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Development of Agents for Reinforcement of Fluorescence on Near-infrared Ray Excitation for Immunohistological Staining

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Abstract—Fluorescence intensity of indocyanine green (ICG) derivative (ICG-sulfo-OSu) was too low for its use to detect microlesions. Therefore, we examined the effects of reinforcement agents on ICG-sulfo-OSu labeled antibodies. Solutions of distearoylphosphatic acid sodium salt (DSPA) and octylglucoside (OG) in physiological phosphate buffered saline (PBS) were found to increase the intensity of fluorescence of ICG-sulfo-OSu labeled antibodies, with shift in the fluorescence peak wavelength from 804 to 821 nm.

Introduction

With recent progress in medical technology, it should now be possible to develop a method for endoscopic detection of microcancers by computer processing of weak electronic signals obtained by video endoscopy of lesions labeled with a fluorescent substance. For this purpose, a non-toxic labeling substance and an imaging system to detect labeled substances, and a method for vital immunohistochemical staining are required.

Attempts have been made to detect minute cancers of the digestive tract,[1] but with little success. Several labeling substances for detection of minute cancers have been reported[2,3] many of which fluoresce when exposed to ultraviolet rays. However, ultraviolet rays may damage living tissues and DNA,[4] and so antibodies labeled with such substances unsuitable for are use in living organisms.

Key words: Reinforcement agents; fluorescence; infrared rays; distearoylphosphatidic acid sodium salt (DSPA); octylglucoside (OG).
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Previously, we developed an indocyanine green (ICG) derivative (ICG-sulfo-OSu) that is fluorescent on excitation with infrared rays and can be used for vital immunohistochemical staining.[5] We also reported an imaging system for use with this labeling substance.[6] However, the intensity of fluorescence of ICG-sulfo-OSu alone is too low for its use in detection of microlesions. Moreover, its fluorescence intensity is reduced on its binding to antibodies. Therefore, improvement of the fluorescence of this dye is necessary for its use for immunohistochemical staining.

We conjugate an antibody with ICG-sulfo-OSu which has a bridging chain with an reactive succinimide group. This conjugate contains many ICG core moieties emitting fluorescence from amino residues of the antibody (Figure 1). As free ICG adheres strongly to albumin, apparently resulting in quenching of the fluorescence of the conjugated antibody, because the flat structure of ICG molecule is essential for π-orbital electron-conjugation and intense fluorescence.

In this study, we compared the fluorescence intensifies of ICG-sulfo-OSu and ICG-sulfo-OSu-labeled antibody,

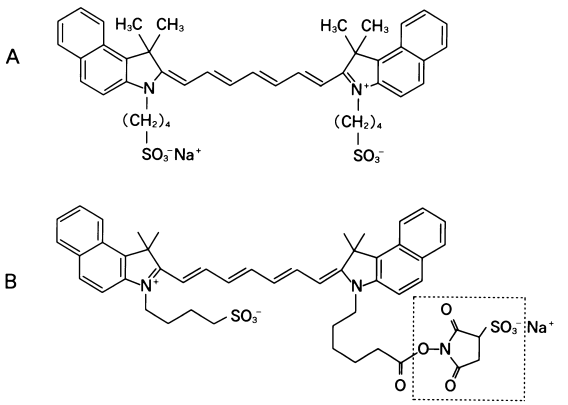

Figure 1. Chemical structures of ICG (A) and ICG-sulfo-OSu (B). ⋯: reactive succinimide group.

and examined effects of Distearoylphosphatidic acid sodium salt (DSPA) and octylglucoside (OG) on the fluorescence intensity of ICG-sulfo-OSu-labeled antibodies.

Results

Infrared fluorescence intensity determined spectrophotometrically

The fluorescence intensity and relevant excitation and emission maxima of ICG-sulfo-OSu and ICG-sulfo-OSu-labeled antibodies, with and without the reinforcement agents were examined (Table 1). In the untreated group (A), the fluorescence intensity of ICG-sulfo-OSu labeled antibody were reduced more than that of ICG-sulfo-OSu. In the treated group (B), the fluorescence intensity of ICG-sulfo-OSu and ICG-sulfo-OSu labeled antibodies were increased, and reinforcement agents had more effect on ICG-sulfo-OSu labeled antibody than on ICG-sulfo-OSu. There was no difference in the excitation maxima in groups A and B, but the fluorescence maximum was shifted from 804 nm to 821 nm (group B). There were no differences in the excitation or emission maxima of the labeled-anti-CEA antibody and labeled-anti-MUC-1 antibody.

Figure 2 shows the effects of the reinforcement agents on the excitation and the emission spectra of ICG-sulfo-OSu-labeled MUC-1 antibody. These agents increased the fluorescence intensity from 0.15 to 0.68 when excited at 768 nm, and shifted the emission maximum from 804 nm to 821 nm when excited at 768 nm.

The effects of various concentrations of OG on the fluorescence intensity and fluorescence wavelength of ICG-sulfo-OSu-labeled anti-MUC-1 antibody are shown in Table 2. Without OG treatment, the fluorescence intensity was 0.153 and the fluorescence peak wavelength was 804 nm whereas on treatment with 150 mM OG, the fluorescence intensity was 0.588, and the fluorescence peak wavelength was 821 nm. The effect of OG was dose-dependent.

Images of labeled substances excited by near-infrared rays

Cotton threads treated with solutions of various concentrations of ICG-sulfo-OSu and ICG-sulfo-OSu-labeled anti-CEA antibody with (B) and without (A) reinforcement agents were excited by near-infrared rays (Table 3). In group A (without agents), there was no difference between the fluorescence intensity with concentrations of 120 μg/mL ICG-sulfo-OSu and ICG-sulfo-OSu-labeled antibody, but fluorescence was less with 10 μg/mL ICG-sulfo-OSu-labeled antibody than with 10 μg/mL ICG-sulfo-OSu. In group B, the effects of reinforcement agents were observed with solutions of 120 μg/mL ICG-sulfo-OSu and 0.1, 1, 10, and 120 μg/mL ICG-sulfo-OSu-labeled antibody. However, the extent of reinforcement was only one level with 120 μg/mL ICG-sulfo-OSu solution, and no reinforcement was observed with 0.1, 1, or 10 μg/mL ICG-sulfo-OSu solution. On the other hand, two levels of reinforcement were observed with 10, and 120 μg/mL ICG-sulfo-OSu-labeled antibody solutions, and reinforcement was

Table 1. Fluorescences of ICG-sulfo-OSu and ICG-sulfo-OSu-labeled antibodies without (A) and with (B) reinforcement agents

	(A)			(B)		
	Excitation maximum (nm)	Emission maximum (nm)	Fluorescence intensity (arbitrary)	Excitation maximum (nm)	Emission maximum (nm)	Fluorescence intensity (arbitrary)
ICG-sulfo-OSu	768	804	0.25	768	821	0.52
ICG*-labeled anti-CEA antibody	768	804	0.15	768	821	0.68
ICG*-labeled anti-MUC antibody	768	804	0.15	768	821	0.68

ICG*, ICG-sulfo-OSu.

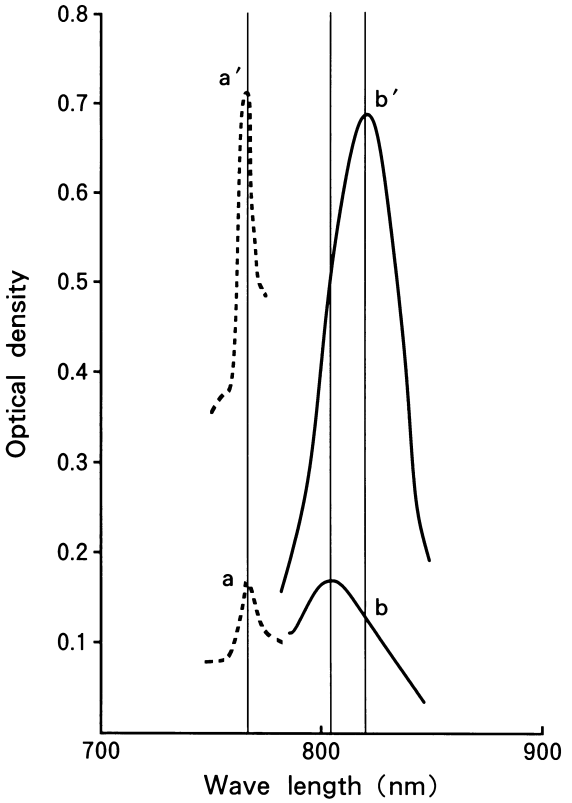

Figure 2. The excitation and emission spectra of ICG-sulfo-OSu labeled anti-MUC-1 antibody before (a,b) and after (a′,b′) treated with reinforcement agents for fluorescence. ------, Excitation spectra: ———, emission spectra.

observed even with 0.1, 1 and 10 μg/mL ICG-sulfo-OSu-labeled antibody solutions, with which no fluorescence was observed in group A. The reinforcement was greater with ICG-sulfo-OSu-labeled antibody than with ICG-sulfo-OSu.

Figure 3 shows images with near-infrared rays of cotton threads treated with 120 μg/mL of ICG-sulfo-OSu-labeled anti-CEA antibody with and without reinforcement agents. Fluorescence is faint in A, and clear in B.

Discussion

Analysis using fluorescence is widely used in various fields because it has high selectivity and sensitivity. However, most reagents used for fluorescence labeling reported to date are toxic and require ultraviolet rays for excitation, so they have not been used for vital immunohistochemical staining. on the other hand, near-infrared rays do not cause tissue damage,[4] and few fluorescent compounds in organisms show fluorescence

Table 2. Effects of various concentrations of OG on spectrum of ICG-sulfo-OSu-labeled anti-MUC-1 antibody

Concentration of OG (mM)	Intensity of flourescence (arbitrary)	Peak wavelength of fluorescence (nm)
0.0	0.153	804
1.0	0.150	806
5.0	0.150	804
10.0	0.189	809
20.0	0.436	817
37.5	0.520	817
50.0	0.515	820
75.0	0.525	816
100.0	0.600	816
150.0	0.588	821

OG, octylglucoside.

Table 3. Effects of the reinforcement agents on spectra of ICG-sulfo-OSu and ICG-sulfo-OSu-labeled anti-CEA antibody on near-infrared ray irradiation

	Concentration of ICG (μg/mL)	A	B
ICG-sulfo-OSu	120.0	+	+ +
	10.0	+	+
	1.0	–	–
	0.1	–	–
ICG-sulfo-OSu labeled antibody*	120.0	+	+ + +
	10.0	–	+ +
	1.0	–	+
	0.1	–	+
Water		–	–

A, without reinforcement agents.
B, with reinforcement agents.
Antibody*, anti-CEA antibody.

on excitation by near-infrared rays.[7] Therefore, a fluorescence dye excited by near-infrared rays should be useful for vital immunohistochemical staining. However, there are few reports on fluorescent labeling substances that show fluorescence on excitation with near-infrared rays.

We synthesized ICG-sulfo-OSu, which can combine with antibodies,[5] because unlike ICG, it has an active succimide moiety. The absorption maximum of ICG-sulfo-OSu in buffer solution is 795 nm. The excitation maximum of its fluorescence spectrum in buffer solution is at 768 nm, and its emission maximum is at 807 nm. Therefore, we constructed an imaging system with an excitation filter transmitting wavelengths of 710–790 nm and a barrier filter transmitting wavelengths of 810–920 nm for examination of ICG-sulfo-OSu fluorescent images.[6]

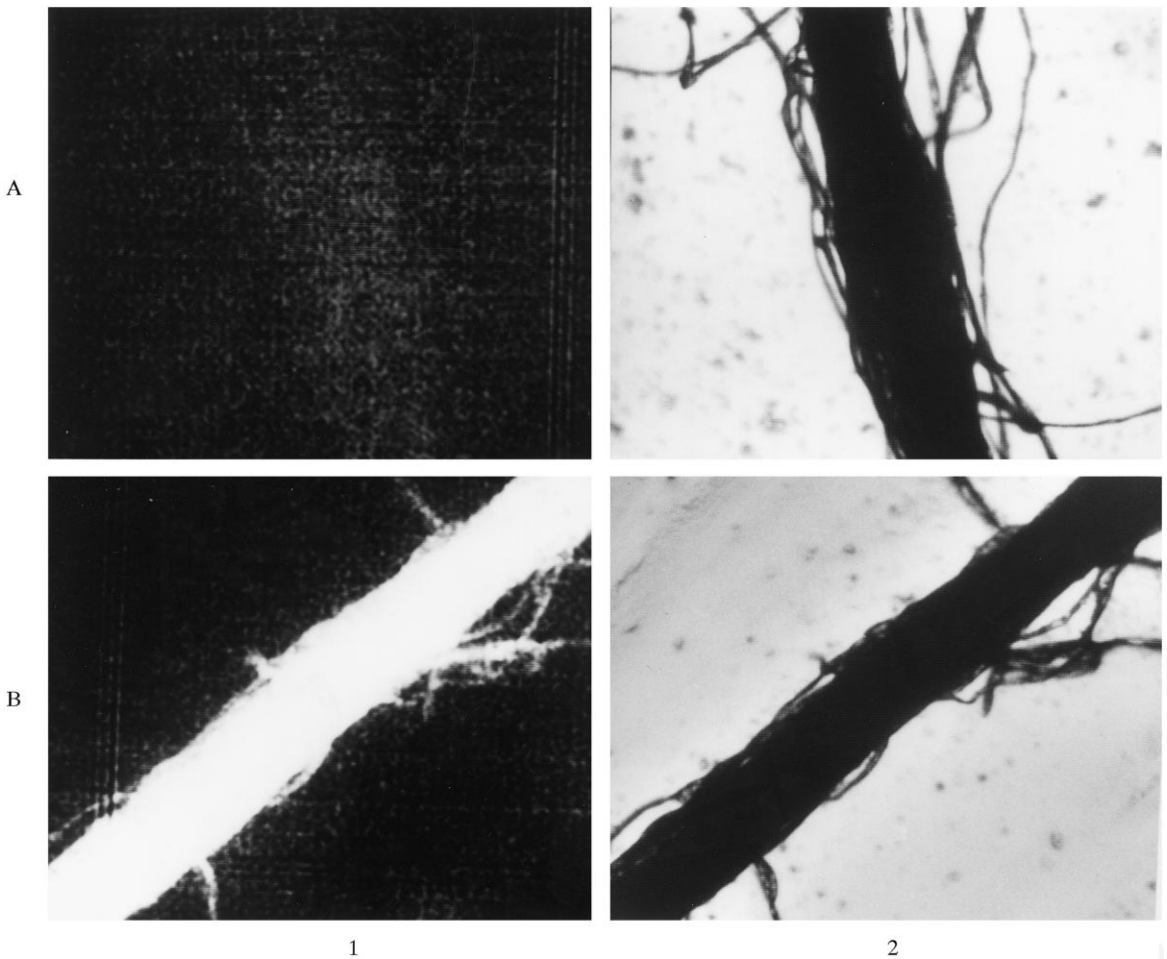

Figure 3. Image with infrared ray of ICG-sulfo-OSu-labeled anti-CEA antibody before (A) and after (B) treated with reinforcement agents for fluorescence. Images with infrared rays (1) and visible rays (2).

However, this imaging system could detect the fluorescence of ICG-sulfo-OSu only at concentrations of 10 μg/mL or more. Thus a more sensitive system was required for vital immunohistochemical staining of microcancers. This could be achieved by improvement of the labeling substances and further amplification of the imaging system.

In this study, we investigated the effects of reinforcement agents on the fluorescence emitted on excitation with near-infrared rays. The ICG-moiety contains highly hydrophobic aromatic rings, like ICG itself, and probably these adhere strongly to the antibodies and interact with the antibody electronically or transform the flat structure of the π-orbit electron conjugation, resulting in the decrease of infrared fluorescence intensity.

The nature of high adhesion of ICG molecule to proteins like as skin and albumin have been known during its synthetic production among us, so we suspected the same character in case of immunoglobin (IgG) conjugated with ICG-sulfo-OSu. When the ICG residues attach to the proteins after the conjugation, ICG core responsive to fluorescence were considered to be deformed to some extent by the adhesion of aromatic two-domains of ICG to IgG by each side and the deformation of ICG seemed to by the case as its lowered fluorescence activity was occurred. This decrement in fluoresence radiation hypothetically seemed to be resulted from the disturbance by the smooth and flat π-electron orbit conjugation and/or the electron-uneven distribution which seems to be keenly related to the activity of the fluorescence radiation.

We therefore decided to examine the effects of separator action to conjugated ICG aromatic core from IgG protein. As the candidate for a separating model, we thought about anion-coated IgG, because ICG core

aromatic domains has bilaterally anionic π-electrons vertically extended from ICG aromatic core. So we selected several phospholipids like as DSPA together with a detergent like as OG among several compounds showing the same nature because those phospholipids are natural compounds and OG is an actually used as a pharmaceutical additives. These compounds seemed to be essentially harmless to a human body as the separator and a possible cleaner for the expected clinical usage.

OG is a considerably safe detergent. OG hardly causes aggregation of proteins and therefore has often been used to solubilize membrane proteins[8] and to reconstitute artificial membrane vesicles (liposomes) having the proteins in the membrane structure.[9] In general, non-ionic detergents, such as OG are less toxic than anionic or cationic detergents. The reconstituted vesicles prepared with OG have been used as vaccines.[10]

We observed less fluorescence of ICG-sulfo-OSu-labeled antibody than of ICG-sulfo-OSu, both free and in combination with cotton, on excitation with near-infrared rays. At concentration of 120 μg/mL, there was no difference in the fluorescences of free ICG-sulfo-OSu and ICG-sulfo-OSu-labeled antibody, but at concentrations of 10 μg/mL, the fluorescence of the latter was lower.

We therefore examined the effects of detergents that could coat the antibody with a cation-layer or cation-coat, preventing the adhesion and maintaining the flat molecular structure of ICG moieties. For this purpose, we chose glycerophosholipids, carbonic acids and sugar derivatives because the former is a natural compound and seems harmless in humans, and the both are probably less toxic than other detergents. We found that, DSPA and OG were effective for reinforcement of fluorescence.

The effects of these agents in reinforcing fluorescence were greater with ICG-sulfo-OSu-labeled antibody than with ICG-sulfo-OSu. They caused not only increase in fluorescence intensity but also a shift in the fluorescence peak from 807 nm to 821 nm. Therefore, fluorescence could be observed with an imaging system for labeled substances excited by near-infrared rays using a barrier filter with transmission wavelengths of 810–920 nm.

The reasons for reinforcement of the fluorescence of the ICG-sulfo-OSu conjugated antibody seem to be (1) more efficient absorption of infrared waves of 700 nm to 790 nm, especially 768 nm, (2) shift of the emission peak of the ICG moiety from 807 nm to 821 nm which is not blocked appreciably by a barrier filter and (3) higher fluorescence. The first and third reasons support our idea of the flat molecular structure of ICG, while the second reason, shift of the emission peak, allowed amount more efficient and convenient filter settings for the systems.

There are still many problems to be over come before practical use of this system for detection of micro-cancers. But this work shows that the sensitivity of fluorescence on vital immunohistochemical staining can be increased by certain agents.

Experimental

Materials

ICG-sulfo-OSu (Daiichi Pure Chemicals Co., Tokyo, Japan)[5] and the imaging system for infrared rays[6] reported previously were used. In addition, we used a microscope (BX-60, Olympus Optical Co., Ltd. Tokyo, Japan), an excitation filter and a barrier filter (Olympus Optical Co., Ltd.), an intensified charge-coupled device (ICCD) camera (ICCD-500/DF, Hamamatsu Photonics, Hamamatsu, Japan) from which the infrared barrier filter was removed, an image capturing device (EVIP-230, Olympus Optical 12 Co., Ltd.), and a magneto-optical disk drive subsystem (MOS321S Olympus Optical Co., Ltd.), serving as an image storage device. Based on the characteristics of the absorption spectrum of ICG-sulfo-OSu, band pass filters with transmission wavelengths of 710–790 nm and 810–920 nm were used as an excitation and a barrier filter, respectively.

Mouse anti-human carcinoembryonic antigen (CEA) monoclonal antibody was obtained from Chemicon International Inc., California, USA (Lot number 31696285). A novel monoclonal antibody of MUC-1 mucin glycoprotein (MUC-1) was obtained from Tatsuro Irimura, Division of Chemicals, Faculty of Pharmaceutical Sciences, The University of Tokyo.[11] In human, eight distinct epithelial mucin genes (MUC1-MUC7) have been identified. MUC-1 mucin is a transmenbrane glycoprotein with an extra cellular domain, and like other epiterial mucins, seems to be expressed in an organ-specific manner. Anti-CEA and anti-MUC-1 antibodies were labeled with ICG-sulfo-OSu as reported previously.[5]

Mixtures of human IgG (2.8 mg) dissolved in 4 mL of 100 mM sodium bicarbonate buffer (pH 8.5) and 40 μL of 6 mM ICG-sulfo-OSu dissolved in dimethylsulfoxide (DMSO) solution were incubated at 37 °C for 1 h. The ratio of ICG residue to IgG were kept 16 residues/IgG when the conjugation procedure was reacted.

DSPA and OG were obtained from Dojindo Laboratories (Kumamoto, Japan) for reinforcement of fluorescence.

Fluorescence emitted on near-infrared ray excitation was measured with a spectrophotometer (650-40, Hitach Electronics Inc. Tokyo, Japan)

Reagents

OG (880 mg) was dissolved in 10 mL of physiological phosphate buffered saline (PBS) (pH 7.4) at 40 °C. DSPA (148.8 mg) was dissolved in PBS at 60 °C, and then PBS was added to give a final volume of 20 mL. The solutions were stored as 1 mL aliquots at −10 °C. Final concentrations of 10 mM DSPA and 150 mM OG were used.

ICG-sulfo-OSu-labeled antibody (54 μg) was dissolved in 20 μg of 10% (v/v) dimethylsulfoxide (DMSO) in PBS (pH 7.4) solution, and 5 μL portions of the solution were mixed with an equal volume of reinforcement agent for 30 min at 40 °C before use. Mixtures of ICG-sulfo-OSu and water with the same volumes of reinforcement agents served as controls. Mixtures of ICG-Sulfo-OSu labeled antibody, ICG-sulfo-OSu and water with equal volumes of PBS, were used as test and control samples without reinforcement agents.

For examination of the effect of reinforcement on the fluorescence of OG alone, various concentrations of OG (0–150 mM) were also prepared. A volume of 1.5 mL of ICG-sulfo-OSu labeled anti-MUC-1 antibody (400 μg) dissolved in 16.0 mL of PBS (pH 7.4) solution was incubated with 1.5 mL of OG solution for 30 min at 50 °C.

Intensity of fluorescence

Spectrophotometric estimation of optical density. The excitation and emission spectra of the fluorescence of ICG-sulfo-OSu-labeled anti-MUC-1 antibody before (A) and after (B) treatment with the reinforcement agents were measured spectrophotometrically.

One ppm sulfo quinine and water were used as a standard and control, respectively.

Observation of images

For examination of the intensity of fluorescence of labeling substances, pieces of cotton thread stained with solutions of ICG-sulfo-OSu and ICG-sulfo-OSu-labeled antibody with and without reinforcement agents were photographed under infrared rays using the imaging system, and contrast intensified images were produced and compared. A cotton thread treated with water alone was used as a control.

The fluorescence intensity was graded as; no fluorescence (−), fluorescence discernible (+), fluorescence of intermediate intensity (++) and clear fluorescence (+++).

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