EFFECT OF WATER IMMERSION STRESS ON THE BIOSYNTHESIS OF RAT GASTRIC GLYCOPROTEINS WITH OR WITHOUT SULFATE

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The biosynthesis of rat gastric glycoproteins with or without sulfate was investigated in rats subjected to restraint and water immersion stress. Studies were carried out in vitro in rat glandular stomach using \(^3\)H-glucosamine and \(^35\)S-sulfate. Labeled glycoproteins were extracted with 2% Triton X-100 and fractionated on Bio Gel A-1.5 m. Radioactivity incorporated into glycoproteins was estimated in the tissue as well as in the medium. The incorporation of \(^3\)H-glucosamine into the tissue was unchanged during the experimental period, while the release of \(^3\)H-labeled glycoproteins into the medium was markedly increased at 12 h after the onset of stress. The incorporation of \(^35\)S-sulfate into the tissue was decreased at 6 h and increased at 12 h. The release of \(^35\)S-labeled glycoproteins into the medium was not changed significantly. However, the change in the total radioactivity (tissue plus medium) of \(^3\)H was similar to that of \(^35\)S. These results suggest that the remarkable increase in the biosynthesis of glycoproteins and sulfated glycoproteins was closely related to reinforcement of defensive response.

Furthermore, we investigated the effect of anti-ulcer agents on the biosynthesis of mucus glycoproteins. Cimetidine and atropine decreased the incorporation of radioactive precursors and the release of labeled glycoproteins into the incubation medium in vitro. AAHA (N-(N-acetyl-\(\beta\)-alaninyl)-L-histidine aluminum complex) and sofalcone (SU-88; 2-carboxymethoxy-4,4'-bis (3-methyl-2-butenyloxy) chalcone) increased the incorporation of radioactive precursors and the release of labeled glycoproteins into the medium. These observations indicate that anti-ulcer agents having different modes of action show different effects on the glycoprotein biosynthesis.

Keywords — glycoprotein; sulfated glycoprotein; stress-induced lesion; gastric ulcer; cimetidine; atropine; N-(N-acetyl-\(\beta\)-alaninyl)-L-histidine aluminum complex; sofalcone

INTRODUCTION

Gastric mucus glycoproteins are known to play an important role in protecting the gastric epithelium from peptic digestion.\(^1\)–\(^4\) Macromolecular glycoproteins, especially sulfated glycoproteins, have an apparent peptic inhibitory activity. The inhibition is due to the binding of sulfated glycoproteins to the protein substrate for pepsin.\(^5\) There are many investigations on the correlation between the change in gastric mucosal glycoproteins and gastric damage.\(^6\)–\(^11\)

These studies indicate that an alteration in the metabolism of mucus glycoproteins may be a factor in causing gastric mucosal damage. Our previous investigation\(^12\) have revealed that the synthetic activity of gastric sulfated glycoproteins does not decrease with stress load, but rather increases at 2 and 12 h after the onset of stress in the in vitro system. This suggests that sulfated glycoproteins play an important role in the restoration of the gastric mucosa damaged by stress.

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From this point of view, the present paper reports the effect of restraint and water immersion stress on the biosynthesis of rat gastric glycoproteins with or without sulfate in vitro in further detail.

MATERIALS AND METHODS

Induction of Gastric Lesions by Restraint and Water Immersion Stress — Male Donryu rats (230—280 g) were deprived of food but allowed access to water for 24 h prior to the experiments. The animals were placed in a stress cage and immersed in a water bath (22 ± 0.5°C). Stress began at 10 AM in all experiments. The animals were then sacrificed by a blow on the head at the time intervals of 6 and 12 h after the onset of stress. The stomach of each animal was excised immediately and cut along the greater curvature. The stomach content was washed cut with a cold saline solution. Gastric mucosal damage was examined according to the following arbitrary scale: 0; no damage; 1; pin point round erosions; 2; 1—5 small erosions (<2 mm); 3; >5 small erosions or 1—5 large erosions (>2 mm); 4; >5 large erosions. After the mucosal damages were observed, the forestomach was removed and the glandular stomach was cut into two pieces. They were used for the incubation experiment.

Atropine sulfate monohydrate (Wako Pure Chemical; 15 mg/kg/0.5 ml of saline) was given subcutaneously 30 min before stress load. Cimetidine (Smith, Kline and French; 200 mg/kg/0.5 ml of 0.5% carboxymethyl cellulose (CMC)), AAHA (N-(N-acetyl-β-alanyl)-L-histidine aluminum complex; Nippon Chemipher Co. Ltd.; 1 000 mg/kg/0.5 ml of distilled water) and solfalcon (SU-88; 2-carboxymethoxy-4,4′-bis (3-methyl-2-butenyloxy) chalcone; Taisho Pharmaceutical Co. Ltd.; 500 mg/kg/0.5 ml of 0.4% CMC) were given orally 30 min before the stress load.

Incubation of Gastric Tissue with Labeled Precursors — The glandular stomach was preincubated for 30 min at 37°C under 95% O2/5% CO2 in 20 ml of Krebs-Ringer bicarbonate buffer containing antibiotics (Penicillin G; Meiji Seika 65 U/ml, Streptomycin; Kaken Chemical 65 U/ml) pH 7.4, and then incubated for another 6 h at 37°C in the presence of 50 μCi of D-[6-3H] glucosamine hydrochloride (New England Nuclear 31.3 Ci/mmol) and H2 35SO4 (carrier free; Radioisotope Association) and/or 14C-serine (Amersham 165 mCi/mmol). After the incubation was terminated, the medium was reparted from the tissue by centrifugation at 1 000 × g for 10 min at 4°C.

Extraction of Rat Gastric Glycoproteins — The extraction of glycoproteins was carried out by some modification of the method of Azuumi et al.10 The labeled tissue was placed in a boiling water bath for 3 min to inactivate the enzymes. The labeled tissue was then homogenized in 2 ml of 50 mM Tris-HCl buffer containing 2% Triton X-100 pH 7.2 using a motor-driven glass homogenizer. The resulting homogenate was centrifuged at 10 000 × g for 30 min at 4°C, and the supernatant was dialyzed against running tap water and then distilled water. The dialysate was applied to a column of Bio Gel A-1.5 m (100—200 mesh, 1.4 × 55 cm; Bio Rad Laboratories) previously equilibrated with 50 mM Tris-HCl buffer containing 2% Triton X-100 pH 7.2 and was eluted with the same buffer. The incubation medium was dialyzed exhaustively against running tap water and then distilled water. The dialysate was concentrated by lyophilization, and applied to a column of Bio Gel A-1.5 m. Fractions of 2.3 ml were collected and monitored for radioactivity and hexose. Hexose was determined by the phenol/H2SO4 method.14 The radioactivity was measured in an Aloka 903 liquid scintillation counter. Statistical analysis was made using the Student’s t-test.

Isolation of Sulfated Glycoproteins by DEAE-Cellulose Column — The macromolecular glycoprotein fraction (peak I) obtained by gel filtration was further applied to diethylaminoethyl (DEAE)-cellulose column (DE-52, 0.5 × 9.5 cm, Whatman) equilibrated with 50 mM Tris-HCl buffer containing 1 mM ethylenediaminetetra acetic acid (EDTA) pH 7.2. The column was
TABLE I. Effect of Cimetidine, Atropine, AAHA and Sofalcone (SU-88) on Water Immersion Stress-Induced Gastric Lesions in Rats

<table>
<thead>
<tr>
<th></th>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>No. of rats</th>
<th>6 h stress lesion index (mean ± S.E.)</th>
<th>Inhibition (%)</th>
<th>No. of rats</th>
<th>12 h stress lesion index (mean ± S.E.)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>p.o., s.c.</td>
<td>6</td>
<td>3.8 ± 0.2</td>
<td></td>
<td>6</td>
<td>3.8 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Cimetidine</td>
<td>200</td>
<td>p.o.</td>
<td>6</td>
<td>1.0 ± 0.3</td>
<td>74</td>
<td>6</td>
<td>1.2 ± 0.3</td>
<td>68</td>
</tr>
<tr>
<td>Atropine</td>
<td>15</td>
<td>s.c.</td>
<td>6</td>
<td>0.33 ± 0.2</td>
<td>91</td>
<td>6</td>
<td>0.83 ± 0.3</td>
<td>78</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>p.o., i.p.</td>
<td>6</td>
<td>3.2 ± 0.4</td>
<td></td>
<td>6</td>
<td>3.8 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>AAHA</td>
<td>1000</td>
<td>p.o.</td>
<td>6</td>
<td>1.8 ± 0.4</td>
<td>44</td>
<td>6</td>
<td>2.2 ± 0.4</td>
<td>41</td>
</tr>
<tr>
<td>Sofalcone</td>
<td>100</td>
<td>i.p.</td>
<td>6</td>
<td>1.5 ± 0.2</td>
<td>53</td>
<td>6</td>
<td>2.5 ± 0.6</td>
<td>32</td>
</tr>
</tbody>
</table>

All drugs were given 30 min before the stress load. Significantly different from control. a) \(p < 0.05\), b) \(p < 0.01\), c) \(p < 0.001\). Gastric mucosal damage was examined according to the following scale. 0; no damage, 1; pin point erosions 2; 1-3 small erosions (< 2 mm), 3; > 5 small erosions or 1-5 large erosions (> 2 mm), 4; > 5 large erosions.

Washed with 30 ml of the same buffer and eluted with a linear gradient of 0-0.4 M NaCl in the same buffer. Fractions of 2.3 ml were collected and assayed for radioactivity and hexose.

**Isolation and Determination of Hexosamines by Ion-Exchange Chromatography** — Each fraction obtained by gel filtration was hydrolyzed in 1.6 ml of 3 N HCl in a sealed tube for 16 h. HCl was removed in a vacuum rotator at 50 °C and dissolved in 0.3 N HCl. Isolation of hexosamines was performed by the method of Gardell. The hydrolysate was applied to Dowex 50-X 8 (H+ form) 200-400 mesh and eluted with 0.3 N HCl. Fractions of 1 ml were collected and assayed for hexosamine and radioactivity. Hexosamine was analyzed by the Elson-Morgan reaction.

**Alkali Degradation** — Alkali degradation was carried out by treatment with 0.3 M NaOH for 24 h at room temperature. The \(^3\)H-glucosamine- and \(^{14}\)C-serine-labeled sample was then neutralized by the addition of HCl and applied to a column of Bio Gel A-1.5 m (1.4×55 cm) equilibrated with 50 mM Tris-HCl buffer pH 7.2. The column was washed with the same buffer. Fractions of 2.3 ml were collected and monitored for radioactivity.

**FIG. 1. Bio Gel A-1.5 m Column Chromatography of \(^3\)H/\(^{35}\)S-Labeled Glycoproteins of Rat Gastric Tissue**

Rat glandular stomach was isolated and incubated with \(^3\)H-glucosamine and \(^{35}\)S-sulfate for 6 h at 37 °C. \(^3\)H/\(^{35}\)S-labeled glycoproteins were extracted with 50 mM Tris-HCl buffer containing 2% Triton X-100 (pH 7.2). The extract was applied to a column of Bio Gel A-1.5 m (1.4×55 cm) and eluted with the same buffer. Fractions of 2.3 ml were collected. Hexose was detected by the phenol/\(\text{H}_2\text{SO}_4\) method and radioactivity counted by a liquid scintillation counter.
RESULTS
Assessment of Gastric Mucosal Damage
Gastric mucosal damage is shown in Table I. Cimetidine markedly inhibited the formation of stress-induced lesions; the inhibition was 68–74% at 200 mg/kg (p.o.) at 6 and 12 h after the onset of stress. Atropine at 15 mg/kg (s.c.) also markedly inhibited it; inhibition was 78–91% at 6 and 12 h. AAHA at 1 000 mg/kg (p.o.) and sofalcone (SU-88) at 100 mg/kg (i.p.) showed a weak inhibition; the inhibition was 32–53%.

Incorporation of Labeled Precursors into Glycoproteins in Rat Gastric Mucosa

1. Incorporation in the Tissue — Figure 1 shows a typical elution profile of glycoproteins from rat gastric tissue on Bio Gel A-1.5 m column. The glycoproteins were divided into three fractions. The fractions were designated as peak I, II and III from the side of the void volume. Figure 2 shows the radioactivity (3H) of macromolecular glycoprotein fraction (peak I) obtained by gel filtration at 6 and 12 h after the onset of stress. In control, the incorporating activity of 3H-glucosamine was unchanged at 6 and 12 h compared with that at 0 h. Cimetidine and atropine decreased the 3H-incorporation at 6 h (atropine; p < 0.05) and 12 h compared with control. AAHA and sofalcone (SU-88) significantly increased the 3H-incorporation at 12 h (AAHA; p < 0.05, SU-88; p < 0.05). No significant change was observed in peak II and peak III.

The incorporation of 35S-sulfate into peak I in the tissue is shown in Fig. 3. In control, 35S-incorporation into macromolecular glycoproteins (peak I) was significantly decreased at 6 h (p < 0.05 or 0.01) and significantly increased at

FIG. 2. Effects of Stress and Anti-ulcer Agents on the Incorporation of 3H-Glucosamine into the Macromolecular Glycoprotein Fraction (Peak I)
Rats were stressed for 0, 6 or 12 h. Each bar represents mean ± S.E. of 5–12 samples. C: control; CI: cimetidine; AT: atropine; AA;AAHA, SU: sofalcone (SU-88) treated. Significantly different from control at each stress time; a) p < 0.05.

FIG. 3. Effects of Stress and Anti-ulcer Agents on the Incorporation of H35SO4 into the Macromolecular Glycoprotein Fraction (Peak I)
Rats were stressed for 0, 6 or 12 h. Each bar represents mean ± S.E. of 5–12 samples. C: control; CI: cimetidine; AT: atropine; AA; AAHA, SU: sofalcone (SU-88) treated. Significantly different from 0 h-control; a) p < 0.05, b) p < 0.01, Significantly different from control at each stress time; c) p < 0.05, d) p < 0.01.
12 h (p < 0.01) compared with 0 h. Cimetidine decreased $^{35}$S-incorporation at 12 h compared with control. Atropine decreased $^{35}$S-incorporation at both 6 h (p < 0.01) and 12 h (p < 0.05). AAHA and sofalcone (SU-88) had no effect at 6 h, but significantly increased $^{35}$S-incorporation at 12 h (AAHA; p < 0.01, SU-88; p < 0.05). No significant change was observed in peak II and peak III.

2. Release of Labeled Glycoproteins into the In-

FIG. 4. Bio Gel A-1.5 m Elution Profiles of $^3$H/$^{35}$S-Labeled Glycoproteins Released from Rat Gastric Tissue into the Incubation Medium at 0 or 12 h after the Onset of Stress

Fractions of 2.3 ml were collected and radioactivity measured by a liquid scintillation counter. C; control, CI; cimetidine, AT; atropine, AA; AAHA, SU; sofalcone (SU-88) treated.
cubation Medium — Figure 4 shows the elution profiles of the incubation medium. ³H-Labeled glycoproteins in peak I, II and III were all markedly increased at 12 h after the onset of stress. The pretreatment with AAHA or sofalcone (SU-88) caused a further increase of ³H-labeled glycoproteins. Figures 5—7 show the release of ³H-labeled glycoproteins into the medium. ³H-labeled macromolecular glycoproteins (peak I) in the medium were significantly increased at 12 h (4—5 fold, p < 0.01 or 0.001) compared with those at 0 h in control. Both cimetidine and atropine had little effect at 6 h, but significantly decreased the ³H-incorporation at 12 h (p < 0.05). Both AAHA and sofalcone (SU-88) had little effect on the ³H-incorporation.

In peak II, ³H-labeled glycoproteins showed a marked increase at 12 h (12—16 fold, p < 0.01 or 0.001) compared with that at 0 h in control. AAHA and sofalcone (SU-88) increased ³H-labeled glycoproteins at 12 h, but not significantly. Cimetidine and atropine caused a significant decrease in ³H-labeled glycoproteins at 12 h (cimetidine; p < 0.01, atropine; p < 0.001).

In peak III, the increase of ³H-labeled glycoproteins at 12 h was less marked than that in peak I and II. Atropine significantly decreased ³H-labeled glycoproteins at 12 h (p < 0.05). AAHA and sofalcone (SU-88) had little effect on the ³H-incorporation.

FIG. 5. Effects of Stress and Anti-ulcer Agents on the Release of ³H-Labeled Glycoproteins into the Incubation Medium in the Macromolecular Glycoprotein Fraction (Peak I)

Rats were stressed for 0, 6 or 12 h. Each bar represents mean ± S.E. of 5—12 samples. C; control, Cl; cimetidine, AT; atropine, AA; AAHA, SU; sofalcone (SU-88) treated. Significantly different from 0 h-control; a) p < 0.01, b) p < 0.001. Significantly different from control at each stress time; c) p < 0.05.

FIG. 6. Effects of Stress and Anti-ulcer Agents on the Release of ³H-Labeled Glycoproteins into the Incubation Medium in Peak II

Rats were stressed for 0, 6 or 12 h. Each bar represents mean ± S.E. of 5—12 samples. C; control, Cl; cimetidine, AT; atropine, AA; AAHA, SU; sofalcone (SU-88) treated. Significantly different from 0 h-control; a) p < 0.01, b) p < 0.001. Significantly different from control at each stress time; c) p < 0.01, d) p < 0.001.
No significant change was observed in the release of $^{35}$S-labeled glycoprotein into the medium.

**Hexose Content in the Gastric Tissue and the Incubation Medium**

Figure 8 shows the hexose content in the macromolecular glycoprotein fraction (peak I) in the tissue and the medium. The hexose content in the tissue at 6 h was significantly higher than that at 0 h ($p < 0.05$), and was of the same level at 12 h as that at 0 h. Cimetidine and atropine caused a significant decrease in the tissue and a significant increase in the medium at 6 h ($p < 0.01$) compared with that in control. At 12 h, cimetidine and atropine caused a slight decrease in the tissue (not significantly). AHA and sofacline (SU-88) had a little effect on the hexose content.

**Specific Radioactivity in Each Fraction Obtained by Gel Filtration**

The specific radioactivity in peak I, II and III was $1.33 \times 10^{-3}$, $3.95 \times 10^{-3}$ and $0.99 \times 10^{-3}$ dpm/µg hexosamine, respectively in stress-loaded rat at 12 h after the onset of stress. Peak I and III had the same specific radioactivity. In contrast, the specific radioactivity in peak II was 3-fold higher than that of peak I.

**DISCUSSION**

It has been reported that the biosynthesis of gastric sulfated mucosubstance in vivo was markedly reduced in rats subjected to restraint stress. However, we recently reported that the incorporating activity of $^{35}$S-sulfate into gastric macromolecular sulfated glycoproteins in vitro showed a biphasic pattern in restraint and water immersed rats, and that when a sufficient amount of $^{35}$S-sulfate was supplied, it was maintained over the normal level at 12 h after the onset of stress. These changes in the biosynthetic activity of sulfated glycoproteins were considered to be a nonspecific response of the gastric cells to a variety of adverse conditions.

The biosynthesis of rat gastric glycoproteins with or without sulfate was further investigated in rats subjected to restraint and water immersion stress. Rat gastric glycoproteins were extracted with 2% Triton X-100 and fractionated on the Bio Gel A-1.5 m column as previously reported by Azumi et al. The glycoproteins extracted were divided into three fractions on the column, and peak I corresponded to the mucus macromolecular glycoproteins. The incorporating patterns of $^{35}$S-sulfate and $^{3}$H-glucosamine into gastric glycoprotein were different from each other. $^{3}$H-labeled glycoproteins were unchanged in the tissue, while they were markedly increased in the medium at 12 h. $^{35}$S-
Labeled sulfated glycoproteins showed a significant change in the tissue as previously reported, while they remained unchanged in the medium. The total radioactivity (tissue plus medium) showed a similar change in glycoproteins and sulfated glycoproteins, that is, it was markedly increased at 12 h after the onset of stress. The higher proportion of sulfated glycoproteins in the tissue may be ascribed to the difference in the distribution of glycoproteins and sulfated glycoproteins in gastric mucosa. Neutral mucin was seen in the surface and foveolar epithelium of cardia and corpus. In contrast, sulfomucin existed in the deep foveolar cells. Another possible reason for the higher proportion of sulfated glycoproteins in the tissue is that sulfated glycoproteins strongly bind to the substrate protein, making the substrate resistant to peptic activity.

The increase in the release of $^3$H-labeled glycoproteins was most remarkable in peak II. Further characterization of peak II was made. The molecular weight of peak II was about 70,000. Peak II was converted to the total volume by $\beta$-elimination with alkali, indicating the presence of O-glycosidic linkages. O-Glycosidic linkages were found in human gastric glycoprotein. When applied to the DEAE-cellulose column, a considerable part of peak II was absorbed in the charged fraction as well as peak I. The specific radioactivity of hexosamine was 3-fold higher than that of peak I, suggesting that peak II has a

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FIG. 8. Effects of Stress and Anti-ulcer Agents on the Hexose Content of Tissue and the Incubation Medium in the Macromolecular Glycoprotein Fraction (Peak I)

Rats were stressed for 0, 6 or 12 h. Each bar represents mean ± S.E. of 5–12 samples. C; control, CI; cimetidine, AT; atropine, AA; AAHA, SU; sofalcone (SU-88) treated. Significantly different from 0 h-control; a) $p < 0.05$. Significantly different from control at each stress time; b) $p < 0.05$, c) $p < 0.01$. 

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high rate of turnover and is not the degrading product of peak I. However, it is not clear from these results whether peak II is a precursor of peak I or is a distinct glycoprotein from peak I.

Sakaguchi et al. reported that a histological improvement was observed at 42–60 h in spite of the continuous water immersion stress load, and that acidic mucosubstances were already increased at 15–28 h before the tissue repair was observed. Though severe hemorrhagic erosions were visible at 12 h in our experiment, the biosynthesis of glycoproteins with or without sulfate was markedly increased. This result indicates that the gastric mucosa was directed toward the healing process at 12 h after the onset of stress, and that the marked increase in the biosynthesis of glycoproteins and sulfated glycoproteins was closely related to the wound healing.

We investigated the effect of some anti-ulcer agents on the biosynthesis of glycoproteins with or without sulfate in stress-loaded rat stomach. It is well known that atropine exhibits an anti-ulcer activity mainly by inhibiting gastric motility and gastric secretion, and that cimetidine is a strong inhibitor of gastric secretion. In this experiment, cimetidine and atropine showed a marked prevention in stress-induced lesions. And the increased incorporation of H-glucosamine into glycoproteins in stress-loaded rats was decreased to the normal level by the administration of cimetidine and atropine. Consequently, the effect of these agents on the glycoprotein biosynthesis seems to be the result of the prevention of stress-induced lesions. However, there is a possibility that the inhibitory effect on glycoprotein biosynthesis may be partially due to the direct effect of these drugs, since atropine has an inhibitory effect on the mucus secretion from mucous neck cells mediated by the cholinergic factor, and cimetidine has been reported to weaken the mucosal defensive factor. On the other hand, AAHA and sofalcone (SU-88) have been shown to strengthen the gastric defensive factor and have little effect on acid output. The stimulatory effect of AAHA and sofalcone (SU-88) on the glycoprotein and sulfated glycoprotein biosynthesis observed here would be interpreted as the reinforcement of defensive response against the adverse condition.

Since the gel layer adhering to gastric epithelium should be more protective than the soluble mucin, the adhering capacity of glycoproteins to gastric epithelium is important in protecting gastric mucosa. Then, we estimated the adhering capacity of gastric glycoproteins to surface epithelium by comparing the content of macromolecular glycoproteins in the tissue to that in the medium after 6 h incubation. The incorporation of H-glucosamine and S-sulfate observed in our experimental system does not represent the dynamics of the pre-existing glycoproteins, but represents the biosynthetic activity of glycoproteins. In the present study, the adhering capacity of macromolecular glycoprotein was significantly increased at 6 h after the onset of stress. However, cimetidine and atropine significantly decreased the adhering capacity at 6 h, although these drugs markedly prevented stress-induced lesions. The decrease in the adhering capacity may result in the temporary withdrawal of the defensive force. This observation is of interest in the light of the recurrence and relapse observed after cimetidine treatment is stopped.

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