Vital immunohistochemical staining for a novel method of diagnosing micro-cancer -examination of immunohistochemical staining of non-fixed fresh tissue-

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Abstract : It becomes possible to establish a novel diagnostic method for micro-cancer by modulating the signals from the lesion, if lesions can be labeled with substances which can be detected by video endoscopy. The authors have already succeeded in synthesizing indocyanine green (ICG) derivatives for a fluorescent labeling substance which emits near-infrared rays. Before the antibodies labeled by these substances can be used, it is necessary to establish a method of vital immunohistochemical staining. So, we investigated the responses of antibodies exposed to non-fixed fresh tissue specimens as a basic study on vital immunohistochemical staining. The responses of fresh esophageal and gastric specimens (biopsied or surgically resected) to immunohistochemical staining with anti-epithelial membrane antigen (EMA) antibodies under various conditions using the ABC method were examined. These tissue specimens were stained immunohistochemically, and incubated with anti-EMA antibodies for 10 and 30 minutes (esophagus), and for 60 and 120 minutes (stomach) at 37℃. These results suggest that vital immunohistochemical staining is possible under optimum conditions. If an infrared fluorescent endoscopy catching this excited fluorescence can be developed, it will be possible to establish a new endoscopic diagnostic method on the basis of vital immunohistochemical staining.
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Key words : *micro-cancer, diagnostic method, indocyanine green (ICG) derivatives, vital immunohistochemical staining*

INTRODUCTION

Video endoscopy has made endoscopic diagnosis easier. The ability to detect micro-cancer, however, differs little between video endoscopy and fiberscopy. Thus, no method has been established to make full use of the video endoscope for diagnosis. If lesions can be labeled with substances which can be detected by video endoscope, it will be possible to establish a new method for the diagnosis of micro-lesions, involving computerized processing of electron signals. To establish such a method, safe labeling substances and methods are required. We concentrated on indocyanine green (ICG), which emits near-infrared rays, as a promising candidate fluorescent labeling substance for use in vivo and have succeeded in synthesizing ICG derivatives which can bind to protein (1). If these ICG derivatives can be labeled with antibody specific to cancer tissue, a new method for the diagnosis of micro-lesions making use of antigen-antibody reactions may become available. Before establishing such a method, however, it is necessary to elucidate whether antigen-antibody reactions take place via this method on the surface of mucosa in vivo. We have previously developed an infrared fluorescence imaging system, using these labeling substances (2). Our previous study demonstrated that ICG derivatives conjugated with existing

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antibodies to cancer-associated antigens retained their antibody activity and fluorophotometric features (3). The present study was undertaken to determine whether antigen-antibody reactions would take place on the surface of mucosa in vivo. To this end, responses of fresh tissue specimens (biopsied or surgically resected) to immunohistochemical staining with anti-epithelial membrane antigen (EMA) antibodies were examined. Furthermore, in view of possible interference by mucus or other factors during immunohistochemical staining of the mucosal surface of the digestive tract, the utility of removing mucus during pretreatment of samples was assessed.

MATERIALS AND METHODS

Materials

Biopsied tissue specimens from 8 healthy adult volunteers and fresh resected tissue specimens from one patient with esophageal cancer and 4 patients with gastric cancer were stained immunohistochemically. Biopsy was carried out using biopsy forceps (RADIAL JAW 3, Boston Scientific Corp., Massachusetts, USA), under endoscopic guidance with a GIF-XQ200 endoscope (OLYMPUS Inc., Tokyo, Japan). About 2 mm in diameter mucosal pieces of the central portion of the esophagus and the gastric body were collected by biopsy. In patients with cancer, approximately 1cm square pieces of cancer-free tissue were cut out of the surgically resected specimens of the esophagus or stomach. Paraffin sections of normal esophagus or stomach tissue, which had been confirmed to respond positively to immunohistochemical stain with anti-EMA antibodies, served as controls.

Anti-EMA antibodies (mouse anti-human monoclonal antibodies, Code No. M 613, DAKO Co., Ltd., Glostrup, Denmark) were used for immunohistochemical staining. The color was developed with 3,3′-Diaminobenzidine tetrahydrochloride (DAB, DOJINDO, Co., Ltd., Kumamoto, Japan), using an avidinbiotinylated peroxidase complex (ABC) kit (Vector Laboratories Inc., California, USA).

Methods

Three or more non-fixed fresh tissue specimens of each test organ were pretreated. Subsequently, they were exposed to normal horse serum (blocking serum) for 15 minutes at room temperature. The surface of each mucosal specimen was then treated with anti-EMA antibodies under various conditions and fixed in 10% formalin. Paraffin sections were then prepared. Immunohistochemical staining by the ABC method was performed according to the following procedure. First, the prepared paraffin sections was exposed to biotinylated horse anti-mouse IgG for 30 minutes at room temperature and then to ABC for 40 minutes. This was followed by immersion in DAB for 1~5 minutes, and by color development. Between steps, the sample was washed with phosphate-buffered saline (PBS) solution for 5 minutes.

Pretreatment was carried out using the method of Ida *et al*. (4) as follows. The method is often used in pretreatment for endoscopic examination. Some samples were immersed and washed in 50 ml of water containing 20,000 U pronase (Pronase MS, Kaken Pharmaceutical Co., Ltd., Tokyo, Japan), 1 g $NaHCO_3$ and 4 mg Dimethylpolysiloxane (Gascon ; Kissei Pharmaceutical Co., Ltd., Matsumoto, Japan) for 30 minutes at 4℃ (Group A). Other samples were immersed in normal saline for 30 minutes at 4℃ to prevent drying (Group B ; unpretreated group).

After pretreatment, the non-fixed specimens of esophageal and gastric mucosa were exposed to anti-EMA antibodies under various sets of conditions. The chromatic responses of these fresh tissue specimens were compared according to the conditions of exposure to the antibodies. The anti-EMA antibodies used in this study were diluted at a ratio of 1 : 25 and to pH 7.2 with PBS before use. The following sets of conditions were employed : exposure for 90 minutes at room temperature, exposure overnight at 4℃, and exposure for 10, 30, 60 and 120 minutes at 37℃ with incubation (conditions similar to those prevailing in vivo).

Chromatic responses were assessed as follows. Control paraffin sections were exposed to anti-EMA antibodies for 90 minutes at room temperature, and the color developd by the ABC method under conditions similar to those used for test specimens (fresh specimens). Control sections which showed adequate chromatic responses were rated as (+++), those without chromatic responses as (-), and intermediate degrees of chromatic responses as (++) and (+). The chromatic responses of test specimens were assessed by comparing them to the control sections and they were rated on the same four-point scale. When no sections exposed to the antibodies under a given set of conditions showed chromatic responses, this set of conditions was rated as (-).

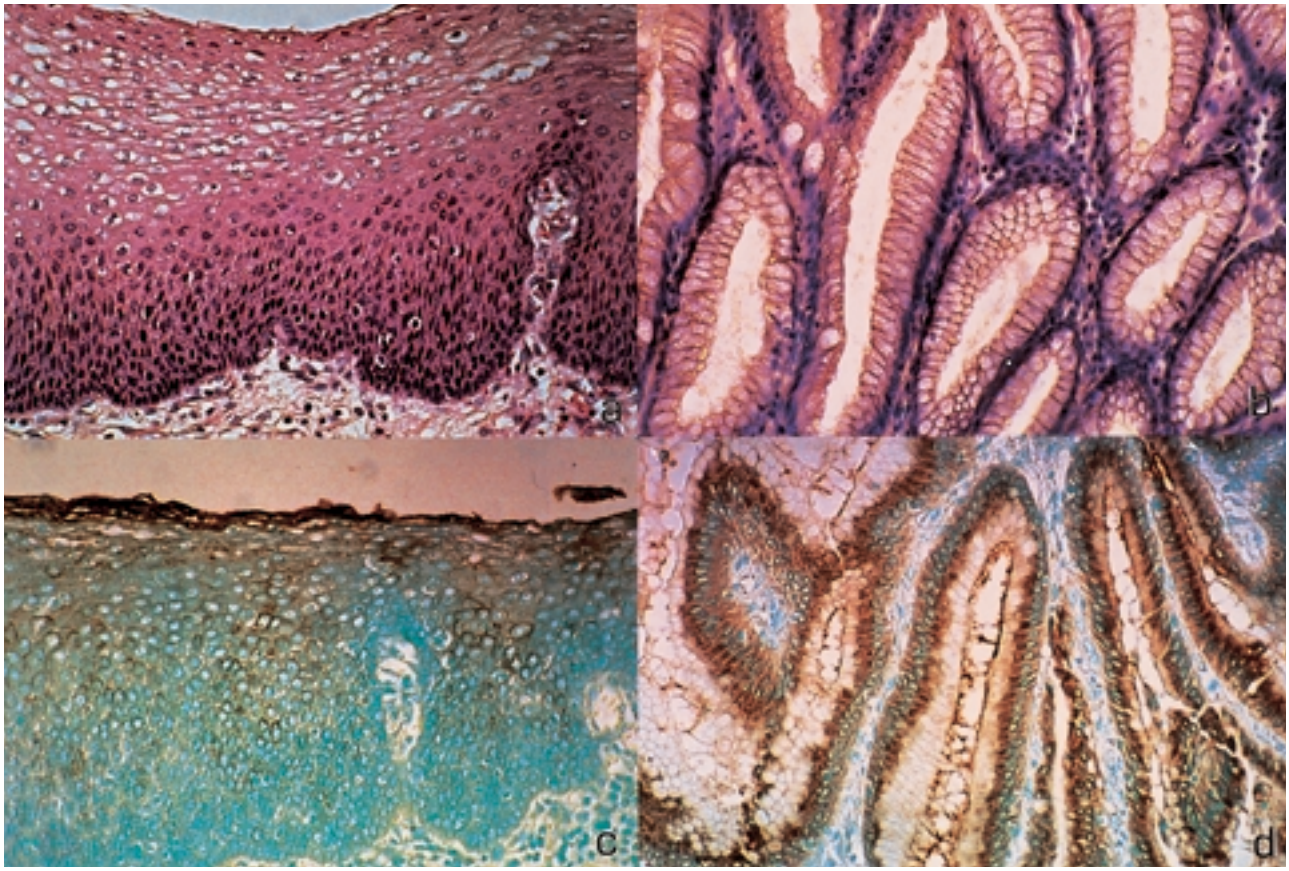

Fig.1. Control sections (10 x 20). a and b ; HE stained. c and d ; immunohistochemically stained with anti-EMA antibodies. a and c ; esophageal tissue. b and d ; gastric tissue.

When one or two sections exposed to the antibodies under a given set of conditions were positively stained, the rating for the most strongly stained section was adopted as the rating for this set of conditions. Figure 1c and 1d shows an example of control sections rated as (+++).

RESULTS

Chromatic responses of biopsied and resected specimens to the stain under different sets of conditions

Test specimens of the esophagus and stomach were exposed to anti-EMA antibodies under different sets of conditions. These specimens were fixed in formalin and paraffin sections made for color development using the ABC method. The chromatic responses obtained under different sets of conditions are shown in Table 1.

Of the specimens without pretreatment, biopsied esophageal specimens showed weak chromatic responses after incubation with antibodies for 30 minutes at 37°C, but the other specimens without pretreatment showed no chromatic responses. Of the pretreated specimens, biopsied esophageal specimens incubated with antibodies for 10 or 30 minutes at 37°C showed adequate chromatic responses. However, as the reaction time was prolonged, transformation and destruction of tissue took place, and no chromatic responses were seen in pretreated biopsied esophageal specimens incubated with antibodies for 60 or 120 minutes at 37°C. Pretreated resected esophageal specimens showed chromatic responses when incubated with antibodies for 10 or 30 minutes at 37°C, but their responses were weaker than those observed in biopsied esophageal specimens. Of the pretreated resected gastric specimens, those incubated with antibodies for 60 or 120 minutes at 37°C showed chromatic responses, but other resected gastric specimens when incubated with antibodies for 10 or 30 minutes at 37°C showed no chromatic responses. However, prereated biopsied gastric specimens showed no chromatic responses except for the specimens incubated with antibodies for 60 minutes at 37°C.

Figure 2 shows chromatic responses of biopsied esophageal specimens after 10 (Figure 2a) or 30

Table 1. Results of Immunohistochemical Staining

	Methods of Pretreatment	Conditions of Exposure	Grades of Staining	
			Resected specimen	Biopsied specimen
Esophagus	A : (normal saline for 30 min. at 4°C)	exposed for 90 min. at room temp.	-	-
		exposed overnight at 4°C	-	-
		incubated for 10 min. at 37°C	-	-
		incubated for 30 min. at 37°C	-	+
		incubated for 60 min. at 37°C	-	-
		incubated for 120 min. at 37°C	-	-
	B : (50 ml of water containing 20,000 U pronase, 1 g $NaHCO_3$ and 4 mg dimethylpolysiloxane for 30 min. at 4°C)	exposed for 90 min. at room temp.	-	-
		exposed overnight at 4°C	-	-
		incubated for 10 min. at 37°C	++	+++
		incubated for 30 min. at 37°C	+	++
		incubated for 60 min. at 37°C	-	-
		incubated for 120 min. at 37°C	-	-
Stomach	A : (normal saline for 30 min. at 4°C)	exposed for 90 min. at room temp.	-	-
		exposed overnight at 4°C	-	-
		incubated for 10 min. at 37°C	-	-
		incubated for 30 min. at 37°C	-	-
		incubated for 60 min. at 37°C	-	-
		incubated for 120 min. at 37°C	-	-
	B : (50 ml of water containing 20,000 U pronase, 1 g $NaHCO_3$ and 4 mg dimethylpolysiloxane for 30 min. at 4°C)	exposed for 90 min. at room temp.	-	-
		exposed overnight at 4°C	-	-
		incubated for 10 min. at 37°C	-	-
		incubated for 30 min. at 37°C	-	-
		incubated for 60 min. at 37°C	++	+
		incubated for 120 min. at 37°C	+	-

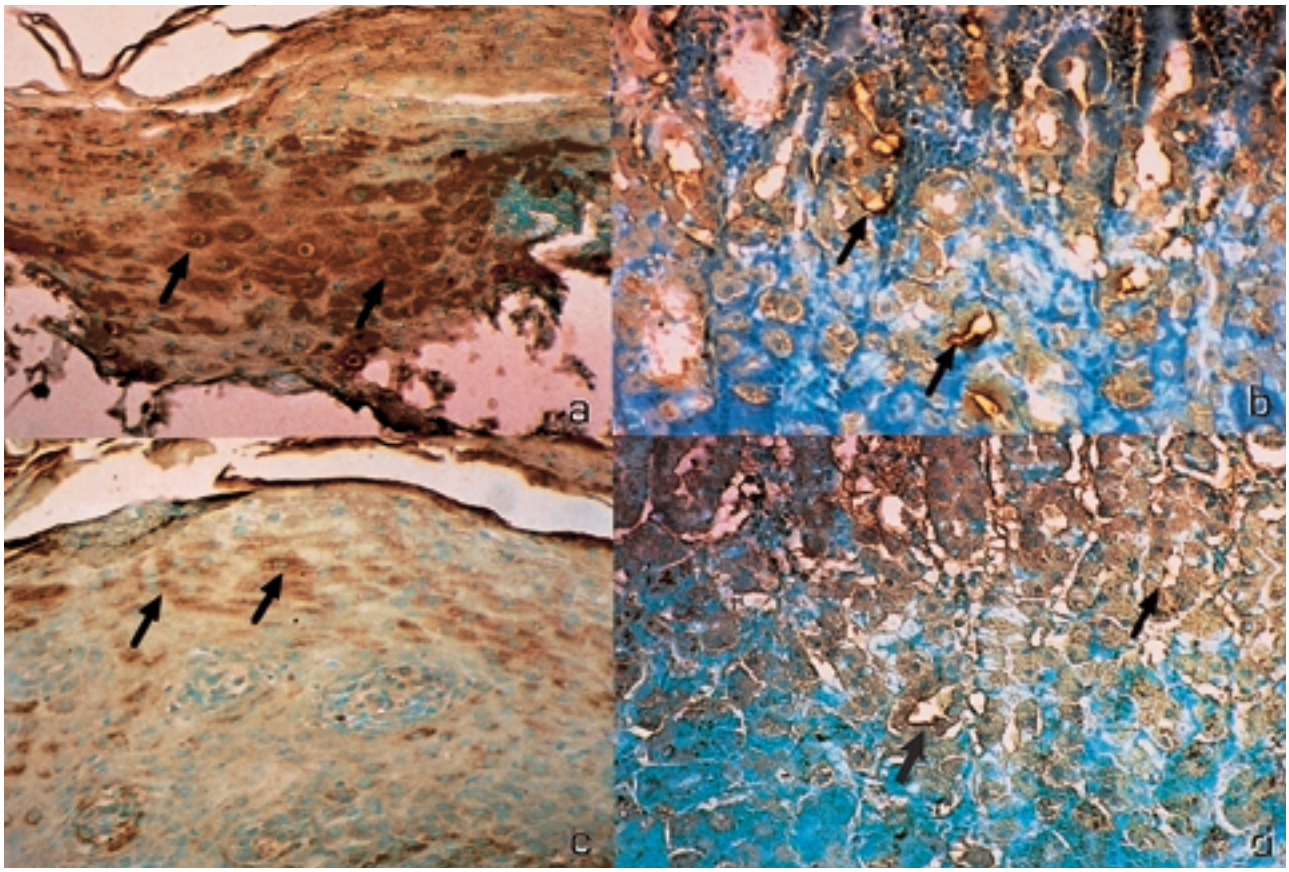

Fig. 2. Immunohistochemically stained views of non-fixed fresh sections (10 x 20). Epithelial cells of each specimen were stained (see arrows). a and c ; biopsied specimen of esophagus. b and d ; resected specimen of stomach.
Incubated with anti-EMA antibodies at 37°C. a ; for 10 minutes. b ; for 60 minutes. c ; for 30 minutes. d ; for 120 minutes.

(Figure 2c) minutes at 37°C of incubation and resected gastric specimens after 60 (Figure 2b) or 120 (Figure 2d) minutes at 37°C of incubation with antibodies. Stratified squamous epithelial cells of the esophagus and columnar epithelial cells of fundic glands of the stomach were positively stained in these specimens.

Distribution of EMA in control sections and non-fixed fresh specimens

In both control sections and non-fixed fresh specimens, positive chromatic responses, as determined using DAB as a color developer, were seen in the cytoplasm of stratified squamous epithelial cells of esophageal mucosa and in the cytoplasm of columnar epithelial cells of gastric mucosa. Thus, the responses to staining with anti-EMA antibodies were consistent between control sections (Figure 3a and 3b) and non-fixed fresh specimens (Figure 3c and 3d).

DISCUSSION

Immunohistochemical staining was first attempted in 1941 by Coons *et al.*, who used anthracene isocyanate as a labeling substance (5). This staining technique has been used primarily for paraffin or frozen sections. At the present time, no reports of its use in vivo (*i.e.*, vital immunohistochemical staining) have been published. Since current methods of immunohistochemical staining use toxic substances for color development, their use in vivo presents problems. Furthermore, since these methods involve many reactive steps and take much time, they have seldom been used on fresh specimens (6).

Immunohistochemical staining is based on antigen-antibody reactions ; these phenomena usually take place in living tissue. Attempts have been made to conjugate anti-cancer agents or products of gene expression to antibodies which have an affinity for lesion-specific antigens, and to administer the conjugates to living bodies for therapeutic purposes. These attempts have proven to some extent effective (7, 8). In all these previous attempts,

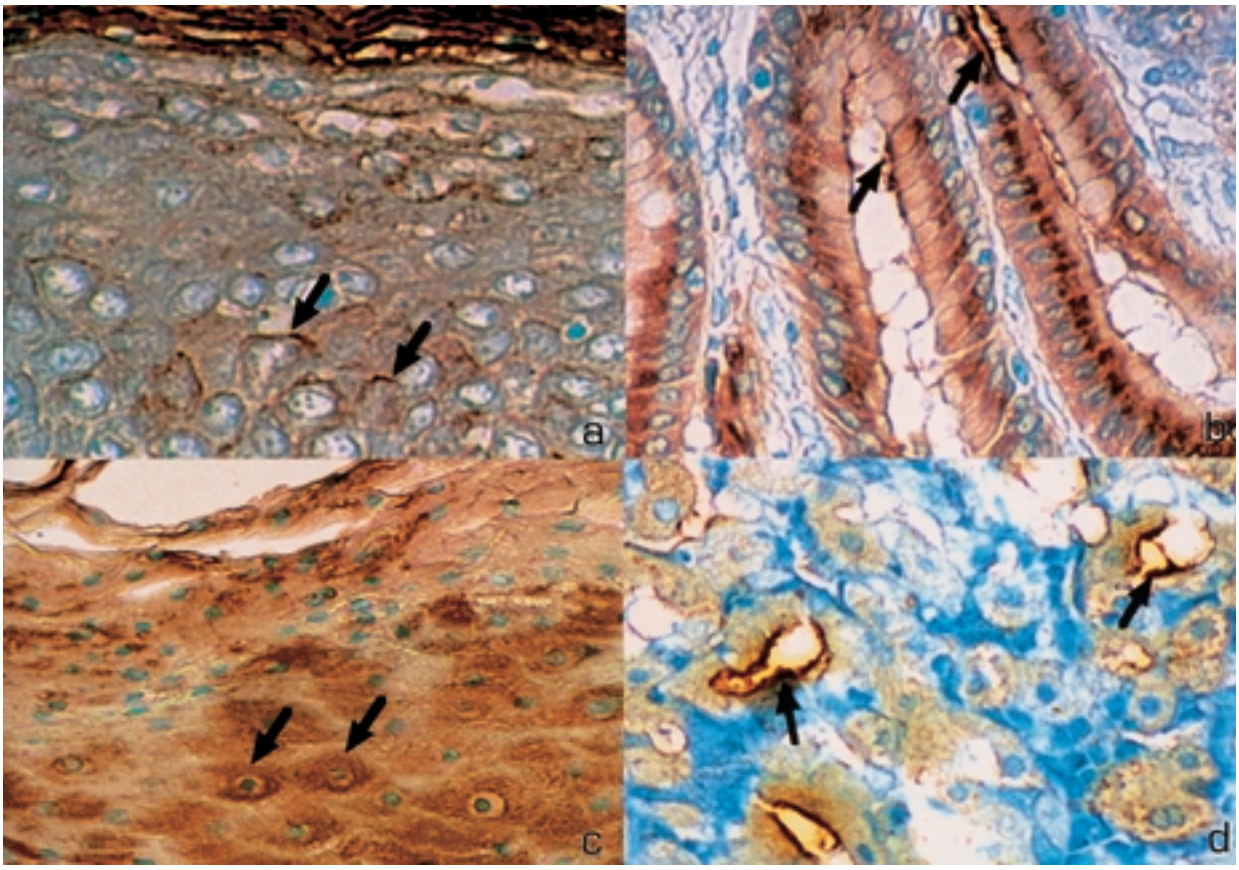

Fig. 3. Comparison of stained areas in non-fixed fresh sections and control sections with anti-EMA antibodies (20 x 20). In fresh sections, the cytoplasm of epithelial cells is colored brown same as in control sections (see arrows). a and b ; control sections. c and d ; non-fixed fresh sections. a and c ; esophageal tissue. b and d ; gastric tissue.

the conjugates were administered into blood vessels. However, considering that antigen-antibody reactions can take place throughout the living body, the establishment of a staining method by which labeled antibodies are administered via mucosal surfaces is expected to lead to the development of a new method of diagnosis and treatment. However it is unknown whether or not the various barriers present on mucosal surfaces suppress antigen-antibody reactions following the administration of such antibodies via the mucosa of the digestive tract.

The authors therefore examined in the present study whether or not vital immunohistochemical staining with antibodies administered via the mucosa of the digestive tract would be possible. To this end, the chromatic responses of non-fixed fresh tissue of biopsied esophageal and gastric specimens and resected tissue of the same organs to anti-EMA antibodies were investigated.

Serial sections of fixed tissue specimens (containing cancer-free tissue in addition to cancerous tissue), resected surgically from 3 patients with esophageal cancer, 4 patients with gastric cancer and 3 patients with colorectal cancer, were subjected to ordinary immunohistochemical staining, using antibodies to cytokeratin, EMA, and carcinoemborynic antigen (CEA) which are representative immunohistochemical markers of epithelial tumors. The chromatic responses of these sections (control sections) were examined.

Both the cancerous and cancer-free areas of each section were stained with anti-cytokeratin and anti-EMA antibodies. The chromatic responses to anti-cytokeratin antibodies were insufficient, while those to anti-EMA antibodies were satisfactory. The chromatic responses to anti-EMA antibodies were particularly strong in the normal mucosal area in all cases. Chromatic responses to anti-CEA antibodies were observed in the cancerous area of a section from a patient with gastric cancer and a patient with colorectal cancer.

Because it was difficult to obtain many non-fixed specimens from the same patient, we evaluated the chromatic responses of specimens from different patients to anti-EMA antibodies, because the chromatic responses to these antibodies did not differ between different cases.

Anti-EMA antibodies were first reported by Ceriani *et al*. as polyclonal antibodies to human milk fat globule membranes (HMFG) (9). Later, these antibodies were found to respond to a number of normal epithelia and epithelial tumors and were called antibodies to 'epithelial membrane antigen.' The antigen has been partially purified, and shown to belong to a heterogeneous group of heavily glycosylated proteins with a MW in the range of 265-400 kDa (10). The antibodies used in the present study were reported by Heyderman *et al*. as new monoclonal antibodies to EMA which had higher reactivity than did the polyclonal antibodies reported by Ceriani *et al* (11). Cordell *et al*. reported that these antibodies responded to the epithelium of normal gastro-intestinal tract (tongue, esophagus, gastric parietal cells, small intestine, colon, and rectum) (12).

The chromatic responses of the mucosal epithelium of the esophagus and stomach to antibodies were analyzed in relation to the conditions under which non-fixed fresh tissue specimens of these organs were exposed to antibodies. Specimens of esophageal mucosa pretreated with a mixture of pronase and $NaHCO_3$ showed positive responses to the 10-minute incubation with anti-EMA antibodies, when the ABC method was used to develop colors. Specimens of gastric mucosa also showed chromatic responses on incubation with antibodies for 60 minutes. As the incubation time was extended beyond 60 minutes, chromatic responses weakened, and transformation or destruction of mucosal tissue occurred, making the evaluation of chromatic responses difficult. This probably indicates that since the current method of immunohistochemical staining involves many reactive steps and takes much time, tissue destruction occurred during the reaction period. Unpretreated specimens of esophagus and stomach showed few chromatic responses, suggesting that mucus affected antigen-antibody reactions in these specimens. The time required for pretreated specimens to show chromatic responses was shorter for esophageal tissue than for gastric tissue. This difference is probably due to the difference in the structure of the two organs, i.e., probably because the columnar epithelial cells of the gastric mucosa are present on the narrow lumen of the fundic glands. It is also attributable to the greater amount of mucus produced in the stomach than in the esophagus. These findings, combined with the knowledge that the gastric mucosa is always exposed to gastric acid, suggest that antigen-antibody reactions do not occur in the stomach in vivo due to low pH levels, or the antibodies administered via the gastric mucosa undergo denaturation. The anti-EMA antibodies used in this study had a pH of 7.2, and the PBS used as a buffer had a pH of 7.4. The optimum pH level for

pronase, used for pretreatment in this study, has been reported to be between pH 6 and 8 (13, 14). It has been reported that pronase has greater mucolytic effects under alkaline conditions, and that the combined use of pronase and $NaHCO_3$ resulted in a significant decrease of mucus in clinical cases (4, 15). In the present study, pronase was used in combination with $NaHCO_3$ for pretreatment, and it probably manifested mucolytic effects when the pH was over 7. The low pH level of gastric tissue seems to interfere with the vital immunohistochemical staining of gastric mucosa. A valid countermeasure against this factor is needed to establish vital immunohistochemical staining of gastric mucosa.

When control esophageal sections were exposed to anti-EMA antibodies, stratified squamous epithelial cells showed positive chromatic reactions. When control gastric sections were used, columnar epithelial cells of fundic glands showed chromatic responses. Fresh specimens of stomach and esophagus also showed responses similar to those observed in control sections of the corresponding organ. These results allow us to conclude that the chromatic responses observed in fresh sections of these organs were an outcome of immunoreactions.

These results suggest that antigen-antibody reactions can take place even on the mucosal surface of fresh specimens if these specimens are exposed to antibodies under optimum conditions. This means that localization of lesions in vivo is also possible with labeled antibodies if these antibodies are administered under optimum conditions. However, if the current methods of immunohistochemical staining are used without modifications, it will be difficult to develop a valid method of endoscopic diagnosis making use of immunohistochemical staining. The development of fluorescent labeling substances which can be used safely in vivo and a fluorescent endoscope which can detect these substances in vivo is indispensable for this purpose.

A laser fluorescent endoscope, designed to observe the mucosal surface structure using laser-excited fluorescence, has recently been developed. Several reports on its use in checking for tumors of digestive tract have been published (6, 16). These endoscopes, however, use ultraviolet rays, which are hazardous to living tissue, for excitation. Furthermore since these endoscopes use short wavelength regions, which involve much background noise, it seems difficult to obtain clear images of lesions with these endoscopes. It is therefore desirable to develop a technique by which only lesions are excited to emit fluorescence.

Ito *et al*. have developed ICG derivatives which are excited by infrared rays to emit fluorescence. They have also developed an imaging system for these ICG derivatives (1, 2). The ICG derivatives and the imaging system they developed appear to satisfy the aforementioned requirements. Therefore, if a fluorescent endoscope using infrared rays is developed to catch the fluorescence emitted from ICG derivatives in vivo, it will be possible to establish a new technique of endoscopic diagnosis on the basis of vital immunohistochemical staining with antibody-conjugated ICG derivatives.

Marked advances have recently been made in medical fields, especially fields related to molecular biology and electronic medical apparatus. It is plausible to imagine that antibodies to cancer-specific antigens will become available in the near future thanks to further advances in molecular biology, and that the accuracy of fluorescent endoscopy will be improved to a level allowing the detection of very small amounts of fluorescence-labeled antibodies. We may expect that a method of diagnosing macroscopically invisible micro-cancer with the use of antibodies to cancer-specific antigens will be established before long.

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