight). Control rats were pretreated with vehicle followed by the injection with saline. Samples were collected at 18 h after injection. Data are the mean ± SD for six rats per group. #, significantly different from the control group ($P < 0.05$). Groups not sharing the same letter (a, b) are significantly different ($P < 0.05$).

controls rats, endotoxin injection significantly elevated the levels of both CINC-1 and ICAM-1 in the intestinal mucosa ($p < 0.05$). Pretreatment with garlic oil significantly lowered ICAM-1 induced by endotoxin in a dose-dependent manner, but did not

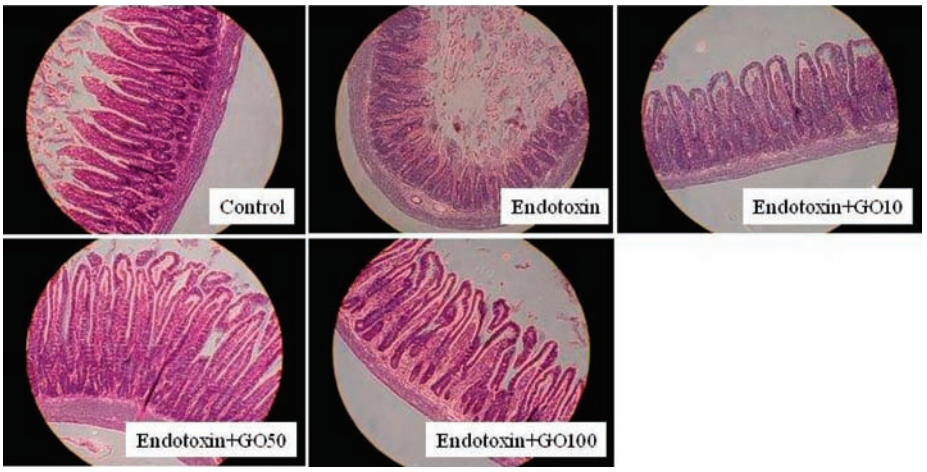

Figure 3. Cross sections of ileum stained with hematoxylin and eosin. Rats received by gavage 10 mg/kg garlic oil (GO10), 50 mg/kg garlic oil (GO50), 100 mg/kg garlic oil (GO100), or the vehicle (corn oil) for 2 weeks and were then injected with endotoxin from *S. typhimurium* (ip, 5 mg/kg body weight). Control rats were pretreated with vehicle followed by the injection with saline. Samples were collected at 18 h after injection. (Original magnification ×100.)

show a significant effect on CINC-1, although garlic oil at a dose of 10 mg/kg did lower the local CINC-1 level by 44%. Further investigation of levels of proinflammatory cytokines in the intestinal mucosa showed that local IL-1β content induced by endotoxin injection remained unaffected by garlic oil, whereas local TNF-α content was significantly elevated in the group pretreated with 100 mg/kg garlic oil compared with the vehicle-treated rats (Table 4).

Neutrophil Infiltration into Intestinal Mucosa. According to the histochemical staining, there were more gray-black particles that reflected more infiltrated neutrophils in the ileum tissue of the endotoxin-injected rats than in the tissue from saline-injected animals. Pretreatment with garlic oil significantly ameliorated neutrophil infiltration at all doses, but the best effect was seen with 10 mg/kg (Figure 2A). Consistent with the histochemical analysis of MPO, the activity of this enzyme in the intestine was significantly elevated in endotoxin-injected rats compared with that in the saline-injected controls ($P < 0.01$) (Figure 2B). Pretreatment with 10 and 50 mg/kg garlic oil significantly lowered MPO activity in the intestine; however, the garlic oil dose of 100 mg/kg showed a weaker effect on ameliorating the infiltration of neutrophils. These results were consistent with the histochemical staining as shown in Figure 2A.

Histological Analysis. The mucosal structure of the ileum was analyzed by light microscopy. The integrity of the intestinal mucosa was impaired by the injection of endotoxin; large areas of ulceration and overt breaches of the epithelial barrier were apparent, with exposure of the connective tissue, lymphatics, and blood vessels to the lumen (Figure 3). Endotoxin-induced mucosal changes were less severe in rats pretreated with garlic oil; in these rats, the epithelial barrier was largely repaired, although the top of the villi was flat and the lamina propria remained swollen. Although rats treated with 100 mg/kg garlic oil showed unaffected neutrophil infiltration (Figure 2), these animals showed ameliorated integrity compared with the vehicle-treated endotoxin group.

DISCUSSION

The present study made use of endotoxin injection to induce systemic inflammation in rats. After the injection of endotoxin, indicators of acute inflammation, including the blood neutrophil count and serum levels of both sL-selectin and sICAM-1, were increased compared with those of control rats. Thus, our findings were consistent with previous findings in various acute inflammatory conditions.[18,19] Consistent with the elevated sL-selectin levels, which represent the activation of neutrophils, the present study also found increased CD11b/CD18 expression on peripheral neutrophils with endotoxin injection, and this was accompanied by elevated MPO activity in the small intestine and damaged integrity of the small intestinal mucosa compared with that of control rats. These findings suggest an association between the transmigration activation of neutrophils and injury to the small intestine in these endotoxin-injected rats. In addition, the present study found that endotoxin-induced neutrophil infiltration in the small intestine is accompanied by elevated local levels of IL-1β, TNF-α, CINC-1, and ICAM-1. IL-1β and TNF-α are known to be two potent mediators of the endotoxin-induced expression of CINC-1 and ICAM-1,[30,31] and the intercellular adhesion with neutrophils through epithelial ICAM-1 and the chemoattractant role of CINC-1 for neutrophils are two major determinants of neutrophil recruitment in inflammatory lesions.[7,12,18,19] Consequently, one would expect that the suppression of levels of these molecules would be crucial to reverse endotoxin-induced neutrophil infiltration in the intestine. In this context, we investigated the effect of garlic oil on endotoxin-induced elevation of levels of these proinflammatory cytokines/chemokine and ICAM-1 in the intestine but found that only local ICAM-1, but not IL-1β, TNF-α, or CINC-1, content was associated with suppressed neutrophil infiltration in the intestine.

Garlic and its sulfur-containing components have been shown to have diverse biological activities. The inhibitory effects of garlic on the expression of proinflammatory molecules via the regulation of mainly the transcription factor NF-κB have been proposed to play significant roles in its anti-inflammatory actions.[32−35] We previously demonstrated in rats that pretreatment with garlic oil at a dose of 50 mg/kg body weight significantly ameliorated endotoxin-induced intestinal injury and apoptosis of intestinal mucosal cells independent of its effect on peripheral proinflammatory cytokines.[26] The data presented here show that garlic oil reverses the endotoxin-induced neutrophil infiltration and

damage to the intestine and that the effects of doses of 10 and 50 mg/kg are statistically significant. The present finding that garlic oil can suppress neutrophil infiltration into inflammatory lesions is consistent with findings of previous in vivo and in vitro studies. Hobauer et al.[36] demonstrated that garlic extract reduces migration of neutrophils through endothelial cell monolayers in vitro. In vivo studies with animals undergoing thermal injury, ischemia/reperfusion, or biliary obstruction also showed that aqueous garlic extracts could ameliorate neutrophil infiltration in various tissues and organs.[37−39] Nevertheless, such an effect of garlic was mainly attributed to its regulatory effect on proinflammatory cytokines and its antioxidant effects.[21] According to the results of a limited number of in vitro studies that showed the inhibitory effect of garlic preparations on the expression of certain adhesion molecules in endothelial cells, Namazi[40] suggested that an anti-inflammatory role of garlic on neutrophil activity through the regulation of adhesion molecules could not be excluded. However, to the authors' knowledge, there is limited evidence for such an effect of garlic in vivo. In the present study, we for the first time provide in vivo evidence showing that the inhibition of endotoxin-induced intestinal damage by garlic oil is associated with suppressed neutrophil activation reflected by a lowered serum sL-selectin level and CD11b expression on neutrophils and with suppressed ICAM content in the inflammatory lesions.

CD11b/CD18 mediates stable adhesion of activated neutrophils to endothelium and epithelium through interactions with ICAMs, including ICAM-1, and it has been shown that in vivo modulation of these adhesion receptors is effective in reducing neutrophil infiltration and limiting the inflammatory response.[41,42] Up-regulation of CD11b/CD18 on circulating neutrophils has been shown after endotoxin injection.[43,44] In other studies, monoclonal antibodies to CD11b/CD18 effectively protected against endotoxin-induced hepatic failure.[45,46] Similarly, the counter-receptor for CD11b/CD18, ICAM-1, increased during endotoxemia,[44,47] whereas ICAM-1 antibodies were also highly effective in reducing the neutrophil-dependent injury in the liver.[44,48] These experimental data support our finding that lower neutrophil infiltration in the intestine is associated with the decreased local ICAM-1, serum sICAM-1, and cellular CD11b on neutrophil in rats treated with 10 and 50 mg/kg garlic oil. This result is consistent with the findings of Hobauer et al.,[36] who showed that in vitro garlic extract is a potent inhibitor of leukocyte migration through endothelial cell monolayers and that treatment of both cell types has an additive effect. On the other hand, because we did not find the levels of the peripheral proinflammatory cytokines IL-1β and TNF-α to be changed by garlic in our previous study with the same animal model,[26] we excluded the theory that the suppressive effect of garlic oil on CD11b and local ICAM-1 was via a lowering of the proinflammatory cytokine level. Thus, it is very likely that garlic oil directly suppressed CD11b and ICAM-1 expression during the inflammatory response.

In rats that received the high dose of garlic oil (100 mg/kg), even though both the ICAM-1 in the intestine and CD11b expression on neutrophils were significantly suppressed, other factors offset the beneficial effect of garlic oil on endotoxin-induced neutrophil infiltration. Compared with other endotoxin-injected groups, we found that rats that received the high dose of garlic oil showed hypertrophy of organs and significantly higher basal serum levels of sICAM-1, peripheral neutrophil count, and TNF-α in the intestine, which thus might explain the absence of benefit of the higher dose of garlic oil. It is worthwhile to note that we previously reported that the administration of garlic oil in normal rats at doses up to 100 mg/kg every other day for 2 weeks did not affect either liver or spleen weight.[25] The organ hypertrophy at the dose of 100 mg/kg found in the present study is therefore likely to be a synergic effect of the high dose of garlic oil and endotoxin. On the other hand, this result is similar to our previous findings in which the beneficial effect of garlic oil on the integrity of the small intestine and protective effect from apoptosis of the mucosal cells were observed with garlic oil at a dose of 50 mg/kg but not 200 mg/kg.[26] In that study, pretreatment with 200 mg/kg garlic oil significantly increased peripheral concentrations of TNF-α and IL-1β compared with those of the untreated animals, and diallyl trisulfide was identified to provide a toxic effect similar to that of high-dose garlic oil. Despite the known anti-inflammatory effect of garlic, a toxic effect of garlic on gastrointestinal mucosa in vivo has been reported previously.[49] The mechanisms of such adverse effects of garlic remain to be clarified.

In conclusion, the present study showed that pretreatment with garlic oil at appropriate doses suppressed endotoxin-induced neutropil infiltration and ameliorated integrity of intestinal mucosa along with ameliorated serum levels of sICAM-1 and sL-selectin and cellular CD11b expressed on neutrophil. In addition, only local ICAM-1 but not IL-1β, TNF-α, or CINC-1 content was associated with garlic oil suppressed neutrophil infiltration in the intestine. These results suggested suppressed levels of soluble and cellular adhesion molecules a major mechanism underlying which garlic oil providing an anti-inflammatory action. The marginal effect of the high dose of garlic oil, however, was associated with a significantly higher peripheral neutrophil count, a higher basal serum sICAM-1 level, and a worsened local TNF-α level in the intestine induced by endotoxin. Thus, the presence of certain compound(s) in garlic oil may dominate the beneficial effect when garlic oil is given at a high dose. A study with various S-containing compounds of garlic oil will be necessary to further identify the most active compound(s) as well as the composition that may have adverse action for regulating the neutrophil-mediated inflammatory response.

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ABBREVIATIONS USED

CINC, cytokine-induced neutrophil chemoattractant; GO, garlic oil; HBSS, Hanks' balanced salt solution; ICAM, intercellular adhesion molecule; IL, interleukin; ip, intraperitoneal; mAb, monoclonal antibody; MFI, mean fluorescence intensity; MPO, myeloperoxidase; NF, nuclear factor; PBS, phosphate-buffered saline; TNF, tumor necrosis factor.

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