Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 5, No. 22, pp. 2689-2694, 1995

0960-894X/95 $9.50+0.00

0960-894X(95)00452-1

Development of Fluorescence-Emitting Antibody Labeling Substance by Near-Infrared Ray Excitation

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Abstract: Indocyanine green succinimidyl esters, near-infrared labeling dyes, were synthesized. These reagents were indocyanine green derivatives possessing N-hydroxysuccinimidyl groups capable of reacting with proteins. The absorption maximum of indocyanine green-labeled human IgG was 785 nm, and its fluorescent excitation and emission maxima were 768 nm and 807 nm, respectively.

Along with the advancement in immunohistochemistry, the use of labeled antibodies has made it possible to identify small lesions and individual cells. At present, antibody labeling substances possess the structure of chromophores or fluorophores in general and are able to link with antibodies. These substances are suited for detection by photometrical methods, such as absorption spectrophotometry, fluorophotometry, and chemiluminescence spectroscopy. Recently, in order to increase detection sensitivity, many attempts have been made to emit fluorescence by ultra violet (UV) ray excitation. Since tissues and DNA can be damaged by UV rays, such fluorescence emitting substance can not be administered to the human body upon excitation with UV. So far, no appropriate antibody labeling substances suitable for vital immunohistochemical staining have been developed. Only the observation of blood vessels in the stomach by infrared endoscopy using indocyanine green (ICG) has been reported[1,2].

Due to recent developments in medical devices, minute cancers that can not be detected by the naked eye could possibly be detected-if an antibody labeling substance detectable under electron endoscope could specially label the lesion, it would be possible to establish a novel diagnostic method for the minute cancers by modulating the signal from an electron endoscope assisted by a computer.

In order to realize the above objective, an antibody labeling substance for vital immunohistochemical staining needs to be developed. We developed an ICG-labeled antibody that emits fluorescence upon excitation with infrared (IR) ray and studied the possibility of its application for vital immunostaining.

Method and Materials

ICG derivatives were prepared as shown in Fig. 1. Both indocyanine green N-hydroxy-succinimide ester (ICG-OSu) and indocyanine green N-hydroxysulfosuccinimide ester (ICG- sulfo-OSu) were derived from 1,1,2-trimethyl-[1H]-benz[*e*]indole. The initial materials-1,1,2-trimethyl-[1H]-benz[*e*]indole and 3-(4-sulfobutyl)-1,1,2-trimethyl-[1H]-benz[*e*]indolium inner salt-were obtained from Daiichi Pure Chemicals Co. Ltd.(Tokyo, Japan), and N-hydroxysulfosuccimide sodium salt was purchased from Fluka Chemical AG(Switzerland). Glutaconaldehyde dianil hydrochloride was obtained from Tokyo Chemical Industry Co., Ltd.(Tokyo, Japan). Other reagents for synthesis were purchased from Wako Pure Chemical Inc.(Osaka, Japan). Human IgG was obtained from Sigma (St. Louis, USA), and PD-10 packed column for the purification of labeled protein was purchased from Pharmacia (Sweden). Phosphate buffered saline (PBS) was prepared(50 mM phosphate, 0.1 M sodium chloride, pH 7.5). Absorption and fluorescence spectra were measured on a UV 260 Shimadzu spectrophotometer and 650-60 Hitachi fluorophotometer, respectively. Intermediates and final compounds were analyzed by a proton NMR spectrometer and a mass spectrometer. Proton NMR spectra were obtained with an AC200 Bruker NMR spectrometer. Mass spectra were measured on a JEOL AX505W mass spectrometer.

Preparation of ICG-sulfo-OSu and ICG-OSu

A synthetic scheme of ICG-sulfo-OSu and ICG-OSu was shown in Fig. 1. A mixture of 1,1,2-trimethyl-[1H]-benz[*e*]indole and ethyl 6-bromohexanoate dissolved in dimethylformamide (DMF) was heated at 100℃ for 40 h. The residue of the reaction mixture was washed with ether several times. The resulting brown residue (compound **2**) was hydrolyzed with sodium hydroxide to make compound **2'** a slightly blue solid. Glutaconaldehyde dianil hydrochloride and compound **6** were dissolved in acetic anhydride, and then the solution was heated for 5 min. The residue of the reaction was washed with saturated sodium chloride aqueous solution, and then with ether. The solid was collected with filtration to render compound **7** into a red-purple powder. A methanol solution of compound **2'** and compound **7** was stirred at ambient temperature for 20 h. The reaction mixture was purified with flush chromatography (Silicagel 60, 5-30% methanol/chloroform) to make compound **5** as dark green powder. N-hydroxysulfosuccinimide sodium salt and compound **8** were linked by dicyclohexylcarbodiimide (DCC) in dry DMF at 0℃. The reaction mixture was poured into ether, and the resulting solid was washed with ether several times, and then collected by centrifugation to produce ICG-sulfo-OSu as green powder. ICG-OSu was synthesized in a same manner as ICG-sulfo-OSu indicated in figure legend of Fig. 1.

Preparation of ICG-labeled IgG

Human IgG was labeled with ICG-OSu or ICG-sulfo-OSu. 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℃ for 1 h. The molecular ratio of human IgG to ICG

derivative was 1 to 12. The reaction mixture was purified with PD-10, and eluted with 50 mM PBS buffer. Dye-conjugated IgG was well-separated from the free dye. ICGsulfo-OSu-labeled human IgG as green solution was immediately freeze-dried. As water solubility of ICG-OSu was lower than that of ICG-sulfo-OSu, six parts of ICG-OSu was used to one part of human IgG. The stability of ICG-labeled protein was determined by measuring 790 nm absorption spectrum of PBS solution of ICG-sulfo-OSu-labeled human IgG.

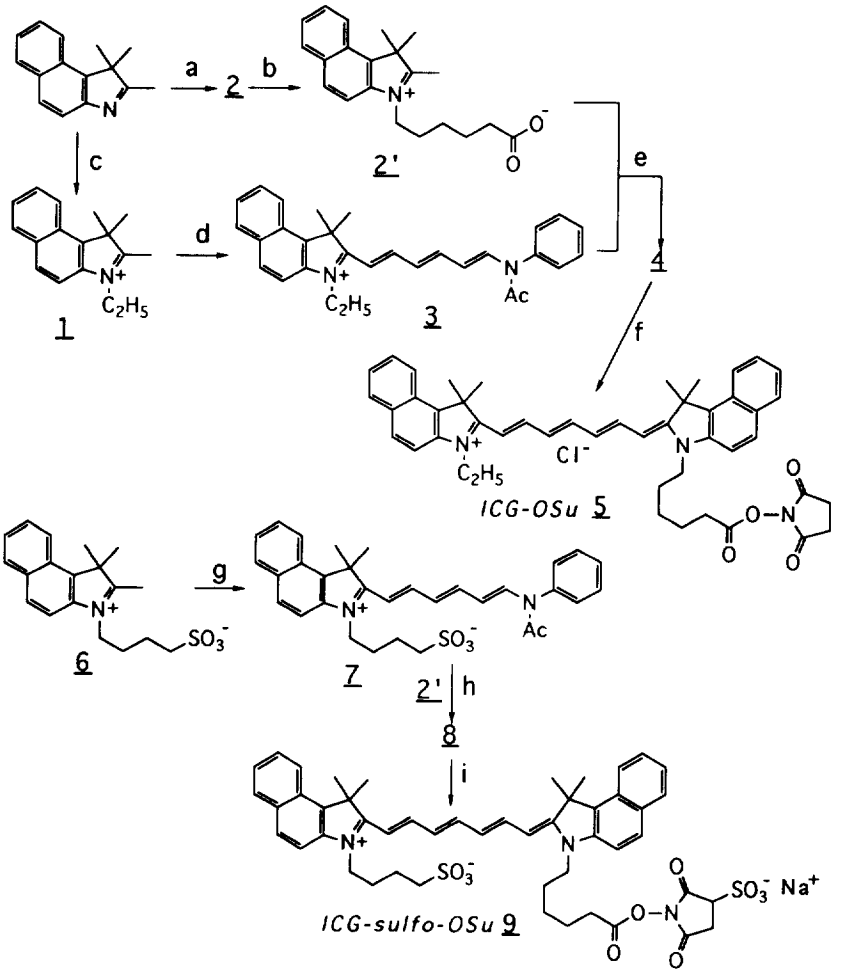

Fig. 1 Synthetic Scheme of ICG-OSu and ICG-sulfo-OSu. a) Ethyl 6-bromohexanoate, DMF, 100℃; b) NaOH aq., room temperature, yield 58%; c) Ethyl iodide, CH3CN, reflux, yield 94%; d) Glutaconaldehyde dianil HCl, acetic anhydride, 80℃, yield 18%; g) Glutaconaldehyde dianil HCl, acetic anhydride, 80℃, yield 32%; e) Pyridine, 50℃, yield 75%; h) MeOH, room temperature, yield 70%; f) N-Hydroxysuccinimide, DCC, DMF, 0 ℃-room temperature, yield 41%; i) N-Hydroxysulfosuccinimide, sodium salt, DCC,DMF, 0℃-room temperature, yield 77%.

Results and discussions

ICG has been used as a diagnostic reagent for a liver function test; the indocyanine green loading test. The absorption maximum of ICG in buffer solution at physiological pH is at 785 nm; also, ICG emits maximum fluorescence at 800 nm. In immunohistochemistry, fluorescence-labeled antibodies has been utilized for the determination of an antigen on a cell surface or a tissue, in order to sensitively detect a target material. Recently, several near-infrared dyes, such as polymethine compounds, have been developed and used for the labeling of biomaterials[3-5]. Reactive moieties of these labeling reagents are succinimidyl esters or isothiocyanates able to link with amino groups. For diagnosis using these dye-labeled antibodies, the spectrum of the labeling reagents is important for the precice detection of stained parts by the antibody. To avoid the interference of blood, a longer wavelength of the labeling reagent is required. ICG seems to be a suitable molecule for preparing a labeling dye for immunohistochemical diagnosis of the digestive tract-such as esophagus, stomach, large intestine, and rectum because of its spectrum property and low toxicity.

As formation of protein-protein crosslinking by a labeling reagent is a disadvantage for effective immunohistochemical staining with the dye-labeled protein, the labeling reagent should have a single reactive group with a protein molecule. Succinimidyl ester was selected as a reactive site to amine moiety of protein. In order to prepare ICG derivative, which has a single reactive group, the 4-sulfobutyl group of ICG was substituted for the sulfosuccinimidyloxycarbonylpentyl group. 1,1,2-trimethyl-[1H]-benz[*e*]indole was used as a starting material to produce 3-(5-carboxypentyl)-1,1,2-trimethyl-[1H]-benz[*e*]indolium inner salt (**2'**), then compound **2'** and 3-(4-sulfobutyl)-1,1,2-trimethyl-[1H]-benz[*e*]indolium inner salt were linked with glutaconaldehyde dianil. As the resulting polymethine dye **8** has one carboxyl group, ICG-sulfo-OSu was derived from compound **8** with N-hydroxy sulfosuccinimide and DCC. As the dye moiety of an ICG-labeled protein is identical with ICG itself, the toxicity of the labeled protein may not be increased by the dye-labeling.

As water solubility of ICG-OSu was extremely poor, the additon of sulfonate was expected to make water-soluble ICG-OSu. ICG-sulfo-OSu has two sulfonate groups, but it is not sufficient to dissolve in water or buffer. In the labeling reaction, ICG-sulfo-OSu was added to the buffer solution of protein as DMSO solution. As shown in the experimental section, about twelve molecules of ICG could link with one molecule of human IgG. Spectral properties of ICG derivatives were the same as that of ICG. The absorption maximum of ICG-sulfo-OSu/DMSO solution was 795 nm, and its molar absorptivity was 1.28×10^5 $M^{-1}cm^{-1}$. The excitation maximum of the fluorescence spectrum of ICG derivatives dissolved in 10v/v% DMSO/PBS was 768 nm, and the emission maximum was 807 nm. The absorption and fluorescent spectra of ICG-labeled human IgG are shown in Fig. 2 and Fig. 3, respectively. In general, the absorption maximum of a dye tends to cause red-shift by making an adduct with a protein; however, spectrum shift after labeling with protein was not observed. It seems that the ICG-moiety of the labeling dye may not be surrounded by the hydrophobic site of IgG.

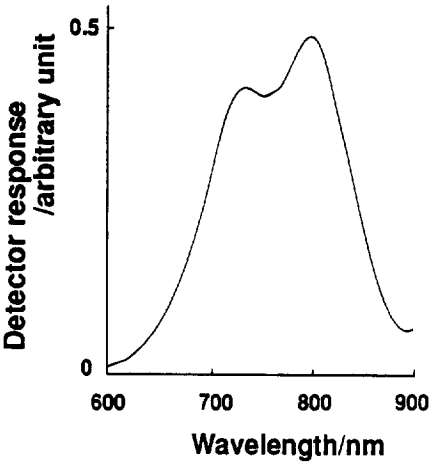

Fig. 2 Absorption spectrum of ICG-sulfo-OSu labeled human IgG.

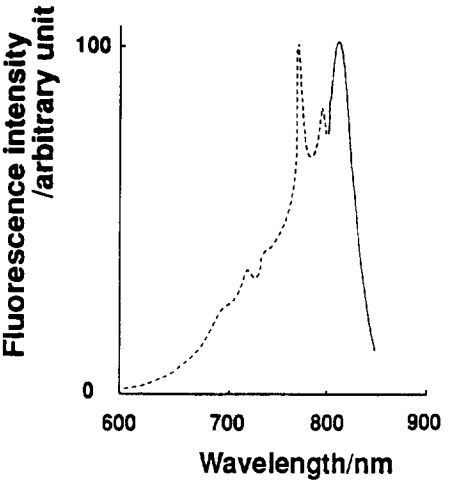

Fig. 3 Fluorescent spectrum of ICG-sulfo-OSu-labeled human IgG.
Excitation spectra(---), Emission spectra (—)

The stability of the buffer solution of ICG-labeled IgG is shown in Fig. 4. Fig. 4 shows that the half life time of the ICG dye in a PBS buffer at physiological pH was about three hours, so ICG-labeled protein must be freeze-dried for long-term storage.

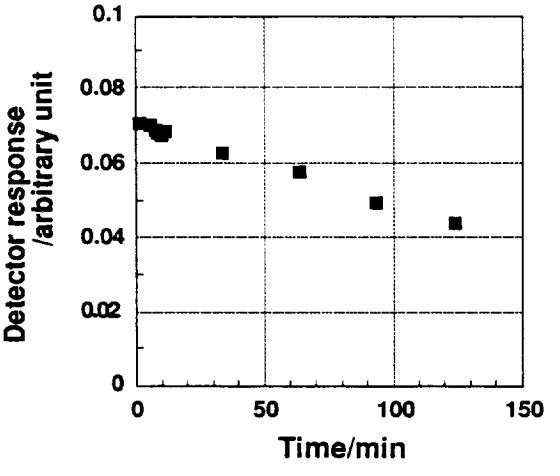

Fig. 4 Time dependency of absorption intensity of ICG-sulfo-OSu-labeled human IgG PBS solution. The absorption intensity of the 1 μM ICG-sulfo-OSu-labeled human IgG in PBS buffer was measured at 790 nm.

In order to realize detection of minute cancer in a digestive tract utilizing these ICG-labeled antibodies by an electron endscope, it is necessary to develop a filter system. From the spectral property of ICG-labeled IgG, excitation must be in the range between 700 and 790 nm, and the emission must be in the range between 800 and 860 nm. Our next issue is to develop an electron endoscope for fluorescent analysis in infrared regions.

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(*Received in Japan* 20 *September* 1995; *accepted* 9 *October* 1995)