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CHARACTERIZATION OF ANTICHEMOTACTIC FACTOR EXTRACTED FROM THE GASTRIC MUCOSA OF RATS

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The gastric mucosa of normal rats exhibits no detectable inflammation or visible damage. We examined the effect of the gastric mucosal extract of rats on neutrophil chemotaxis and tried to purify antichemotactic factor. The chemotaxis of neutrophils was examined by the modified Boyden's method. After mucosal layer was scraped and then homogenized and centrifuged at 20,000 × g for 30 min, the supernatant was used as rat gastric mucosal extract (RGME). Prior exposure of neutrophils to the gastric mucosal extract caused a dose-dependent reduction in the neutrophil migration induced by formyl-methionyl-leucyl-phenylalanine (FMLP), leukotriene B_4 (LTB_4) and interleukin 8 (IL-8) without affecting the cell viability. The antichemotactic factor was partially purified by lectin affinity chromatography on wheat germ lectin (WGL)-Sepharose, anion exchange chromatography on Mono-Q and gel filtration on Superose 12. The molecular weight of the antichemotactic factor was estimated to be around 60 k by gel filtration. The activity was markedly abolished by boiling for 5 min, heating at 60°C for 30 min, and treatment with 1% acetic acid, 0.1 M Na_2CO_3 or trypsin. Furthermore, the FMLP-induced migration of neutrophils pretreated with the antichemotactic factor for 5 min followed by washing with fresh medium was inhibited, although the factor was not added to the chamber. These results suggest that the gastric mucosa of rats intrinsically generates an antichemotactic factor which might play a crucial role in maintenance of the integrity of the gastric mucosa.

Key words: *gastric mucosal extract, antichemotactic factor, neutrophils, FMLP, LTB_4, IL-8.*

INTRODUCTION

The gastric mucosa is constantly exposed to acid and pepsin, which are potent cytotoxic agents. However, visible mucosal lesions and/or inflammation seldomly occur, if at all, since the stomach has the ability to protect and repair its mucosa. It is generally considered that increases in mucus-bicarbonate

secretion, mucosal blood flow, inherent resistance of gastric mucosal cells and daily renewal of epithelial cells greatly contribute to mucosal protection and antiinflammation (1—6). Furthermore, many studies have revealed that these physiological responses are mediated through several substances, such as prostaglandins, nitric oxide and the calcitonin gene-related peptide (7—9). However, it is possible that other so far unidentified substances exist in the gastric mucosa which protect the mucosa itself or prevent the occurrence of inflammation. In the present study, we searched for novel substances in the gastric mucosa of rats. As a result, we found a new factor which potently inhibits neutrophil chemotaxis induced by FMLP, LTB_4 and IL-8, and characterized it.

MATERIALS AND METHODS

Animals

Male Wistar rats (Oriental Bioservice, Kyoto, Japan), weighing 350—450 g, were used.

Drugs

FMLP (Sigma Chemicals, St. Louis, MO), LTB_4 (provided by Ono Pharmaceutical Co., Osaka, Japan), and IL-8 (R & M Systems, Minneapolis, MN) were all dissolved in MEM. N-Acetyl glucosamine (GlcNAc) (Nacalai Tesque, Kyoto, Japan) was dissolved in 10 mM sodium phosphate buffer (pH 7.2) containing 0.5 M NaCl. MTT (Sigma), trypsin (DIFCO, Detroit, MI), and antipain (Peptide Institute, Osaka, Japan) were dissolved in distilled water.

Preparation of neutrophils

Rat peritoneal neutrophils were purified according to the method of Kudo *et al.* (10) and Watt *et al.* (11). In brief, 15 ml of 3% casein (Nacalai Tesque, Kyoto, Japan) was injected into the peritoneal cavity of a rat. Fifteen hours later, peritoneal exudated cells were collected and suspended in a Krebs-Ringer solution containing 1% glucose. The cells were treated with a hypotonic buffer to lyse erythrocytes and then washed twice with the Krebs-Ringer solution. The cells were then placed on a Percoll density gradient and centrifuged at 1,500 × g for 15 min. Cells at the boundary between specific gravities of 1.04 and 1.08 were collected and washed with MEM containing 1% bovine serum albumin. The purity and viability of the neutrophils thus obtained were both over 95%, as estimated by Diff Quick (International Reagents, Kobe, Japan) staining and the trypan blue dye exclusion test (12), respectively.

Preparation of RGME

Normally fed rats were anesthetized with ether and then perfused systemically *via* the heart with saline (200 ml). Their stomachs were excised, opened along the greater curvature, and washed with saline. The mucosal layer was scraped with a glass slide, and then homogenized with 10 mM sodium phosphate buffer (pH 7.2) containing 1 mM EDTA, 0.1

mM phenylmethylsulfonylfluoride and centrifuged at 20,000 xg for 30 min. The supernatant was used as RGME.

Chemotaxis assay

The chemotaxis assay was performed by the modified Boyden's method (13, 14) using a membrane filter of 5 μm pore size (Neuro Probe Inc., Cabin John, MD). Chemotactic factors were added to the lower compartment of the chemotaxis chamber. Neutrophils were placed in the upper compartment at 1×10^6 cells/well in MEM. The chamber was stood for 60 min at 37°C to allow neutrophil migration. The filter was then removed and the cells were stained with Diff Quick. The numbers of neutrophils which migrated to the lower face of the filter in five randomly chosen fields were determined under a light microscope (×400; Olympus, Tokyo, Japan). Data are expressed as the average number in five fields. In preliminary experiments, we examined the maximal effects of FMLP, LTB_4 and IL-8 on rat neutrophil migration. FMLP, LTB_4 and IL-8 caused chemotaxis of neutrophils in the ranges of 5 nM to 100 nM, 500 pM to 50 nM, and 1 ng/ml to 500 ng/ml, respectively, the maximal responses being observed at 50 nM FMLP, 5 nM LTB_4 and 50 ng/ml IL-8. Therefore, we used the above concentrations of the chemotactic factors in the present study.

WGL-Sepharose 6 MB column chromatography

RGME was applied to a WGL-Sepharose 6 MB column (Pharmacia Biotec, Uppsala, Sweden) preequilibrated with 10 mM sodium phosphate buffer (pH 7.2) containing 0.5 M NaCl. The column was washed with the equilibration buffer and then the absorbed materials were eluted with 0.1 M GlcNAc at the flow rate of 0.2 ml/min. The active fractions were pooled and used as the partially purified factor for determination of biochemical property.

Mono-Q column chromatography

Partially purified factor was subjected to a Mono-Q column (Pharmacia Biotec, Uppsala, Sweden) preequilibrated with 10 mM sodium phosphate buffer. The column was washed with the equilibrated buffer and then eluted by a linear gradients of NaCl (0—0.3 M) at the flow rate of 1.0 ml/min. Fraction size was 0.5 ml/fraction.

Superose 12 gel filtration

An aliquot (0.5 ml) of the Mono-Q active fraction was applied to a column of Superose 12 (Pharmacia Biotec, Uppsala, Sweden) preequilibrated with 10 mM sodium phosphate buffer (pH 7.2) containing 0.2 M NaCl. The antichemotactic factor was eluted with the same buffer at the flow rate of 0.5 ml/min.

Determination of biochemical properties

The partially purified factor was boiled for 5 min or heated at 60°C for 30 min. After preincubation of the partially purified factor with 1% acetic acid or 0.1 M Na_2CO_3 for 10 min, each fraction was neutralized with 0.1 M Na_2CO_3 or 1% acetic acid, respectively. The partially purified factor was treated with trypsin (10 μg/ml) for 30 min at 37°C, and then antipain (10 μg/ml) was added.

Estimation of cell viability

Cell viability was examined by dye exclusion and MTT methods (12, 15). Neutrophils (2×10^6cells/ml) were exposed to RGME at 150 μg/well for 5 min and then further incubated with the vehicle, i.e. FMLP at 50 nM, LTB_4 at 5 nM or IL-8 at 50 ng/ml, for 1 h. In the case of the dye exclusion assay, 50 μl of a trypan blue solution (0.05 % in PBS) was added after the incubation. Three minutes later, the numbers of stained and non-stained cells were determined under a microscope.

$$\text{viability (\%)} = \frac{\text{non-stained cells}}{\text{non-stained cells} + \text{stained cells}}$$

Alternatively, 0.1 ml of an MTT solution (5 mg/ml in distilled water) was added after the incubation. Thirty minutes later, the MTT was extracted with 1.5 ml of isopropanol containing 0.04 N HCl and the color change of the extract was measured at 595 nm.

Protein assay

Protein concentrations were determined by the method of Bradford (16) using a protein assay kit (Bio-Rad, Hercules, CA).

Statistical analysis

Statistical differences were evaluated using the Dunnett's multicomparison test or Student's t-test, a P value of <0.05 being regarded as significant.

RESULTS

Effect of the gastric mucosal extract on neutrophil migration

To determine whether or not RGME contain some chemotactic factors, the effect of RGME alone on neutrophil migration was examined. When the extract (15—150 μg protein) was added to the lower compartment in the absence of any stimulus, neutrophil migration was not induced at all (data not shown).

After mixing neutrophils in the upper compartment, FMLP at 50 nM induced neutrophil migration about 3-fold as compared with the control (*Fig. 1A*). In contrast, RGME significantly inhibited cell migration induced by FMLP in a dose-dependent manner. The extraction buffer alone did not affect neutrophil chemotaxis (data not shown). The inhibition by RGME was $37.0 \pm 9.7\%$ at 15μg protein, $74.4 \pm 8.1\%$ at 75μg protein, and $91.8 \pm 7.4\%$ at 150 μg protein.

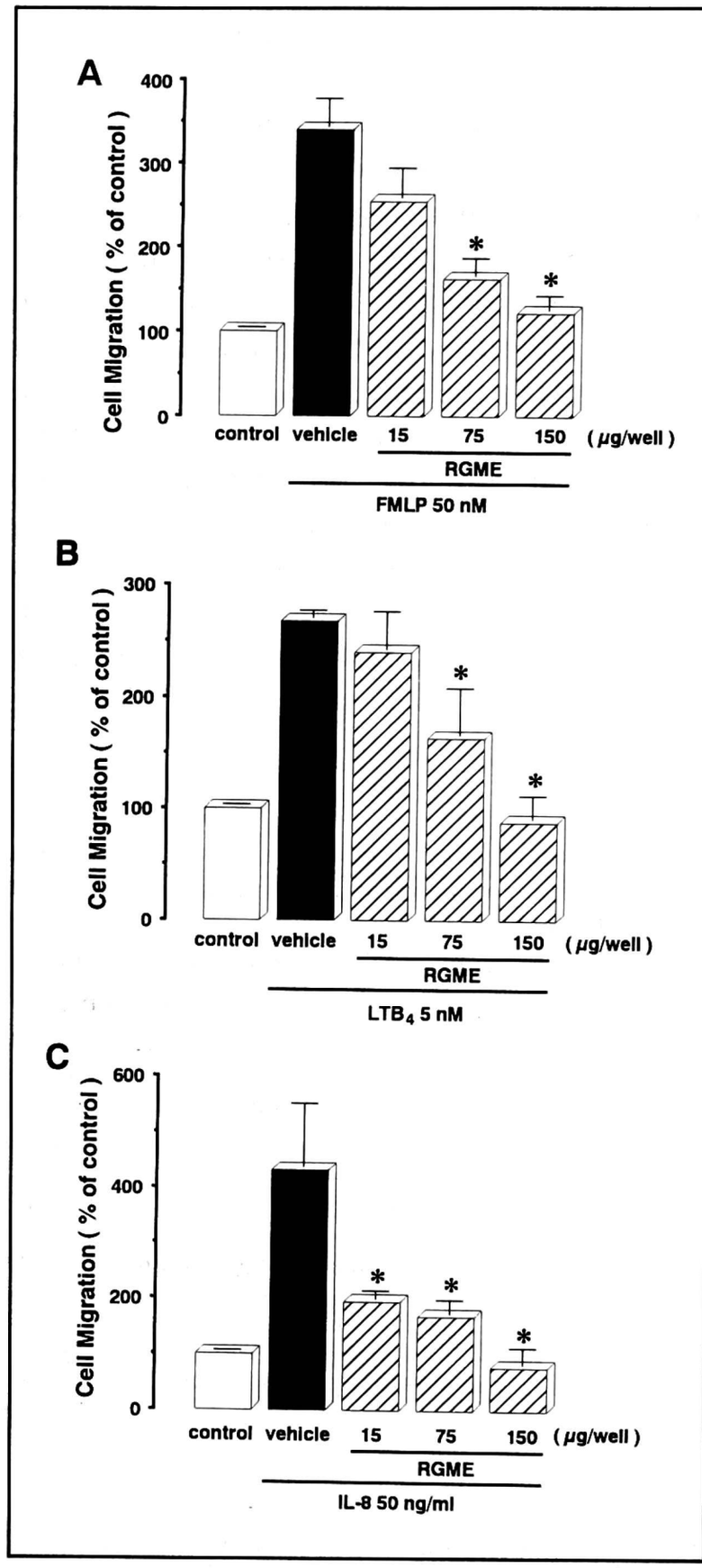

Fig. 1. Inhibitory effect of RGME on FMLP(A), LTB_4(B), IL-8(C)-induced neutrophil chemotaxis. Neutrophils were pretreated with the indicated concentrations of RGME for 5 min. FMLP, LTB_4, IL-8-induced chemotaxis of neutrophils during incubation for 60 min was determined. Data are expressed as percentages of the control in the absence of stimulus, and are means $\pm$ 1 S.E.M. for six experiments. *Significantly different from FMLP or LTB_4 or IL-8-stimulated neutrophil migration in the absence of RGME (vehicle) at $P < 0.05$.

Similar results were obtained in the case of stimulation by LTB_4. RGME dose dependently caused significant inhibition of neutrophil migration induced by 5 nM LTB_4, which stimulated the migration about 2.5-fold as compared with the control (*Fig. 1B*). The inhibition by RGME was

18.5±16.9% at 15 μg protein, 64.7±23.2% at 75 μg protein, and 108.2±13.1% at 150 μg protein.

Furthermore, IL-8 at 50 ng/ml stimulated neutrophil migration about 4-fold as compared with the control. RGME also significantly inhibited IL-8-induced neutrophil migration in a dose-related manner (*Fig. 1C*). The inhibition by RGME was 63.8±9.0% at 15 μg protein, 78.1±6.5% at 75 μg protein, and 108.8±7.4% at 150 μg protein.

The inhibitory activity was even maximally expressed on exposure of neutrophils to the extract even for 5 min. It was confirmed that FMLP-induced neutrophil migration was not affected by the addition of bovine serum albumin, at 3 mg/ml, instead of the extract (109.4±0.9% compared with FMLP-induced migration).

On the other hand, the viability of neutrophils was not affected by treatment with RGME for 60 min despite the absence or presence of FMLP, LTB_4 or IL-8, as determined by the dye exclusion and MTT methods (*Table 1*).

Table 1. Effect of RGME on the viability of neutrophils. Neutrophils were pretreated with RGME (150 μg protein) for 5 min, and then incubated with a vehicle, FMLP (50 nM), LTB_4 (5 nM) or IL-8 (50 ng/ml), for 1 h. Cell viability was assessed by the MTT and dye exclusion methods. Data are expressed as percentages of the control in the absence of RGME, and are means±1 S.E.M. for six experiments.

Treatment	Dye Exclusion Method (% of control)	MTT Method (% of control)
non-stimulated neutrophils	108.8±9.3	99.5±0.3
FMLP-stimulated neutrophils	123.1±8.4	106.1±5.4
LTB_4-stimulated neutrophils	124.8±16.1	112.3±6.4
IL-8-stimulated neutrophils	118.8±6.4	92.9±2.0

Purification of the antichemotactic factor

Since RGME was found to contain the antichemotactic factor, we tried to purify the antichemotactic factor. RGME was loaded to WGL-Sepharose and eluted by 10 mM phosphate buffer containing 0.1 M GlcNAc. The inhibitory activity against FMLP-induced neutrophil migration was low in the pass-through fractions, but the antichemotactic factor, having potent activity, was eluted with 0.1 M GlcNAc. The antichemotactic activity decreased as the

elution proceeded (*Fig. 2*). However, when the active fractions were added to the lower compartment of the chemotaxis chamber without any stimulus, neutrophil migration was not induced at all (data not shown).

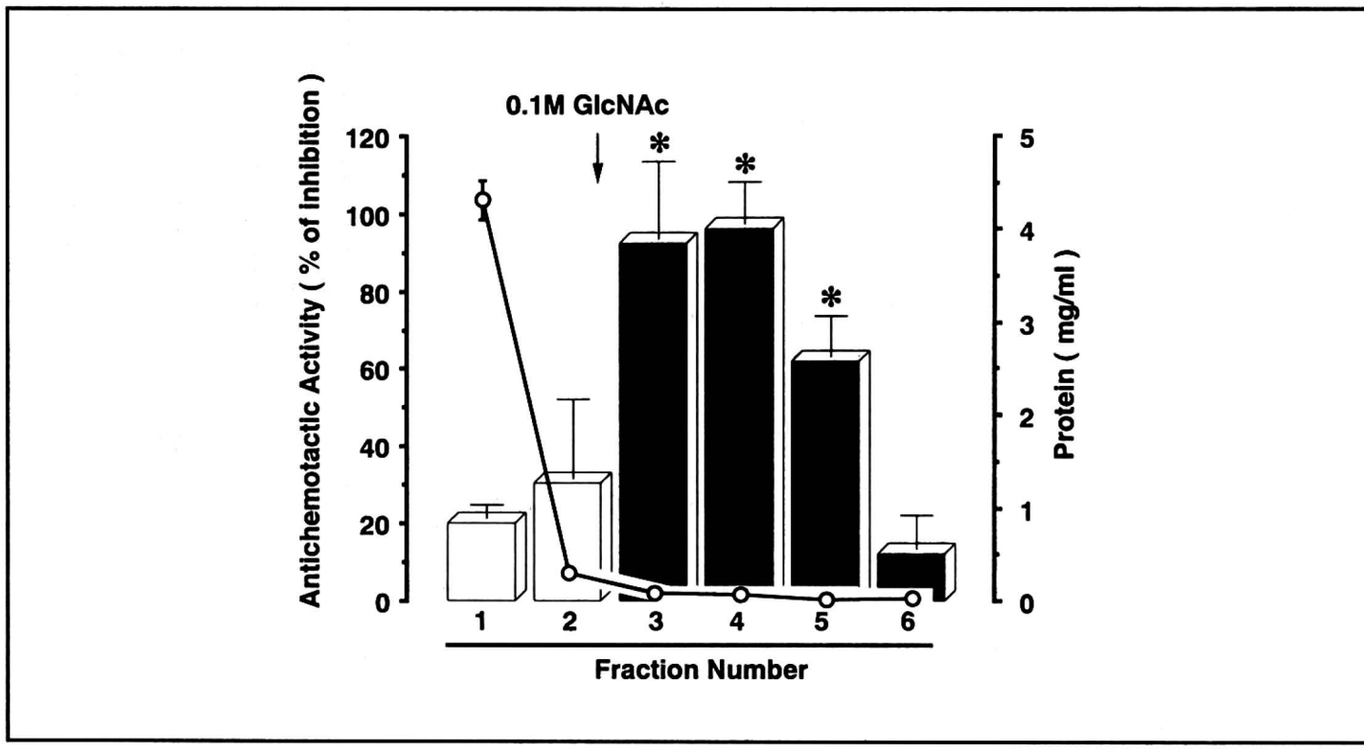

Fig. 2. WGL-Sepharose 6 MB column chromatography of the antichemotactic activity. RGME was applied to a WGL-Sepharose 6 MB column, and the materials bound to the resin were eluted with 0.1 M GlcNAc. Antichemotactic activity in an aliquot (10 μl) of each fraction was determined. Data are expressed as percentages of inhibition of FMLP-stimulated neutrophil chemotaxis, and are means $\pm$ 1 S.E.M. for three experiments. *Significantly different from FMLP-stimulated neutrophil migration in the absence of RGME (vehicle) at $P<0.05$.

The active fractions obtained from WGL-Sepharose column was dialyzed with 10 mM phosphate buffer and applied to a column of Mono-Q and eluted by a linear gradient of 0—0.3 M NaCl. The antichemotactic activity was eluted as a broad peak at an concentration of 0.1 M NaCl (*Fig. 3*).

The molecular mass of the antichemotactic factor was determined by gel filtration (*Fig. 4*). The active fractions from Mono-Q column was pooled and concentrated to 500 μl by ultrafiltration and then fractionated on a column of Superose 12. Two peaks of the absorbance at 280 nm were detected in the Superose 12 column and antimigration activity matched in the former peak. The molecular mass of the factor was estimated to be around 60 k, as calibrated with protein standards of known molecular mass. Thus by these isolation steps, the antichemotactic factor was purified about 600 relative to RGME (*Table 2*).

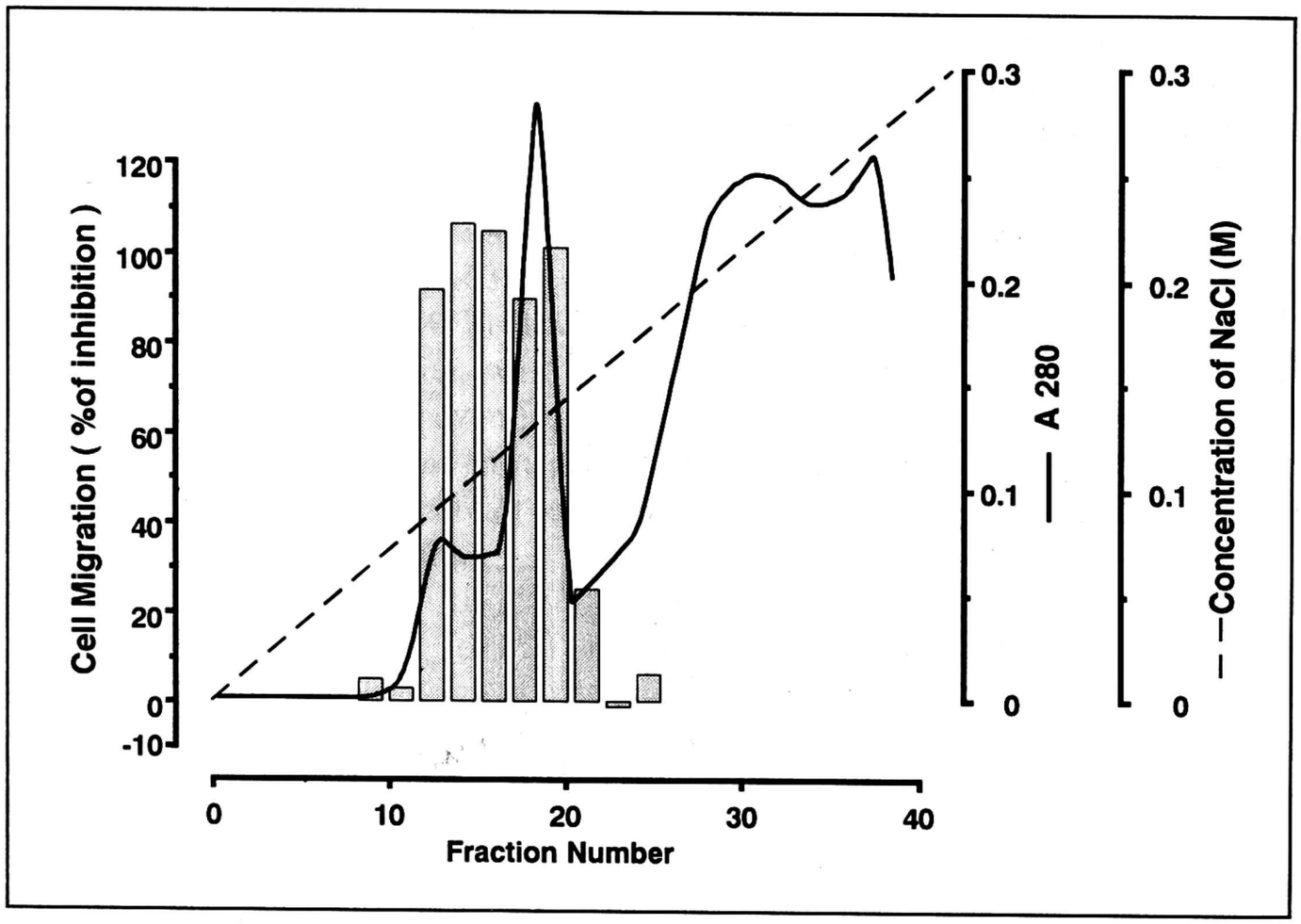

Fig. 3. Mono-Q column chromatography of the antichemotactic activity. Active fraction of antichemotactic factor from WGL-Sepharose was loaded to a Mono-Q column preequilibrated with 10 mM phosphate buffer and eluted by a linear gradient of NaCl (0M—0.3 M in 30 min). Flow rate was 1.0 ml/min and 0.5 ml-fractions were collected. The antichemotactic activity in an aliquot (10 μl) of each fraction was determined. Data are expressed as percentages of inhibition of FMLP-stimulated neutrophil chemotaxis.

Table 2. Purification of antichemotactic factor. One unit activity was defined as half maximal inhibition of FMLP-induced neutrophil migration.

Purification Steps	Protein (mg)	Activity (units)	Specific Activity (units/mg)
RGME	900	337500	0.38
WGL-Sepharose	100	12500	1.25
Mono-Q	0.52	143	276.6
Superose 12	0.05	30	600.0

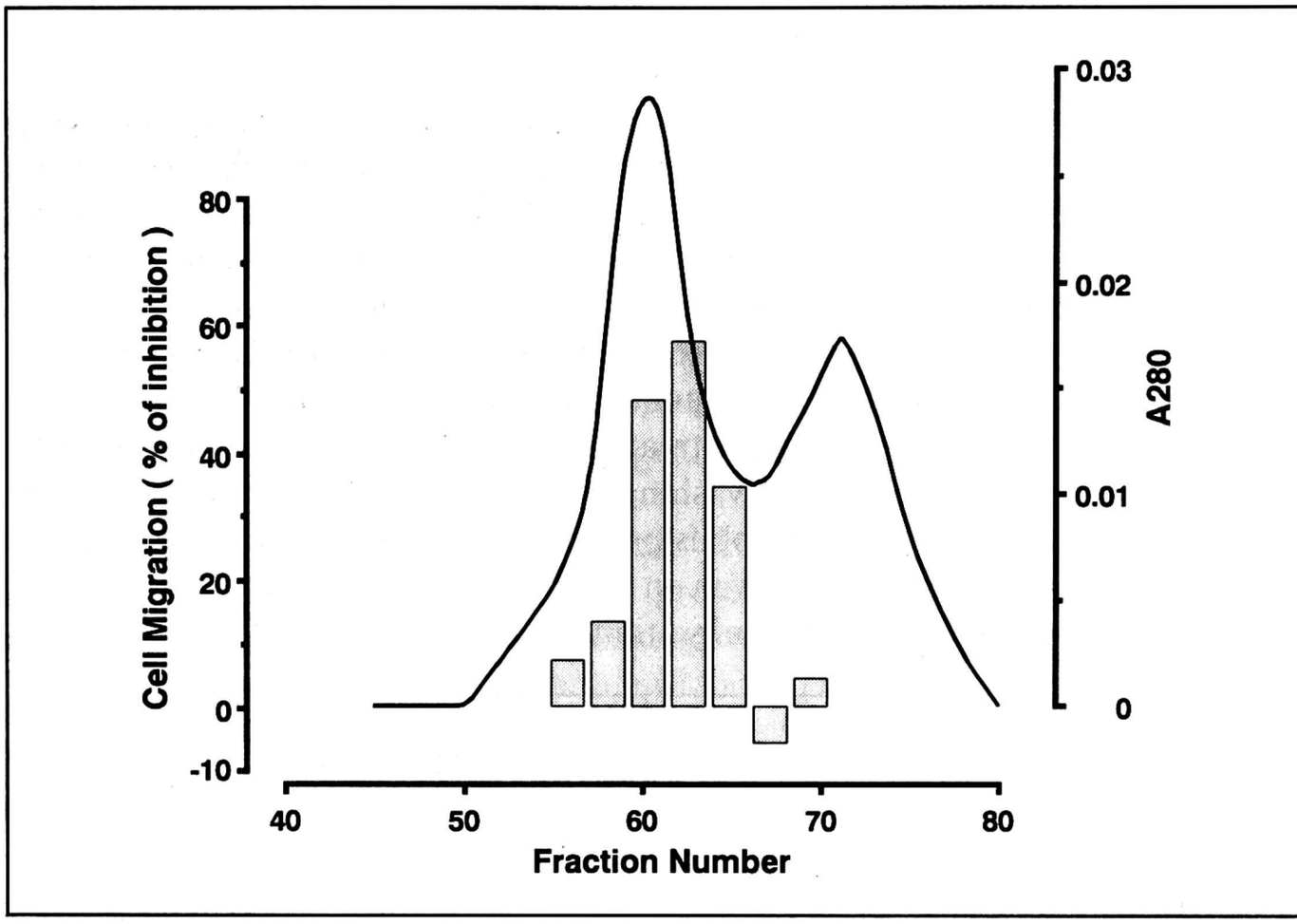

Fig. 4. Superose 12 column chromatography of the antichemotactic activity. Mono-Q active fraction was concentrated and applied to a Superose 12 column and then eluted with 10 mM phosphate buffer (pH 7.2) containing 0.2 M NaCl. Flow rate was 0.5 ml/min and 0.2 ml-fractions were collected. The antichemotactic activity in an aliquot (20 μl) of each fraction was determined. Data are expressed as percentages of inhibition of FMLP-stimulated neutrophil chemotaxis.

Table 3. Effects of various treatments on the antichemotactic activity. The partially purified factor was subjected to boiling for 5 min, heating at 60°C for 30 min and treatment with 1% acetic acid for 10 min, 0.1 M Na_2CO_3 for 10 min or trypsin (10 μm/ml) for 30 min. Thereafter, antichemotactic activity was determined. Data are expressed as percentages of inhibition of FMLP (50 nM)-stimulated neutrophil chemotaxis or percentages of the antichemotactic activity of the non-treated factor, and are means $\pm$ 1 S.E.M. for three eyperiments. *Significantly different from FMLP-stimulated neutrophil migration in the absence of RGME at $P<0.05$.

	Antichemotactic Activity	
Treatment	(% of inhibition)	(% of non-treatment)
none	65.1 $\pm$ 4.9*	100.0
boiling	-8.9 ± 10.8	-11.2
60°C	13.5 $\pm$ 26.9	-16.5
acetic acid	12.9 $\pm$ 15.3	25.0
Na_2CO_3	18.8 $\pm$ 16.5	23.8
trypsin	0.9 $\pm$ 11.4	3.5

Characterization of the antichemotactic factor

Table 3 shows the effects of various treatments on the inhibitory activity of the partially purified factor. The activity almost completely disappeared on boiling for 5 min and on heating at 60°C for 30 min. It was labile on treatment with 1% acetic acid for 10 min and 0.1 M Na_2CO_3 for 10 min. In addition, the activity was abolished on trypsin digestion at 10 μg/ml for 30 min.

After neutrophils had been exposed to the partially purified factor for 5 min and then washed with the fresh medium, the effect of FMLP on their migration was examined (*Fig. 5*). In the vehicle-treated cells, FMLP-stimulated neutrophil migration was not affected by washing as compared with untreated cells. However, the migration of neutrophils pretreated with the factor in response to FMLP was potently inhibited, even in the absence of the factor during the incubation to allow neutrophil migration.

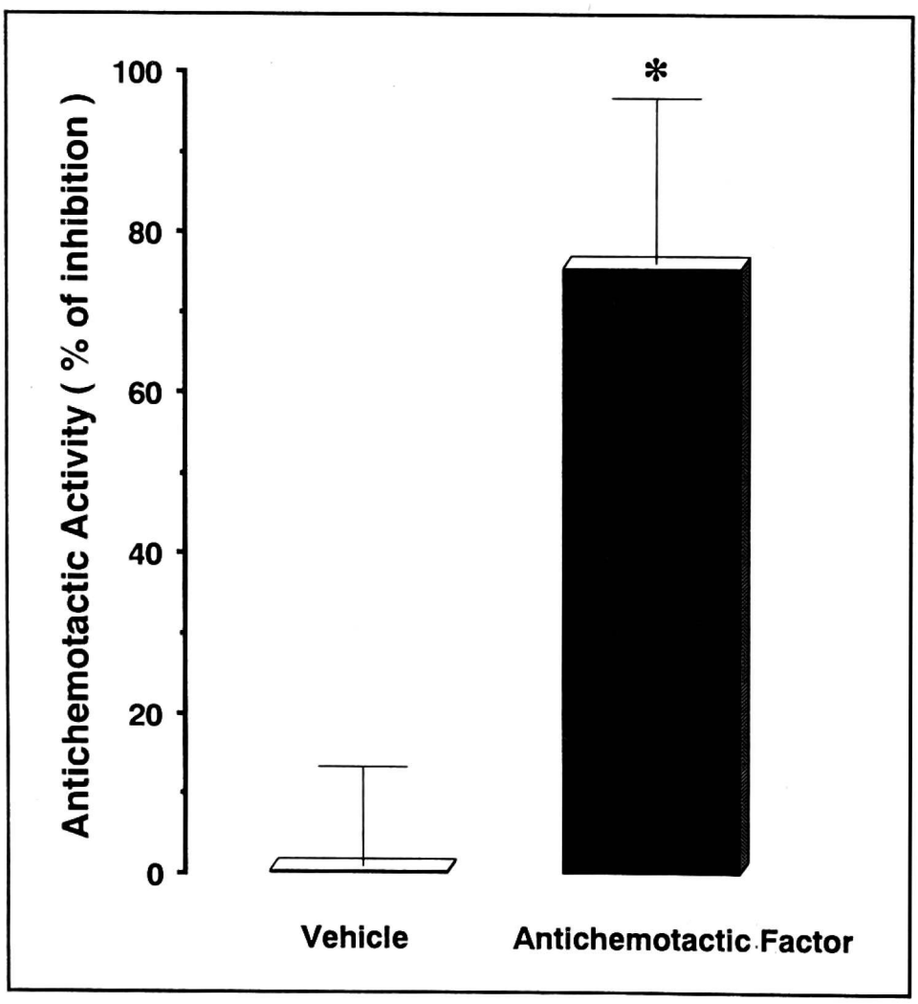

Fig. 5. Neutrophils were pretreated with the antichemotactic factor or vehicle for 5 min, and then washed with fresh medium. FMLP (50 nM)--induced chemotaxis of neutrophils during incubation for 60 min was determined. Data are expressed as percentages of inhibition of FMLP-stimulated neutrophil chemotaxis, and are means ± 1 S.E.M. for three experiments.
*Significantly different from FMLP-stimulated neutrophil migration in the absence of RGME (vehicle) at $P<0.05$.

DISCUSSION

This study clearly indicated that, in the gastric mucosa of rats, there exists a novel antichemotactic factor. The factor rapidly (<5 min) and potently inhibited neutrophil chemotaxis caused by FMLP, LTB_4 or IL-8. It was apparent that the inhibitory effect of the factor is not due to cytotoxicity

toward neutrophils as the cell viability was not affected. In addition, it is unlikely that the extract acts on the chemotactic agents directly, resulting in inhibition of chemotaxis. As a matter of fact, the extract had no effect on the chemotactic properties of FMLP itself, since the inhibition of neutrophil chemotaxis was observed even after washing of neutrophils with fresh medium.

Of interest was that the mucosal extract inhibited the chemotaxis in response to FMLP, LTB_4 or IL-8. It has already been determined that neutrophils have specific receptors for these chemotactic factors (17—19). Therefore, it is possible that the factor blocked all their receptors nonspecifically or the factor have affected the signal transduction in neutrophils activated by the chemotactic factors.

Our determination of biochemical property indicate that the factor is a heat-, acid- and alkali-labile protein. Since the factor binds to WGL-Sepharose resin, the factor is suggested to contain a variable composition and content of carbohydrate residues. This would suggest our factor contains carbohydrates residues. The activity was eluted as one broad peak on gel filtration, suggesting that the factor contains a variable composition and content of carbohydrate residues.

Several proteins have been reported to act directly on neutrophils and to exert antichemotactic activity, i.e., α 1-antitrypsin, α 2-macroglobulin (20), neutrophil immobilizing factors (21, 22), and leukocyte inhibitory factor (23). Judging from the properties and molecular masses of the latter, the factor in the gastric mucosa appears to be different from them.

Weisbart *et al.* (24, 25) reported that neutrophil inhibitory factor (NIF-T), produced by T lymphocytes, inhibits the chemotaxis of human peripheral neutrophils. However, NIF-T is insensitive to heating at 100°C for 5 min and 60°C for 30 min, indicating that our factor is apparently different from NIF-T.

Recently, Sikiric *et al.* (26, 27) purified a novel 40 k-protein with a wide range of organoprotective effects, termed BPC, from gastric juice. However, they did not examine the effect of BPC on neutrophil migration. It is unlikely that our antichemotactic factor found in the gastric mucosa is identical to BPC, since the activity of our factor was easily lost on acid treatment. Therefore, the antichemotactic factor in the gastric mucosa may be a novel protein.

Kozol *et al.* (28) found a neutrophil chemotactic factor in the rabbit gastric tissue. However, we could not detect such a chemotactic activity in RGME. The difference is thought to be mainly due to the preparation of the extract. In their report, the rabbit gastric tissue was incubated at 37°C for at least 1 h and the medium was then collected for assaying chemotactic activity. In our study, however, the mucosal layer was scraped immediately after stomachs had been isolated and then homogenized. It can be considered that a chemotactic factor discovered by the above authors is released from the tissue during the incubation period.

It is known that both prostaglandins E_2 and I_2, important defensive factors in the gastric mucosa, have inhibitory effects on neutrophil functions, including chemotaxis (29, 30). Nitric oxide has also been reported to inhibit the activation of neutrophils (31). It is suggested that activation of neutrophils is related to the pathogenesis of gastric mucosal damage induced by ischemia-reperfusion systems (32). It is likely that our antichemotactic factor plays a crucial role in maintaining gastric mucosal integrity in concert with other factors such as prostaglandins and nitric oxide.

It will be important to study distribution of this factor in order to its physiological role in gastric mucosa. It will also be necessary to purification. Determination of its complete primary structure will establish its molecular identity and relationship with other factors and cytokines.

We conclude that the gastric mucosa in rats intrinsically generates the antichemotactic factor (possibly an about 60 k glycoprotein), which maintains the gastric mucosal integrity.

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