FLUORESCENCE HISTOCHEMICAL FINDINGS OF THE STOMACH WALLS IN RESPONSE TO ULCEROGENIC STIMULI IN RATS

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Depending upon the origin of the stimuli which signal the secretion of gastric juice, three distinct aspects of secretion have been elucidated: the cephalic, gastric and intestinal. The efferent cholinergic mechanism is involved in the cephalic phase of gastric secretion, because cutting of the vagi just above the stomach completely eliminates the secretion. On the other hand, participation of gastrin to the gastric phase of secretion and its chemical structure have recently been demonstrated (1). However, the gastric secretion mechanism is more complicated. It is possible that the intact cholinergic innervation (2) and the presence of endogenous monoamines in the gastric wall (3) are the prerequisite to the gastrin-induced gastric secretion.

There are controversial reports regarding the effect of adrenergic stimuli on the gastric secretion (4–7). In the mucous membrane of the glandular portion of the rat stomach, dopa-decarboxylase activity is high (8), but the high activity is not explained by the number of mast cells (9) and adrenergic nerves (10, 11) in the gastric mucosa. The distribution of the enterochromaffin cells is also different from that of gastric dopa decarboxylase (8). Intraperitoneal injection of dopa or 5-hydroxytryptophan (5-HTP) to rats causes a marked increase in the concentration of dopamine and 5-hydroxytryptamine (5-HT) in the gastric mucosa, and the amines are located to an enterochromaffin-like cell system in the pyloric part of the gastric mucosa (8). However, the physiological significance of the uptake and decarboxylation of dopa or 5-HT remains to be settled.

In the present experiments the distribution of the endogenous monoamines in the structures of the gastric wall of the rat was studied using a formaldehyde fluorescence technique (12, 13). The temporal changes of the monoamine fluorescence were also followed in the course of formation of the gastric ulcers in the Shay rat and the restraint, cold-exposed rat.

METHODS

Male and female Wistar rats weighing 150 to 200 g were used. They were housed in an individual cage at the room temperature of 22±1˚C at least for one week prior to
experiment and were allowed to free access to the commercial laboratory diet, CLEA CA-1, and drinking water *ad libitum*.

1. Ulcerogenic stimuli
   a) Shay rat

   Pylorus-ligated rats were prepared according to the method of Shay *et al.* (14, 15). The animals were fasted but allowed to take drinking water 24 or 48 hours before surgery. Under ether anesthesia the abdominal cavity was opened and the pyloric end of the stomach was ligated tightly with silk thread. At various time intervals after surgery the animals were killed by head amputation and the whole stomach was removed immediately. Each group consisted of 3 to 5 rats.

   b) Cold-exposed rat

   Stress stimuli were given to rats according to the modification of the method of Brodie and Hanson (16). The fasted animals were lightly anesthetized with ether inhalation and fixed on the wood plate in a supine position. Then the animals were soaked vertically to the upper abdomen in the cold water bath (18±0.5°C). The room temperature was 22±1°C. Ten groups of 3 animals each were exposed to the cold for various time-length. After killing by head amputation the whole stomach was removed.

2. Macroscopic observation of the gastric mucosa

   The gastric cavity was opened by cutting the wall with a scissor along the greater curvature from the cardia to the pylorus. After removing the gastric juice and mucinous secretion, the gastric wall was extended with the inner surface uppermost on a glass plate. The number and size of ulcers in the rumen, body and antrum were checked. Other macroscopic abnormalities of the mucous membrane, particularly in the vicinity of ulceration, were also examined.

3. Fluorescence histochemical demonstration of tissue monoamines

   The stomach isolated from normal and treated rats was divided into the three portions: rumen, body and antrum. Each portion was cut into pieces of about 1.0×1.0 cm. The specimens were frozen in isopentane cooled by liquid nitrogen. For the demonstration of biogenic monoamines according to the method of Falck and Hillarp (12, 13), the specimens were freeze-dried, treated with formaldehyde gas at 80°C for 1 hour, embedded in paraaffin *in vacuo* at 60°C, serially sectioned (6–8 μ) and treated further for fluorescence microscopy (for details of the method see Fujiwara *et al.* (17). The fluorescence was observed and photographed on the Kodak Tri-X film by use of a fluorescence microscope (Zeiss). The exciting light provided by an Osram HBO 200 high pressure mercury lamp was filtered through Schott BG 12 and Zeiss 50 as the primary and secondary filters.

RESULTS

1. Monoamine fluorescent structures of the stomach wall

   The normal stomach of the rat is divided into two portions by a prominent white, somewhat curved, transverse ridge, as described by Berg (18) and Shay *et al.* (14).
upper two-fifths is the rumen and the lower three-fifths is the glandular portion. The glandular portion is composed of two clearly defined areas, the antrum and the body.

Three kinds of specifically fluorescent structures were observed in the stomach wall of the rat. They were as follows: 1) the adrenergic nerve fibers showing the green or yellowish green fluorescence of catecholamines; 2) the mast cells showing the yellow fluorescence of 5-HT in the cytoplasm; and 3) the glandular epithelial cells which also show the yellow fluorescence of 5-HT in the cytoplasm. The distribution of these fluorescent structures differed considerably according to the anatomical division of the stomach.

1. Catecholamine fluorescence

The adrenergic nerve fibers with yellow-green fluorescence showed a considerably uniform distribution in all divisions of the stomach. The specific fluorescent fibers with varicosity were distributed in the following three structures: the blood vessels, especially small arteries and arterioles, the smooth muscles and the myenteric nerve plexus. The specific fluorescent fibers of the arteries and arterioles were present mainly in the external layer. The distribution of vascular fluorescence was considerably abundant in the submucosal layer. The blood vessels crossing or invading the circular and longitudinal muscle layers, the lamina muscularis mucosa and the lamina propria of the mucous membrane also developed the specific green fluorescence. The fluorescent blood vessels in the lamina propria were detected only at the basal layer.

The fluorescent adrenergic nerve fibers with varicose structures were also seen abundantly along or within the circular and longitudinal muscle layers, independent of the blood vessels. But there were not many fluorescent fibers in the lamina muscularis mucosa.

The Auerbach's myenteric plexus located between the circular and longitudinal muscles did not show the cytoplasmic fluorescence. But the cell bodies were surrounded by a dense net of the specific fluorescent fibers with varicose structures. No

![Fig. 1. Fluorescence photomicrograph of transversally sectioned stomach wall of the rumen. x50. Normal. The green-fluorescent adrenergic fibers are seen in the muscle layer proper and in the vicinity of arterioles in the submucosa. The yellow-fluorescent mast cells are present in the submucosal layer and intermuscular tissues.](image-url)
submucous ganglion cell surrounded by the fluorescent fibers was found. The stratified squamous epithelium of the rumen and the glandular epithelia of the body and antrum also did not show the sign of adrenergic innervation. Figs. 1, 2a and 3a illustrate the catecholamine fluorescence in these portions of normal stomach of rat.

**Fig. 2.** Transversally sectioned stomach wall of the body.

- a) Control. ×50. The green-fluorescent adrenergic fibers are seen around the arterioles present at the base of the mucosa and in the submucosa, and they are also seen in the muscle layer proper. The yellow-fluorescent mast cells are located in the submucosal layer and intermuscular tissues.
- b) 6 hours after cold-exposure. ×50. The yellow-fluorescent mast cells and small-sized fluorescent materials accumulate to the submucosa, and later disappear from the muscular layer.
- c) 8 hours later. ×128. The yellow-fluorescent materials accumulate to the mucosal surface.
- d) 24 hours later. ×50. The fluorescent mast cells reappear in the muscular layer.
2. Mast cells with 5-HT fluorescence

A considerable number of yellowish fluorescent cells was present in the serosal, muscular and submucosal layers of the rumen and body (Figs. 1 and 2a). These cells were identified as mast cell by metachromasia of the cytoplasmic granules with toluidine blue. There was no fluorescent mast cell in the mucous membrane, or only a few were present in the lamina propria mucosa. The fluorescent mast cells were absent from all layers of the antrum (Fig. 3a). The mast cells, usually round or oval and 10 to 15 μ in diameter, were distributed near or along the blood vessels. The contour of mast cells present in the serosal and muscular layers was uniform, but some of submucosal mast cells, especially immediately beneath the lamina muscularis mucosa were variously shaped. In addition to such mast cells, much smaller-sized, specific fluorescent materials were also present in the
upper layer of the mucous membrane in the body. As described below, it was probable that these materials represent the cytoplasmic granules released from the broken mast cells.

3. Yellow fluorescence of the glandular epithelium

Many of glandular epithelia in the antrum exhibited dense or light yellow fluorescence of 5-HT (Fig. 3a). The fluorescence faded considerably after a long exposure of tissue specimen to the ultraviolet light. The fluorescence was abundant in the middle layer of the mucous membrane in the antrum. In addition, a spindle-like light yellow fluorescence was found occasionally in the lamina propria. The stratified squamous epithelium of the rumen did not show any specific fluorescence (Fig. 1). In the body, few if any glandular cells in the basal layer developed the specific yellow fluorescence.

II. Macrosopic observation of the gastric ulceration
1. Shay rat
As shown in Table 1, the gastric ulcers in the Shay rat were restricted to the rumen. Four to six hours after the pyloric ligation was placed, a small number of mucous erosions was disseminated diffusely in the rumen. Then the dimension of erosion increased progressively to the formation of definite ulceration with the defect of the gastric mucosa 12 hours later. Twenty-four hours after ligation, the ulcerative lesions increased in dimension and sometimes actual perforation to the serosal layer occurred. However, the mucous membrane surrounding the ulcerative sites usually did not show the macroscopic sign of acute inflammation.

2. Cold-exposed rat

The gastric ulceration due to a cold stress was produced mainly in the body, but after a long exposure to the stress the lesion extended to the antrum and rumen (Table 2). After the exposure of the restraint animals to the cold water for 4 to 5 hours, many various-shaped, different-sized erosions were disseminated on the mucous membrane of the body. The surface of the erosion which was surrounded by an extremely hyperemic reaction was covered with blood-clot. A further exposure to the stress caused the erosion to expand along the longitudinal folds of the mucous membrane. Eight to twelve hours after the exposure, the hyperemia and petechia expanded diffusely to the whole surface of the rumen, body and antrum, but there was no definite ulceration with the defect of mucous membrane. The
definite ulcers, covered with blood-clot and surrounded by hyperemic and edematous reaction, appeared in the body 18 to 24 hours later.

TABLE 1. The incidence of gastric ulcers after ligation of the pylorus in Shay rats.

<table>
<thead>
<tr>
<th>Time after ligation of pylorus (hr)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>8</th>
<th>12</th>
<th>24</th>
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<tr>
<td>Antrum</td>
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<tr>
<td>Average number of lesion per rat</td>
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<td>0</td>
<td>0</td>
<td>3.3</td>
<td>5.3</td>
<td>8.4</td>
<td>13.8</td>
<td>12.8</td>
<td>14.6</td>
<td>12.3</td>
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<td>0.5~1.0</td>
<td>0.5~3.0</td>
<td>1.0~3.0</td>
<td>1.0~5.0</td>
<td>1.0~5.0</td>
<td>1.0~5.0</td>
<td>1.0~5.0</td>
<td>1.0~10.0</td>
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</tr>
<tr>
<td>Remarks</td>
<td>Erosions</td>
<td>Erosions</td>
<td>Erosions</td>
<td>Erosions</td>
<td>Erosions</td>
<td>Erosions, Defect of mucosa</td>
<td>Perforations in 2/5</td>
<td>Perforations in 5/5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- : no lesion, + : slight lesion, ++ : moderate lesion, +++ : marked lesion

Evaluations are based on observation of 3 to 5 rats each.

TABLE 2. The incidence of gastric ulcers after cold-exposure of restraint rats.

<table>
<thead>
<tr>
<th>Time after cold-exposure (hr)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>8</th>
<th>12</th>
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<tr>
<td>Antrum</td>
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<td>-</td>
</tr>
<tr>
<td>Average number of lesion per rat</td>
<td>0</td>
<td>0</td>
<td>5.6</td>
<td>11.3</td>
<td>10.0</td>
<td>9.2</td>
<td>7.6</td>
<td>8.0</td>
<td>6.3</td>
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<tr>
<td>Size (mm)</td>
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<td>0.5~1.0</td>
<td>0.5~3.0</td>
<td>1.0~5.0</td>
<td>1.0~5.0</td>
<td>1.0~5.0</td>
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</tr>
<tr>
<td>Remarks</td>
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<td>Erosions</td>
<td>Erosions, Petechia</td>
<td>Erosions, Petechia</td>
<td>Erosions, Bleeding</td>
<td>Erosions, Bleeding</td>
<td>Erosions, Blood-clot, Bleeding</td>
<td>Defect of mucosa</td>
<td>Defect of mucosa</td>
<td></td>
</tr>
</tbody>
</table>

- : no lesion, + : slight lesion, ++ : moderate lesion, +++ : marked lesion

Evaluations are based on observation of 3 to 5 rats each.

definite ulcers, covered with blood-clot and surrounded by hyperemic and edematous reaction, appeared in the body 18 to 24 hours later.

III. Fluorescence histochemical changes in response to ulcerogenic stimuli

The green fluorescence representing the adrenergic nerve fibers in the blood vessels, myenteric nerve plexuses and muscle layers of the gastric wall was essentially unaffected by ulcerogenic stimuli at least before the definite ulcers were produced. This fact suggests only a minor contribution of the adrenergic nerves to the formation of the gastric ulcers. On the other hand, there was a marked change in the yellow fluorescence of the mast cells and glandular epithelia, particularly after stress stimuli.

1. Shay rat

The distribution and number of fluorescent mast cells as well as the intensity of the yellow fluorescence in the serosal, muscular and submucosal layers of the whole stomach were not significantly affected usually until about 8 hours after pyloric ligation when the peptic erosions were found in the rumen. Differing from the rat subjected to stress stimuli, no
fluorescent mast cell appeared in any layer of the antrum. However, from the 12th hour of pyloric ligation when the definite ulceration appeared, the fluorescent mast cells were slightly increased in number throughout all layers of the body wall. At this stage, the fluorescent materials representing 5-HT increased in the submucosal layer immediately beneath the lamina muscularis mucosa and the same materials also appeared in the lamina propria of the body. When the gastric ulceration expanded to the body and sometimes to the antrum, the fluorescent materials accumulated in the upper layer of the mucous membrane. The fluorescent epithelial cells in the antrum were usually unaffected by the production of the gastric ulceration at least within 12 hours after pyloric ligation.

2. Cold-exposed rat

As the exposure time to cold stress was prolonged, the fluorescent mast cells in the serosal, muscular and submucosal layers of the body increased markedly in number and in intensity of fluorescence. The fluorescent mast cells newly appeared in the layers of the antrum where otherwise no fluorescence was observable. Such increased appearance of the fluorescent mast cells coincided with the manifestation of the hyperemic reaction and the erosion production of the mucous membrane of the body. In addition to the usual mast cell, the uneven, weakly fluorescent one also appeared in the serosal and muscular layers of the body and antrum. These facts suggest that the non-fluorescent mast cells in the serosal and muscular layers take up circulating or tissue 5-HT to show the specific fluorescence. These newly appeared fluorescent mast cells were usually distributed along the blood vessels. After the 5 or 6-hour exposure to cold stress when the hyperemic reaction extended to the surrounding mucous membrane, the fluorescent mast cells accumulated densely to the submucosal layer. The shape of mast cell was not uniform. At the same time, small-sized specific fluorescent materials, probably pieces of broken mast cells, lined or accumulated in the submucosal layer beneath the lamina muscularis mucos (Figs. 2b and 3b). Further prolonged exposure to the stress produced such a histological appearance as if the fluorescent mast cells and the small-sized materials invaded the mucous membrane across the lamina muscularis mucosa. At this stage the fluorescent mast cells disappeared from the muscular and serosal layers or they were seen only in the serosal layer. Thereafter, the fluorescent histochemical pictures were different between the body and antrum. In the antrum the fluorescent glandular epithelia were increased in number and the fluorescence was intensified in accordance with the movement of fluorescent materials toward the mucosal surface (Fig. 3c). A spindle-like light or dense yellow fluorescence was frequently found in the lamina propria. It is likely that some of glandular epithelia take up 5-HT from the fluorescent materials moving in the lamina propria and thus increase or manifest the specific fluorescence.

On the other hand, the number of fluorescent glandular epithelium in the body was much less than that in the antrum. Although the pictures indicated the movement of the fluorescent materials along the capillaries in the lamina propria toward the upper layer of the mucous membrane, the glandular epithelia in the body did not increase the yellow fluorescence. Fluorescent materials rather accumulated to the surface of mucosa
In the rumen where the lesion due to stress was less than other parts, no cytological change of fluorescent mast cell was observed.

About 18 hours after exposing to the stress when the gastric ulceration with hemorrhage began to occur, the fluorescent materials were found to concentrate especially in the upper layer of the mucous membrane of the body where the ulceration occurred most frequently. Once the gastric ulcers were formed, the specific fluorescence deteriorated totally in the area. However, in the vicinity of necrotic tissues there still observed the accumulation of the fluorescent materials in the mucosal surface. At this stage, the fluorescent mast cells appeared again in a line or irregularly in the submucosal, muscular or serosal layer (Figs. 2d & 3d).

**DISCUSSION**

On the basis of fluorescent histochemical findings, it has been proposed that 5-HT present in the glandular portion of the stomach of the rat plays an important role in producing experimental gastric ulcers (19). By combined histochemical and biochemical methods, Håkanson and Owman have described the cellular localization of histamine as well as dopa, dopamine and dopa decarboxylase in the gastric wall of the rat (8, 11, 20). In addition to the mast cells, adrenergic nerves and enterochromaffin cells, another large system of enterochromaffin-like cells, which normally store neither catecholamines nor 5-HT, was found to take up 1-dopa or 5-HT. Results obtained here confirm that there are three monoamine-containing structures in the stomach wall of normal rats: catecholamine-fluorescent adrenergic nerves, 5-HT-fluorescent mast cells and glandular epithelia. The pattern of the cytological distribution was essentially similar to that in the esophagus (21) and the intestine (3, 10, 22). The vascular fluorescence was not detected in the smooth muscle of the media but was in the external layer. The cell bodies of the myenteric plexus were surrounded by the fluorescent varicosities, indicating that the adrenergic nerves in the gastrointestinal tract of several species invest and terminate around the intramural cholinergic ganglion cells. However, unlike in the small intestine, no submucous ganglion cell surrounded by green-fluorescent fibers was detectable in the stomach as well as in the esophagus (21). The catecholamine fluorescence in the gastric wall was essentially unaffected by ulcerogenic stimuli such as the pyloric ligation and cold stress. It is unlikely that the adrenergic nerves contribute primarily to the production of experimental stomach ulcer.

The mast cells in the stomach of the rat developed the intense 5-HT fluorescence as those in other organs (23–25). However, the localization of the fluorescent mast cells was much different according to the division of the stomach. There were many fluorescent mast cells in the serosal, muscular and submucosal layers of the body and rumen, but not in the antrum. On the other hand, a large number of glandular epithelia in the antrum exhibited definite 5-HT fluorescence. In contrast to the catecholamine fluorescence, the cytological distribution of yellowish 5-HT fluorescence of mast cells and glandular epithelia was extensively affected by ulcerogenic, particularly cold stress.
Stress ulcers were primarily produced in the body of the stomach. Fluorescence histochemical studies indicated that the 5-HT containing mast cells normally present in the serosal, muscular and submucosal layers of the body gathered to the submucosal layer, then to the mucosa following the exposure of restraint rats to cold stress. Many small-sized fluorescent materials also appeared along the blood vessels in the serosal and muscular layers, then accumulated to the submucosal layer and finally invaded the mucosa. In the antrum where otherwise no fluorescent mast cell was seen, the mast cells newly appeared in response to cold stress. After a sustained exposure to cold, the fluorescence of glandular epithelium of the antrum was also increased in number and intensity. There are at least two possibilities for explaining such cytological changes of fluorescent materials in the course of development of stress ulcers.

One possibility is that the nonfluorescent mast cells take up circulating 5-HT to develop the specific fluorescence following the exposure to cold stress. Small-sized fluorescent materials might have represented the pieces of broken mast cells produced by a sustained exposure to cold. The ability of the mast cell to take up and concentrate circulating or tissue 5-HT increases with the maturation of cell (26). Thus, it is probable that cold stress stimulates a maturation of the immature, non-fluorescent mast cell to the 5-HT fluorescent one. Another possibility is that the 5-HT containing mast cells migrate from the serosal and muscular layers to the submucosal layer and finally to the mucosal surface along the blood vessels. When the mast cells arrive in the surface, they might be broken into a large number of small-sized pieces. It is assumed that the mast cells exhibit slow ameboid motility (27-29). At any rate, in the site of ulceration particularly in the body where the ulceration occurred most frequently and seriously, a large number of fluorescent mast cells and small sized pieces were present. It is considered that the 5-HT carried by the mast cells in the mucosal surface plays an important role in producing the ulcerative lesions in the stomach. In the antrum many of glandular epithelia were made yellowish fluorescent. It seems probable that the mast cell-5-HT, which otherwise produces ulcerative lesions, is taken up by the glandular epithelium and thus the production of ulceration in the antrum is prevented. No significant change in 5-HT fluorescence occurred in the rumen where ulcerative lesion was less than in other portions. Biochemical determinations revealed that the 5-HT levels were increased in the body and antrum but not in the rumen following the exposure to cold (30). These facts also support the view that there is a definite correlation between the occurrence of stomach ulcers after cold stress and the stomach 5-HT levels. The 5-HT fluorescence does not seem to play an important role in the occurrence of stomach ulcers of the pylorus-ligated Shay rat. The slight change in the late stage might have been due to a long-sustained ligation stress.

**SUMMARY**

Using a fluorescence histochemical technique it was confirmed that there were three monoamine-containing structures in the stomach wall of normal rats: catecholamine-fluorescent adrenergic nerves, 5-HT-fluorescent mast cells and glandular epithelia. The ad-
Renergic fluorescent fibers were seen in the small arteries and arterioles, the smooth muscle layer proper and the myenteric nerve plexus. The fluorescent mast cells were present in the serosal, muscular and submucosal layers of the body and rumen but not of the antrum of the stomach. Glandular epithelia in the antrum exhibited yellow fluorescence of 5-HT abundantly. In the body, few if any glandular epithelia developed the specific fluorescence. The catecholamine fluorescence in the stomach wall was essentially unaffected by ulcerogenic stimuli such as pyloric ligation and cold stress, suggesting that the adrenergic nerves would not contribute primarily to the production of experimental stomach ulcer.

Gastric ulceration in the Shay rat developed most frequently and markedly in the rumen after pyloric ligation. However, the distribution, intensity and number of 5-HT fluorescence were only slightly affected by pyloric ligation. It is unlikely that 5-HT plays an important role in the occurrence of stomach ulcers of the Shay rat.

Gastric ulceration due to a cold-exposure developed first in the body, but after a sustained exposure to the stress the ulcers expanded to the antrum and rumen. Fluorescence histochemical studies revealed that the 5-HT containing mast cells normally present in the serosal, muscular and submucosal layers of the body increased in number, then gathered to the submucosal layer and finally to the mucosa following the exposure of restraint rats to cold stress. A large number of small-sized fluorescent materials also appeared along the blood vessels in the serosal and muscular layers, then accumulated to the submucosal layer and finally invaded the mucosa. In the antrum where otherwise no fluorescent mast cell was seen, the mast cells newly appeared in response to cold stress. After a sustained exposure to cold, the fluorescence of glandular epithelium of the antrum was increased in number and intensity. These changes in fluorescent materials coincided with the manifestation of the hyperemic reaction and the erosion production of the mucous membrane and then the occurrence of definite ulceration with hemorrhage. No significant change of 5-HT fluorescence occurred in the rumen where ulcerative lesion developed least frequently. It is suggested that the 5-HT transported to the mucosal surface by the mast cells plays an important role in producing the ulcerative changes in the stomach of rat after cold stress, and that the ulcerogenic 5-HT is taken up considerably by the glandular epithelium of antrum and thus the production of ulceration is prevented in the antrum.

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