ORIGINAL

Optimal labeling condition of antibodies available for immunofluorescence endoscopy

Yoko Tadatsu[1], Naoki Muguruma[1], Susumu Ito[1], Masaya Tadatsu[1], Yoshihiro Kusaka[1], Koichi Okamoto[1], Yoshitaka Imoto[1], Hiromi Taue[2], Shigeki Sano[2], and Yoshimitsu Nagao[2]

[1]Department of Digestive and Cardiovascular Medicine, [2]Faculty of Pharmaceutical Sciences, Institute of Health Biosciences, The University of Tokushima Graduate School, Tokushima, Japan

Abstract : *Purpose*: In recent years, labeled antibodies have been used for diagnostic imaging in many studies. In this study, we investigated the mode of binding in antibodies labeled with ICG derivatives newly developed for the diagnosis of microcarcinomas, and evaluated the optimal binding molar ratio between the labeling compounds and antibody. *Methods* : MUC 1 antibody and ICG derivatives (ICG-ATT and ICG-sulfo-OSu) were used. ICG derivatives non-covalently bound to the antibody were removed with ethyl acetate, and the ratio of ICG derivatives covalently bound to the labeled antibody was confirmed. During purification of the labeled antibody, the amount of each labeling compound reacting with 1 molecule of the antibody varied as follows: 4, 8, 16, and 32 molar equivalents. Subsequently, the intensity of fluorescence was evaluated by spectroscopy and infrared fluoroscopy. *Results* : The ratio of residual ICG derivative labeling the antibody was 67.4% for ICG-ATT and 65.0% for ICG-sulfo-OSu. When fluorescent antibody labeled with ICG-ATT at an F/P ratio of 2.94 or 4.18 was used, specific and clear fluorescent images of the antigen were obtained. When ICG-ATT-labeled antibody at an F/P ratio of 6.50 or 6.75 was used, the fluorescence intensity decreased and the fluorescent images of antigen became unclear. *Conclusions*: It was found that the ICG-ATT-labeled antibody was a more specific and sensitive marker than ICG-sulfo-OSu-labeled antibody, and that lower binding molar ratios of ICG-ATT were more useful for labeling the antibody. J. Med. Invest. 53 : 52-60, February, 2006

Keywords **: immunofluorescence, infrared fluorescence, labeled antibody**

INTRODUCTION

Although diagnosis using electronic endoscopy has undergone remarkable evolution, no new diagnostic method for microcarcinoma utilizing electronic endoscopy has been developed (1). An accurate diagnosis of small lesions by conventional endoscopy is difficult in some cases, and it depends on the histopathological findings of a biopsy specimen (2). To obtain information on lesions that are not evident by conventional endoscopy, various techniques have been developed (2-5). Labeling of micro lesions with an antibody detectable by electronic endoscopy, followed by computer processing and imaging of the electronic signals of the labeling substance, may allow the diagnosis of such microcarcinomas. Fluorescent labeling substances are highly sensitive. Several kinds of labeling agents have been developed (6, 7), but many of them fluoresce when exposed to ultraviolet

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Address correspondence and reprint requests to Naoki Muguruma, Department of Digestive and Cardiovascular Medicine, Institute of Health Biosciences, The University of Tokushima Graduate School, Kuramoto-Cho Tokushima 770-8503, Japan and Fax:+81-88-633-9235.

rays which are likely to damage the DNA of living tissues (8). Thus, we attempted to develop an infrared fluorescence endoscopic method using an infrared fluorescence-labeled antibody with less background noise (9).

Indocyanine-green, which is known to emit fluorescence in the infrared range (10), is used in fluorescein angiography (11) to determine the depth of burns (12), but it does not bind to an antibody. Thus, we synthesized indocyanine-green-N-hydroxysuccinimide ester (ICG-sulfo-OSu) which can bind to a antibody (13), and developed an infrared fluorescence microscope that can detect ICG-sulfo-OSu (14, 15). Using this system, we investigated the fluorescence characteristics of ICG-sulfo-OSu and labeled antibody activity (16).

Then we immunostained paraffin-embedded sections of human gastric cancer and excised fresh gastric specimens with ICG-sulfo-OSu-labeled anti-human CEA antibody (17). The infrared fluorescence was found to correspond to the area stained with 3-3' diaminobenzidine (DAB) (17, 18). These findings indicated that microcarcinoma may be evidenced by an infrared ray electronic endoscopy using ICG-sulfo-OSu-labeled anti-human CEA antibody. However, the infrared fluorescence intensity of ICG-sulfo-OSu is not sufficient for imaging microcarcinomas (19, 20).

In an attempt to obtain a compound more appropriate for infrared fluorescence labeling, we synthesized 3-indocyanine-green-acyl-1, 3-thiazolidine-2-thione (ICG-ATT)(21) and investigated the differences between the optical characteristics of ICG-sulfo-OSu-labeled and ICG-ATT-labeled antibodies (22). Diagnostic methods using labeled antibodies have been reported to detect specific signals from lesions, and to be of diagnostic value (3, 7, 23-25). However, many aspects regarding the binding of fluorescence-labeled antibodies need to be elucidated to establish an accurate diagnostic method. Details of the binding of ICG derivatives to antibodies are also unknown, although the possibility of non-covalent binding has been reported in some cases (22). The ratio of non-covalent bonds in labeled antibodies is thought to influence fluorescence intensity and background noise (2). Furthermore, the binding molar ratio of the labeling substance to an antibody may also have a marked influence on fluorescence intensity (24). Thus, we investigated the binding of ICG derivatives to an antibody, and the optimum binding molar ratio for a labeled antibody.

MATERIALS AND METHODS

Materials

As an anti-MUC 1 antibody, MY. 1 E 12 provided by Professor Irimura (Department of Cancer Biology and Molecular Immunology, Faculty of Pharmaceutical Sciences, The University of Tokyo, Tokyo) was used. Although this anti-MUC1 antibody reacts with the normal mucosa (26), its sensitivity to cancer tissue is high (27) and staining intensity was significantly higher in cancer tissues than in non-neoplastic tissues (28). Therefore, we thought it may become a useful marker for the diagnosis of microcarcinomas.

As for the labeling substances, the ICG derivatives ICG-sulfo-OSu (DOJINDO LABORATORIES, Kumamoto, Japan) and ICG-ATT were used. For paraffin-embedded sections of human gastric cancer, surgical preparations of adenocarcinoma were used.

Equipment

Absorption of ultraviolet visible light and fluorescence spectrum were measured using N, N-dimethylformamide (DOJINDO LABORATORIES, Kumamoto, Japan and BECKMAN DU 650 spectroscope, and 650-40 spectrophotometer (Hitachi High-Technologies, Tokyo), respectively. The fluorescence microscope, BX-60 (Olympus Optical Co., Ltd., Tokyo) with attached excitation and barrier filters was used. An ICCD-500/DF camera (Hamamatsu Photonics K.K., Hamamatsu, Japan), an EVIP-230 image capture system (Olympus Optical Co., Ltd., Tokyo), and a magneto-optic disc drive S321S (Olympus Optical Co., Ltd., Tokyo) as the image recording system, were used. For imaging infrared fluorescence, a reflected illumination-type infrared fluorescence imaging system (Olympus Optical Co., Ltd., Tokyo)(15) was used. This system is composed of an infrared microscope, an excitation filter with transmission wavelengths of 710-790nm, a barrier filter with transmission wavelengths of 810-920nm and a highly sensitive intensified charge-coupled device (ICCD) camera.

The protein assay kit used to quantify protein was purchased from Bio-Rad Co. (Hercules, CA). The PD-10 column was purchased from Amersham Biosciences K.K. (Piscataway, NJ).

Methods

Labeling of the antibody and investigation of the binding of the labeling substance to the antibody

The anti-MUC1 antibody MY. 1E12 was labeled with ICG-sulfo-OSu and ICG-ATT to obtain two

markers using a PD-10 column as previously reported (13) and the absorbance was measured. To remove the ICG derivative bound to the antibody by non-covalent binding, ethylacetate was added to the labeled antibody solution, followed by stirring at room temperature protected from light for 30 minutes, and the aqueous layer was collected. The absorbance of the collected aqueous layer was measured. The absorbance of the control solution before treatment with ethylacetate was taken as 100% and the percentage of residual ICG derivative-labeled antibody was calculated (Fig. 1, Method A).

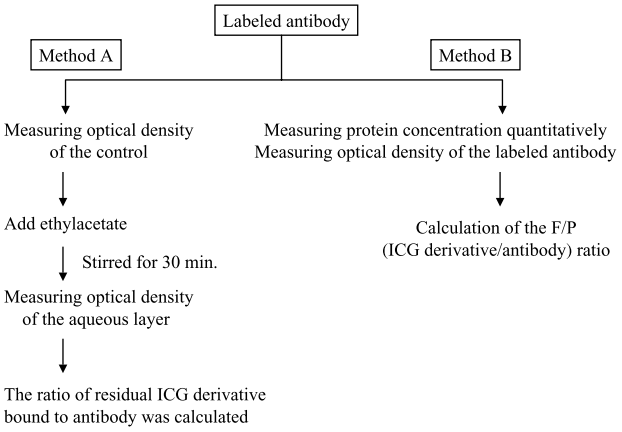


Fig.1. Evaluation of the binding and binding molar ratios between labeling compounds and antibody.

Binding molar ratio of the labeling substance to the antibody

The ICG derivative was reacted with 1.57 mg/ml anti-MUC 1 antibody at 4, 8, 16, and 32 molar equivalents, and the protein content of the labeled antibody was measured using a protein assay kit. Absorbance of the labeled antibody was measured at the maximum absorption wavelength and the content of the ICG derivative-labeled anti-MUC 1 antibody was calculated from the molar molecular extinction coefficient. The binding molar ratio (F/P ratio) of the ICG derivative to anti-MUC 1 antibody was calculated from the above values (Fig. 1, Method B). In addition, the fluorescence intensity of each labeled antibody solution was measured using a spectrophotometer.

Observation of infrared fluorescence in tissue sections

After deparaffinizing thin sections(2-3 μm)of human gastric cancer in xylol, they were covered with normal goat serum and allowed to react at room temperature for 20 minutes to block non-specific binding. After rinsing with 0.1 M phosphate buffered saline (PBS, pH 7.2), ICG-ATT-and ICG-sulfo-OSu-labeled antibodies were separately applied to two adjacent sections of the same tissue, and allowed to react at room temperature in a wet box for 90 minutes. The sections were rinsed with PBS, mounted with glycerin, and observed using a reflected illumination-type infrared fluorescence imaging system(Olympus Optical Co., Ltd.) (15). To keep a constant condition throughout the observation, the laboratory was kept dark at room temperature, not to affect the results. ICG-ATT-labeled anti-MUC 1 antibody was diluted with PBS to investigate its optimum concentration, and reacted with the antibody at various concentrations(1,000, 500, 250, and 125 mg/ml). As controls, the adjacent sections were stained with hematoxylin and eosin (HE staining) and immunostained according to the avidin-biotinylated peroxidase complex (ABC) method using anti-MUC1 antibody.

Investigation of nonspecific reaction with ICG-ATT

To confirm whether the fluorescent images detected in paraffin-embedded sections of human gastric cancer using ICG-ATT-labeled anti-MUC 1 antibody were due to the antigen-antibody reaction, a control experiment was performed. After tissue sections were covered with unlabeled anti-MUC1 antibody and incubated at 4℃ overnight, ICG-ATT-labeled anti-MUC 1 antibody was allowed to react with the sections at room temperature for 90 minutes, and fluorescent images were observed. The unlabeled anti-MUC 1 antibody was diluted in PBS at concentrations of 1,000, 500, 250 and 125 μg/ml. As the control, normal mouse antiserum was used similarly and incubated at 4℃ overnight, followed by the reaction of tissue sections with ICG-ATT-labeled anti-MUC 1 antibody, and the fluorescent images were evaluated.

RESULTS

Investigation of the binding of the labeling substance to the antibody

The residual rates of the ICG derivative ICG-ATT and ICG-sulfo-OSu, after acetate treatment were 67.5% and 65.0%, respectively, showing that covalent binding accounted for nearly 70% of the bonds in either derivative-labeled antibody. Almost no difference was observed in the ratio of covalent bonds to all bonds between the two ICG derivatives.

Binding molar ratio of the labeling substances to the antibody (Table 1)

When 4, 8, and 16 molar equivalents of the ICG derivatives were used, the F/P ratios of ICG-ATT-labeled

Table 1. Relationship between F/P ratio and fluorescence intensity among all binding modes of ICG derivatives

ICG-derivative(mol eq.)	F/P ratio	Fluorescence intensity
ICG-ATT(4)	2.94	14.5
ICG-ATT(8)	4.18	17.4
ICG-ATT(16)	6.50	56.8
ICG-ATT(32)	6.75	69.6
ICG-sulfo-OSu(4)	1.50	24.8
ICG-sulfo-OSu(8)	2.90	40.8
ICG-sulfo-OSu(16)	5.97	125
ICG-sulfo-OSu(32)	10.9	200

fluorescent antibody were 2.94, 4.18, and 6.50, respectively, and those of ICG-sulfo-OSu-labeled fluorescent antibody were 1.50, 2.90, and 5.97, respectively. In contrast, when 32 molar equivalents were used, the F/P ratios of ICG-ATT- and ICG-sulfo-OSu-labeled fluorescent antibodies were 6.75 and 10.9, respectively. However, the fluorescence intensity of the ICG-ATT-labeled antibody was generally lower than that of the ICG-sulfo-OSu-labeled antibody. Furthermore, when the ICG-ATT reacted with anti-MUC 1 antibody was doubled from 16 to 32 molar equivalents, almost no change of the F/P ratio was observed, but an increase of the F/P ratio proportional to the increase in the equivalents of ICG-sulfo-OSu was observed.

Observation of infrared fluorescence in tissue sections

When 1,000 and 500 μg/ml ICG-ATT-labeled anti-MUC 1 antibody were used, distinct fluorescence was observed in the cancerous area on the tissue sections. When 250 μg/ml ICG-ATT-labeled anti-MUC 1 antibody was used, the intensity of fluorescence of the cancerous tissue decreased, and the image was slightly indistinct. When 125 μg/ml was used, almost no fluorescence of the cancerous area was observed. We used 6 tissue sections of 3 gastric cancers, and the results were similar in all tissue sections. There were no significant differences in the fluorescence intensity observed with 1000 and 500μg/ml of ICG-ATT-labeled anti-MUC 1 antibody. Moreover, no fluorescence was observed in the normal areas of the tissue sections. Based on these findings, fluorescence-labeled antibody was used at 500 μg/ml for immunohistochemical staining in the rest of the experiments.

ICG-ATT-labeled antibodies with 4 different F/P ratios were reacted with cancer tissue sections as described above, and the fluorescence in the cancerous area was examined. When ICG-ATT-labeled fluorescent antibody with F/P ratios of 2.94 and 4.18 was used, specific and distinct fluorescent images were observed in the cancerous area of the tissue sections. In contrast, when the F/P ratios were 6.50 and 6.75, the intensity of fluorescence in the cancerous area decreased, and the images were indistinct (Table 2 and Fig. 2). When paraffin-embedded sections of tissues adjacent to the gastric cancer were immunostained using ICG-sulfo-OSu-labeled fluorescent antibody, fluorescence was very weak compared to immunostaining

Table 2. Relationship between F/P ratio and fluorescence intensity on tissue sections.

ICG-derivative-labeled MUC 1 antibody	F/P ratio	Fluorescent image
ICG-ATT-labeled MUC 1 antibody	2.94	+++
	4.18	+++
	6.50	++
	6.75	++
ICG-sulfo-OSu-labeled MUC 1 antibody	1.50	++
	2.90	++
	5.97	+
	10.9	+

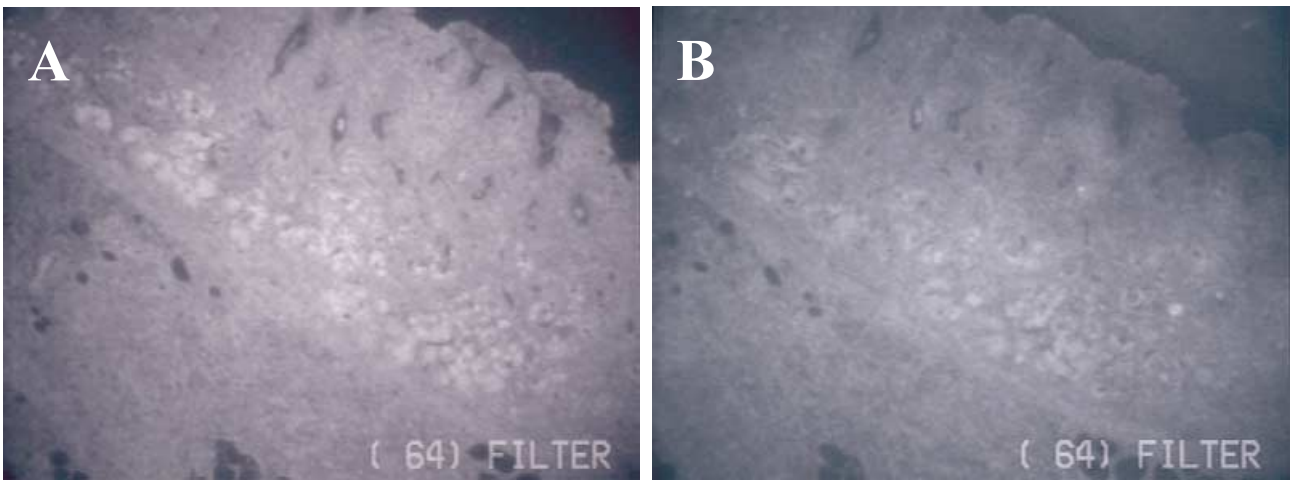


Fig. 2. Infrared fluorescent image of gastric cancer immunostained with ICG-ATT labeled MUC 1 antibody.
A : ICG-ATT/IgG=8 ; B : ICG-ATT/IgG=32. At an F/P ratio of 6.75 images were unclear.

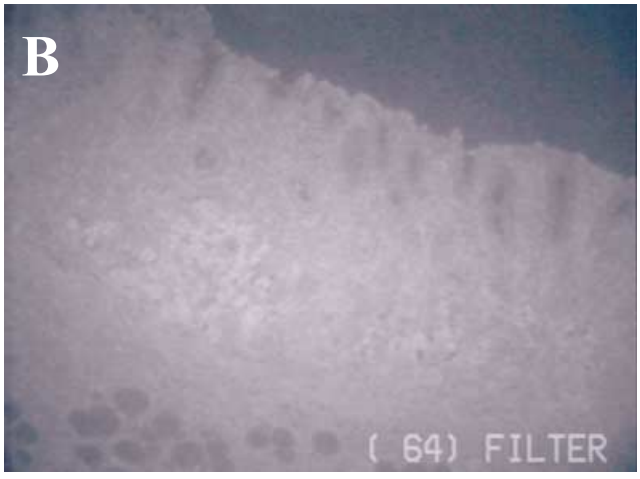
with ICG-ATT-labeled fluorescent antibody, and nonspecific staining of the background was remarkable (Fig. 3 A and B). The regions stained with DAB by the ABC method and the fluorescent regions stained with ICG-ATT-labeled antibody corresponded with each other (Fig. 3 C). Figure 3 shows a tissue section adjacent to the area shown in figure 2.

Investigation of nonspecific reaction with ICG-ATT

After pretreatment with unlabeled anti-MUC 1 antibody, tissue sections were reacted with ICG-ATT-labeled MUC 1 antibody, and infrared fluorescence was measured. When the tissue sections were pretreated with 1,000 or 500 μg/ml of unlabeled anti-MUC 1 antibody, the fluorescence intensity decreased, and no distinct fluorescent image was observed. When the tissue sections were pretreated with 250 and 125 μg/ml of unlabeled anti-MUC 1 antibody, the fluorescence intensity was strong. Distinct fluorescent images were also obtained when the sections were pretreated with normal mouse antiserum (Table 3 and Fig. 4 A-C). We used 6 tissue sections of 3 gastric cancers, and the fluorescence results were similar in all tissue sections. Control experiments on adjacent sections were performed for all immunostained sections. Fluorescence detection and imaging were performed in the dark at the same room temperature, as already described.

Table 3. Infrared fluorescence intensity according to the proportion of labeled and unlabeled antibodies.

ICG-ATT-labeled MUC 1 antibody(μg/ml)	Unlabeled MUC 1 antibody(μg/ml)	Infrared fluorescent image
500	1000	-
500	500	-
500	250	+
500	125	++
500	Mouse lgG	+++



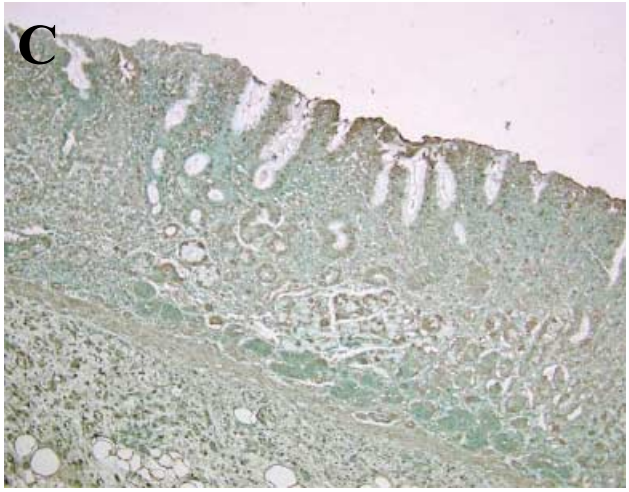


Fig.3. Infrared fluorescent image of gastric cancer immunostained with ICG-derivative labeled MUC 1 antibody.
A:ICG-ATT;B:ICG-sulfo-OSu, ICG derivative/IgG=8. The fluorescence intensity of the antigen stained with the ICG-sulfo-OSu labeled antibody was markedly lower than that stained with the ICG-ATT labeled antibody. Nonspecific fluorescence was predominantly observed in the background region with ICG-sulfo-OSu labeled antibody compared with C stained according to the conventional ABC method using DAB.

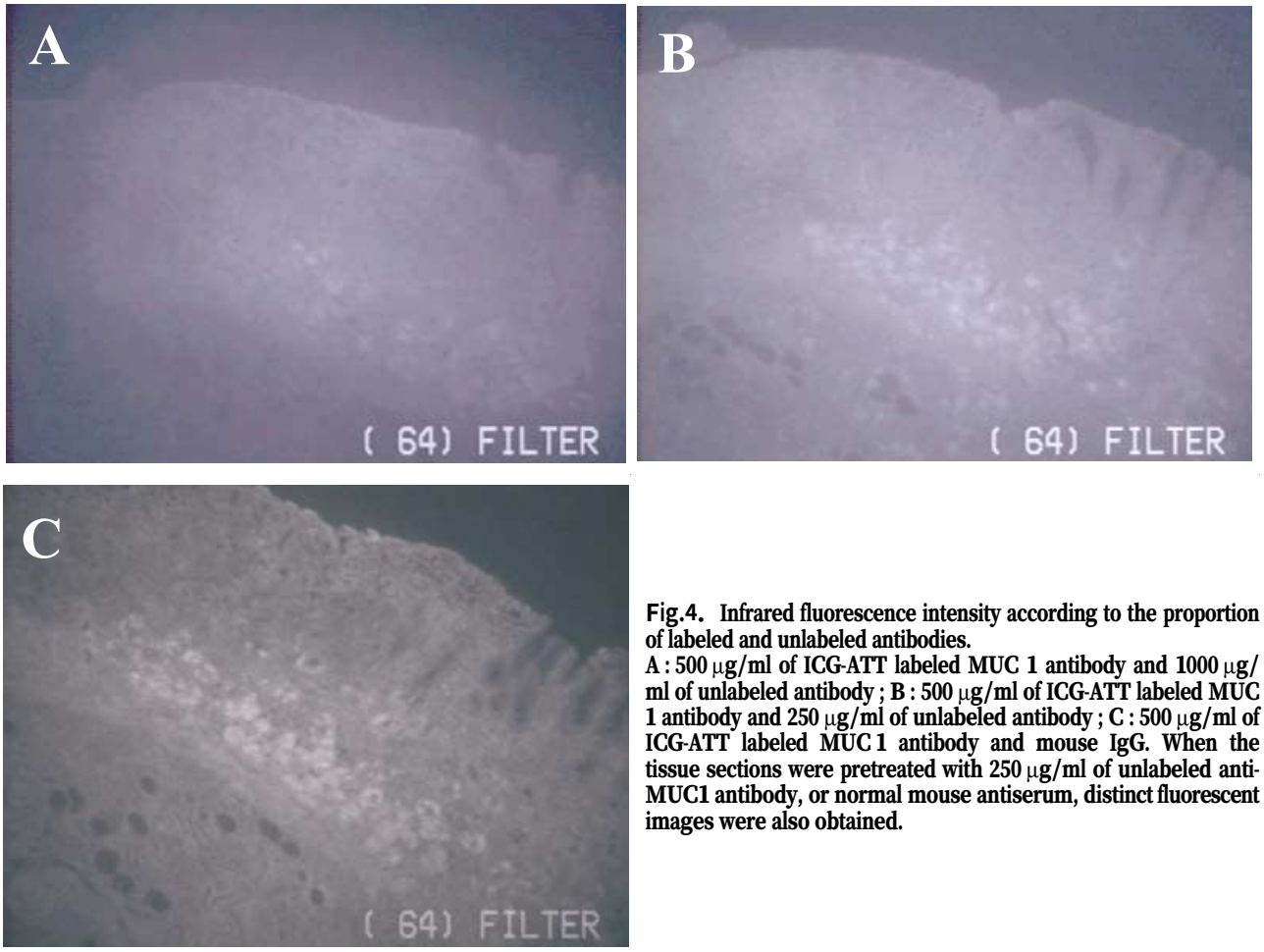


Fig.4. Infrared fluorescence intensity according to the proportion of labeled and unlabeled antibodies.
A : 500 μg/ml of ICG-ATT labeled MUC 1 antibody and 1000 μg/ml of unlabeled antibody ; B : 500 μg/ml of ICG-ATT labeled MUC 1 antibody and 250 μg/ml of unlabeled antibody ; C : 500 μg/ml of ICG-ATT labeled MUC 1 antibody and mouse IgG. When the tissue sections were pretreated with 250 μg/ml of unlabeled anti-MUC1 antibody, or normal mouse antiserum, distinct fluorescent images were also obtained.

DISCUSSION

To date, several procedures using labeled antibodies have been developed for the diagnosis of gastrointestinal carcinoma (3, 7, 23-25). These procedures were reported to be useful because lesion-specific signals were obtained. However, the mode of binding between the fluorescent compound and the antibody remains unclear. Fluorescence in the field of infrared rays has not been used in gastroenterology as yet (29). However, the markedly low biological background noise in the field of infrared rays may facilitate specific diagnosis, differing from diagnostic procedures using autofluorescence (9).

We have previously developed ICG derivatives that can be used to label antibodies for infrared fluorescence imaging (13, 16). However, the mode of binding between those ICG derivatives and antibodies has not been elucidated, although it was suggested that the binding included non-covalent bonds. The results of this study showed that covalent bonds accounted for approximately 70% of all binding between antibodies and ICG-ATT or ICG-sulfo-OSu. In addition, the data shown in Table 3 demonstrate that fluorescence observed on paraffin-embedded tissue sections was produced by the reaction between the labeled antibody and the antigen in the tissue.

However, in our previous study fluorescence intensity was not sufficient to detect micro-lesions. It was considered that fluorescence intensity decreased due to the following reasons:(a) the fluorescent dye itself bound or adhered to the antigen-binding site (hyper-variable region) in the Fab region of the antibody, or (b) the specificity of the antibody for the antigen decreased during the procedure. This was similar to when the binding of a fluorescent dye changes the tertiary structure of an antibody. If a fluorescent dye is electrically charged, the binding of the fluorescent dye alters the entire electric charges in fluorescent-labeled antibody. This may result in nonspecific staining of areas other than the antigenic region due to static electric binding and unclear fluorescent images due to an antigen-antibody reaction. If there is unlabeled fluorescent antibody, the binding of the fluorescent-labeled antibody to the antigen is competitively inhibited by the unlabeled antibody, probably resulting in decreased fluorescence. Therefore, it is important to calculate the binding molar ratio of

the fluorescent dye to the antibody (F/P ratio). The decreased fluorescence intensity of the antigen cannot be avoided when the F/P ratio is markedly high or low. From this point of view, the labeling of the antibody at an optimal F/P ratio is very important.

In this study, we compared ICG-sulfo-OSu and ICG-ATT labeled antibodies regarding optical characteristics, properties, and immunohistological staining (16, 22). As for the optical characteristics of ICG-ATT- and ICG-sulfo-OSu-labeled antibodies, the intensity of fluorescence obtained with ICG-sulfo-OSu-labeled antibody was stronger than that obtained with ICG-ATT-labeled antibody at a uniform concentration of the antibody. Actually, however, when these fluorescent antibodies were reacted with the antigen on tissue sections, results contrary to those expected based on the fluorescence intensity of the labeled antibody were obtained. Fluorescent images of gastric cancer obtained with ICG-ATT-labeled antibody were far clearer than those obtained with ICG-sulfo-OSu-labeled antibody.

One plausible reason for this finding was that the reduction of the MUC 1 antibody specificity may be less when labeled with ICG-ATT than when using ICG-sulfo-OSu. However, it remains unclear whether this finding was due to differences in the absolute number of fluorescent dye molecules bound to the hyper-variable region of the antigen, to differences in the alteration of the tertiary structure of the antibody resulting from the binding to either ICG derivative, or to other unknown factors.

Moreover, differences in nonspecific fluorescence between ICG-ATT- and ICG-sulfo-OSu-labeled antibodies might have influenced the reactivity of the antigen (22). Since carbon chains bound to the framework of ICG differ between ICG-ATT and ICG-sulfo-OSu, the negatively charged terminal side chain of ICG-sulfo-OSu (SO_3) alters all the electric charges in the antibody. Subsequently, if static electricity enhances nonspecific binding of the labeled antibody to surrounding tissues, background tissues are nonspecifically stained when the ICG-sulfo-OSu-labeled antibody is used, probably resulting in decreased reactivity due to the antigen-antibody reaction. In this study, however, the results of conventional immunostaining of tissue sections using fluorescent-labeled antibodies did not show any significant differences in the level of nonspecific staining of background tissues due to differences in fluorescent labeling compounds or F/P ratios. To investigate the cause of differences in fluorescence intensity, experiments facilitating a direct comparison of the numerically evaluated specificity of fluorescent-labeled antibodies and the degree of nonspecific fluorescence may be required in the future. We used anti-MUC1 antibody in this study, which is a tumor marker. More investigations using different antibodies which show cross reactivity with cancer tissues (e. g. anti - CEA antibody) are needed. We presume that similar results should be obtained because the basic structure of antibodies is similar. It has been demonstrated that immunostaining can be performed using both fresh specimens (30) and *in vivo* (31) by distributing labeled antibodies. Actually, a strict toxicity test is needed for ICG-derivatives and labeled antibodies for *in vivo* use because they are totally different from ICG. However, the basic structures of ICG and ICG-derivatives are similar, which may suggest low or no toxicity for the living body.

In conclusion, it is quite likely that ICG-derivative-labeled antibodies will become available for use in clinical settings in the near future.

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