Antibody-Indocyanin Conjugates for Immunophotodetection of Human Squamous Cell Carcinoma in Nude Mice

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ABSTRACT

We have recently shown that immunophotodetection of human colon carcinomas in nude mice and in patients is possible by using anti-carci-noembryonic antigen monoclonal antibodies (MAb) coupled to fluorescein. The most common clinical application of photodiagnosis has been for the detection of squamous cell carcinomas (SCC) in the upper respiratory tract, but the free dyes used have a poor tumor selectivity. We selected the known MAb E48 directed against SCC and coupled it to a fluorescent dye: indopentamethinecyanin (indocyanin). This dye has an advantage over fluorescein in that its absorption and emission wavelengths are above the autofluorescence of normal tissues. However, the free dyes used have a poor tumor selectivity. We selected the MAb-dye conjugate strategy because it allows a much higher tissue penetration than fluorescein, with a very minimal decrease in intensity in the tumor, indicating a lack of tumor selectivity for the free dye. The results demonstrate the possibility of improving the efficiency of tumor immunophotodetection by coupling to a MAb directed against SCC, a fluorescent dye absorbing and emitting at higher wavelength than fluorescein, and thus having deeper tissue penetration and lower tissue autofluorescence. Such a demonstration opens the way to a new form of clinical immunophotodetection and possibly to the development of a more specific approach to photodetection of early bronchial carcinomas.

INTRODUCTION

After systemic injection of photoactive dyes, laser-induced fluorescence endoscopy was shown to be helpful for the photodiagnosis and therapy of early bronchial carcinoma (1-8). Such a procedure, although limited to accessible tumor (directly or endoscopically), can be applied to many tumors, including carcinomas of the respiratory, gastrointestinal, and urogenital systems as well as cutaneous and skin cancer (9).

A major limitation of tumor photodetection and therapy, however, is the lack of selectivity for cancerous tissues of the presently available dyes. To overcome this problem, coupling the dyes to MAB directed against tumor-associated antigen has been proposed. The obvious advantage of using a MAb as a vector for tumor localization of a dye is the property of the MAb to bind specifically to a marker which is more abundant in tumor than in normal tissue. Another advantage of the MAb-dye conjugate strategy is that the dye can be selected on the basis of its optimal photochemical properties and not its tumor-localizing capacity, since the tumor selectivity is provided by the MAb specificity.

Dyes such as porphyrins or chlorins have been coupled to MAb directed against various tumor markers, but these conjugates were studied primarily in vitro (10-13) and the few experimental immunophototherapy studies have not yet given highly significant results (14).

Recently, we have shown that MAb directed against the CEA, conjugated with up to 10 molecules of fluorescein per MAb molecule, were able to localize specifically in vivo in human colon carcinomas almost as well as 111-I-anti-CEA antibodies (15, 16). The studies of MAb-fluorescein conjugates performed first in a nude mouse xenograft model (17) and later in a pilot clinical study (18), demonstrated that the small amount of fluorescein (15 to 80 ng/g) specifically localized in the tumor by the antibody was sufficient to allow a clearly detectable laser-induced fluorescent signal in the tumor.

The weakness of the fluorescein anti-CEA-MAB conjugate, however, is that the fluorescein absorption and emission wavelength of 488 and 515 nm, respectively, are too low for satisfactory tissue penetration, and secondly, the exciting laser light used induced an important nonspecific yellowish autofluorescence in several normal tissues such as the retinal mucosa (18), which, despite sophisticated computerized technology (7, 19), can sometimes interfere with optimal detection of the specific green fluorescence due to fluorescein. A third limitation of the fluorescein anti-CEA conjugate is that CEA is not highly expressed in SCC of the upper respiratory and digestive tracts in which most of the pioneer photodiagnosis and therapy work has been performed.

The purpose of the present study is to determine in nude mice grafted with the human SCC A431, whether the MAb E48 directed against SCC (20), which has been shown after radio-labeling both at experimental and clinical levels to localize well in human SCCs (21, 22), could be useful as a vector to bring specifically into the tumor a new dye, indocyanin. This dye has never been used previously for tumor photodetection and was selected on the basis of its long absorption and emission wavelength of 652 and 667 nm, respectively, which allows a much higher tissue penetration than fluorescein, with which it will be compared.

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3 The abbreviations used are: MAb, monoclonal antibody; SCC, squamous cell carcinoma; CEA, carcinoembryonic antigen; indocyanin, indopentamethinecyanin; PBS, phosphate-buffered saline; BSA, bovine serum albumin; % ID/g, percentage of injected dose per g of tissue.

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Materials and Methods

Monoclonal Antibody. Monoclonal antibody E48 (IgG1), kindly provided by Centocor, Inc. (Leiden, the Netherlands), was derived from mice immunized with cells from a metastasis of a moderately differentiated SCC of the larynx (23). The antigen recognized by MAb E48 was reported to be expressed within these tumors. A comparable reactivity pattern was observed in 26 tumor-infiltrated lymph nodes from neck dissection specimens (24). Antibody reactivity with normal tissue is restricted to normal stratified squamous cell epithelium as well as urothelium of the bladder.

In several experiments we used as a control an irrelevant mouse IgG1 (PxlgG1), purified from ascites produced by the secreting mouse myeloma P3X63 (25).

Preparation of Indocyanin-MAb Conjugate. Prior to coupling indocyanin to MAB E48 or control PxlgG1, the MAb and IgG were trace labeled with 125I. One or 2 mg of MAb E48 or PxlgG1 were labeled with 60 or 120 μCi 125I (1 Ci = 37 GBq) by the Iodo-Gen (Pierce, Rockford, IL) method, yielding a specific activity of about 0.05 μCi/μg protein. Free 125I was separated from labeled MAb on a Sephadex-G25 column (Pharmacia, Uppsala, Sweden) in equilibrated in 0.15 M sodium bicarbonate buffer, pH 9.5.

Indocyanin-N-hydroxysuccinimidyl ester was produced under the name of CY5.18 by Biological Detection System, Inc. (Pittsburgh, PA), that we refer to as indocyanin, has the following specifications. It is highly soluble in water, has a molecular weight of around 750, has a maximum absorption at 652 nm, a maximum emission at 667 nm, an absorption molar coefficient of 200,000 M\(^{-1}\) cm\(^{-1}\), and a quantum yield of fluorescence of 0.28.

Twenty μg, representing 26.66 × 10\(^{-9}\) mol of indocyanin N-hydroxysuccinimidyl ester, were dissolved in 25 μl of pure dimethylformamide and were progressively added to 1 mg of 125I-labeled MAb, which represents 6.66 × 10\(^{-7}\) mol of antibody diluted in the sodium bicarbonate buffer at a concentration of 4 mg/ml. The final dimethylformamide concentration did not exceed 10% (v/v) of the reaction volume. After gentle shaking during 4 h at room temperature, the conjugate was filtered through a Sephadex-G200 gel column (Pharmacia) to remove free indocyanin and any aggregated material generated by the labeling process. The indocyanin:MAb molar ratio was determined by using absorbance at 652 nm for the indocyanin concentration and radioactive measurement for MAb quantitation. The same procedure was used for coupling indocyanin to control PxlgG1, trace labeled with 125I. We verified that the conjugation did not change the absorption or emission spectra of the dye, as indicated by the manufacturers. The affinity of the conjugates was almost identical to that of the unconjugated MAb in the range of 4.4 × 10\(^{10}\) M\(^{-1}\) as determined by direct binding to living A431 carcinoma cells and Lineweaver-Burke reverse plot analysis. For the fluorescein conjugate, 50 μg (128.53 × 10\(^{-9}\) mol) of fluorescein isothiocyanate (Isomer 1, Sigma, St Louis, MO) was coupled to 1 mg (6.66 × 10\(^{-9}\) mol) of 125I-labeled MAB E48, as described (17).

In Vitro Immunoreactivity and Stability of Indocyanin-MAb Conjugate. The immunoreactivity of the indocyanin-MAb conjugate was determined in vitro by a direct binding assay to cells from the SCC line A431 (26). Single cell suspension from the A431 cell line, which grew as an adherent monolayer in RPMI culture medium, were obtained by a 20-min incubation at 37°C with PBS containing 0.05% trypsin and 0.02% EDTA (w/v) (Seromed, MO). Briefly, 200 ng representing 10 nCi of 125I-MAb E48 (indocyanin), conjugate or of the 125I-unconjugated MAb E48 were incubated for 4 h at 4°C with 1.6 × 10\(^{6}\) living cells of the A431 carcinoma line in 150 μl of PBS containing 1 mg/ml of BSA. After two washes with cold (4°C) PBS containing 0.02% azide, 20 mm EDTA, and 1 mg/ml of BSA, the radioactivity bound to the cells was counted and expressed as percentage of bound cpm.

The same cell binding assay was used for the 125I-MAb-(fluorescein)\(_2\) conjugate. Nonspecific binding was determined after a similar incubation with the use of the melanoma cell line Me-67 as a negative control.

To test its stability a conjugate sample [920 μg of 125I-MAb-(indocyanin)] diluted in 300 μl of PBS, pH 7.4, was incubated for 24 h at 37°C with 500 μl normal mouse serum containing 0.02% sodium azide. After this incubation the conjugate was filtered on a Sephadex-G200 column (Pharmacia) and the eluent was analyzed for absorption at 652 nm and 125I radioactivity for detection of indocyanin and MAb, respectively. The same procedure was used for the 125I-MAb-(fluorescein)\(_6\) conjugate, except that the absorption was analyzed at 488 nm.

In Vitro Characterization of Indocyanin-MAb Conjugate. Indocyanin was solubilized in pure dimethylformamide and incubated at room temperature with the MAb E48 previously labeled with trace amounts of 125I. After the coupling reaction, the conjugate preparation contained no detectable MAb aggregates as determined by Sephadex-G200 gel filtration. The eluted conjugate gave only a single peak of radioactivity at a retention volume identical to that of the IgG peak of the normal mouse serum, which was in complete correlation with the absorbance peak of indocyanin at 652 nm (Fig. 1A).

The additional labeling of the conjugates with 125I allowed more precise in vitro and in vivo study of the immunoreactivity and biodistribution of the MAb-(indocyanin)\(_2\) or -(fluorescein)\(_2\) conjugates. Table 1 shows that the percentages of binding of the conjugates to A431 SCC line were almost as high as that obtained with the unconjugated MAB, ranging from 79.9 to 82.9%, while the nonspecific...
binding on the melanoma cell line Me 67 used as negative control, ranged from 0.1 to 2.4%.

The stability of a $^{125}$I-MAb-(indocyanin)$_2$ conjugate was studied in vitro after a 24-h incubation at 37°C in normal mouse serum. Subsequent filtration of the conjugate on Sephadex-G200 showed a major peak of absorption at 652 nm correlating with the radioactivity and with the elution of the IgG peak from mouse serum (Fig. 1B). However, a first peak representing about 20% of the total absorbance at 652 nm was detected which correlated with only a very small peak of radioactivity. This peak at 652 nm eluted at the exclusion volume of the column, suggesting the presence of a population of antibody molecules more heavily conjugated with indocyanin, which formed aggregates during the incubation period (Fig. 1B).

The last in vitro study was the determination of the density of the antigen recognized by MAb E48 on A431 cells. This was done by incubating increasing amounts of unconjugated $^{125}$I-labeled MAb E48 to A431 cells in suspension and expressing the cell binding results by Lineweaver-Burke reversed plot analysis. It was found that the number of accessible epitopes per cell was $1.5 \times 10^6$ (Fig. 2).

**In Vivo Tumor Localization and Biodistribution of $^{125}$I-MAb-Indocyanin Conjugate.** Preliminary results showed that an indocyanin:MAb molar ratio ranging from 2 to 5 could be obtained without significantly modifying the conjugate immunoreactivity. However in vivo results showed that conjugate with indocyanin:MAb molar ratio greater than 2 had a markedly shorter whole body half-life and a lower tumor localization. Therefore, the $^{125}$I-MAb-(indocyanin)$_2$ conjugate was selected for a more extensive kinetic study of tumor localization and biodistribution. Groups of four and three mice bearing the SCC A431 xenografts were given injections i.v. of 20 pg of the $^{125}$I-MAb-(indocyanin)$_2$ conjugate, together with unconjugated MAb E48 trace labeled with $^{131}$I as a positive control. Results were obtained by differential radioactivity measurements of the dissected tumor and normal tissues.

As shown in Fig. 3, the tumor localization and biodistribution of the $^{125}$I-MAb-(indocyanin)$_2$ conjugate were almost as good as those of the $^{131}$I-unconjugated MAb. Six h postinjection the %ID/g of tumor were already 11.9 and 13.8%, respectively. At later time points, for both conjugated and native MAb E48, the %ID/g in tumors were not as high as could be expected from the results obtained at 6 h; indeed, usually the antibody tumor uptake in other carcinoma xenograft models increases between 6 and 48 h. This was not the case for the MAb E48 conjugate or the native MAb E48 in xenografts. For instance, the %ID/g tumor of the indocyanin conjugate were 14.8, 15.5, and 14.7 at 12, 24, and 48 h, respectively, and 16.6, 18.2, and 17.7 at the same time points for the unconjugated MAb E48. In contrast, the %ID/g of normal tissues decreased rapidly with values below 3.5% in all normal tissue except blood at 48 h for both the indocyanin conjugate and the native MAb E48. The highest normal tissue values were obtained in blood at 48 h with 7.5 and 7.8% injected dose for the conjugate and the native MAb E48, respectively. The latter results confirm that the conjugate has almost the same circulating half-life as the native MAb E48.

The highest tumor:normal tissue ratios were observed at 96 h for...
Thenonspecific binding, measured in presence of an excess of unlabeled MAbE48, was
of accessible epitopes on the cell surface. with 2 molecules of indocyanin and trace labeled with was
regression line with the ordinate gives the maximum binding corresponding to the number
liver, kidneys, and muscle, respectively. ranged for the indocyanin conjugate from 2.8 for the blood to 40.2 for
the large bowel with representative values of 8.1, 10.1, and 26.8 for
Tumors from mice given injections of fluorescein-MAb E48 conjugate, which were irradiated across the skin by a laser light at 488 nm, were not detectable (data not shown). This was mainly due to a strong yellowish autofluorescence of the mouse skin induced by the irradiation at 488 nm. In contrast, after removal of the mouse skin, an intense specific green fluorescence could be detected almost exclusively in the tumor (Fig. 4C). The stomach and intestines, on the left and right flanks of the mouse, showed a less intense yellowish autofluorescence. In clinic, the problems of autofluorescence can be partially overcome by computerized background subtraction procedures currently applied in modern photodiagnosis technology (7, 29, 30).

The immunophotodetection results after injection of 100 μg of MAB E48-(indocyanin)2 conjugate corresponding to 1 μg of indocyanin were compared to data obtained in control animals given injections i.v. of either 100 μg of the irrelevant IgG1-(indocyanin)2 conjugate or of a 15 times higher amount of free indocyanin (15 μg) (Fig. 4, D and E). Animals which received the irrelevant IgG1-indocyanin conjugate showed weaker and less selective tumor fluorescence signal (Fig. 4D). Animals given injections of a 15-fold higher dose of free indocyanin showed an intense and diffuse red fluorescence involving the entire mouse body and the tumor (Fig. 4E).

To further compare the distribution of the fluorescent signal, tumors from the mice illustrated in Fig. 4, A, D, and E were resected, opened by a longitudinal incision, and exposed to a homogeneous laser irradiation at 640 nm. Fig. 4F shows that the fluorescence signal intensity observed in the mice given injections of the tumor-specific indocyanin-MAb conjugate was as high as that obtained in mice which received a 15 times higher amount of free indocyanin, while the tumor from the mouse given injections of control irrelevant IgG coupled to indocyanin showed only a weak fluorescent signal. An additional control tumor from a mouse which received no indocyanin injection was barely visible, indicating that the tumor tissue presented no autofluorescence after irradiation at 640 nm.

DISCUSSION

The concept of using photoactive dyes coupled to anti-tumor antibodies for photodiagnosis and therapy of cancer has been proposed by several groups, but most studies remained at the stage of in vitro testing of immunon conjugate. The rare in vivo studies were aimed at tumor phototherapy with very preliminary results. No biodistribution data and no follow-up on the development of this strategy has been reported. For instance, in 1983, Mew et al. (14) reported on an experimental therapy using anti-miosarcoma MAbs coupled to a hematoporphyrin derivative. However, 2 years later, the same group reported on an entirely in vitro study using the same photoactive dye coupled to a MAb specific for a leukemia-associated antigen (10). Among the numerous in vitro studies the most important were from Oseroff et al. (11), who by using a dextran polymer intermediate coupled to up to 30 molecules of chlorin e6 to an anti-T-cell marker MAb, showed that the conjugate had selective phototoxic properties against leukemia cells; and from Hasan et al. (12), who also coupled chlorin e6, but to an anti-bladder carcinoma MAb through a polygluta mamic chain intermediate. In addition, Jiang et al. (13) used another intermediate linker, polyvinylalcohol, to couple benzoporphyrin to a MAb directed against human SCC.

One of the reasons for the absence of reports of in vivo results with these new conjugates is that the photoactive dyes used, hematoporphyrins, chlorins, and benzoporphyrins, all had hydrophobic properties which may have been responsible for their weak intrinsic tumor-localizing properties, but which decrease the solubility and the circulating half-life of the antibodies.
One of the advantages of the use of antibody as a vector for tumor localization is that we can select entirely new photoactive dyes primarily on the basis of their photochemical properties. Our experience, however, showed that for satisfactory in vivo use of immunoconjugates, more hydrophilic dyes should be selected such as fluorescein (17, 18) and the presently described indocyanin.

In nude mice, we have already shown that murine anti-CEA MAb substituted with as many as 10 to 12 fluorescein isothiocyanate molecules could localize efficiently in human colon carcinoma xenografts and that their in vivo half-life was only reduced by 50% as compared with the same unconjugated 125I-labeled anti-CEA MAb (17). This experience was confirmed in patients, in whom a mouse-human chimeric anti-CEA MAb (CGP44290) also substituted with 10 to 14 fluorescein isothiocyanate molecules was shown to localize with excellent specificity in human primary colon and rectum carcinoma with a moderate decrease of circulating half-life (30 to 50%) as compared to unconjugated chimeric MAb. In this study the feasibility of clinical immunophotodiagnosis was demonstrated (18).

Despite the advantages of fluorescein-MAb conjugate in vivo, in terms of circulating half-life and some of the favorable photophysical properties of fluorescein for diagnosis such as a high absorption coefficient and quantum yield of fluorescence (17), we think that the excitation and emission wavelength of fluorescein of 488 and 515 nm, respectively, are too low for efficient tissue penetration. Furthermore, we observed a relatively high tissue autofluorescence induced by the excitation laser light at 488 nm, which can interfere with optimal immunophotodiagnosis (17, 18).

In the present experimental study we introduced into this field a new dye, indocyanin, with a higher excitation and emission wavelength than a fluorescein and a new MAb E48 directed against an antigen expressed in SCC which has been shown after radioactive labeling to localize well in head and neck SCC (21, 22).
The major finding was that the conjugate with an indocyanin:MAb molar ratio of only 2 was more efficient in tumor detection than a fluorescein-MAb conjugate with a molar ratio of 6, which was used for comparison. This improvement is most likely due to the higher excitation and emission wavelengths of indocyanin (640 and 667 nm, respectively) over fluorescein (488 and 515 nm, respectively), which allowed a better penetration of the laser light and of the emission signal across the tissues. Thus, an increased number of dye molecules localized more deeply into the tumor could be excited and could transmit their signal, resulting in an overall amplification of the red fluorescent intensity detectable on the tumors. This is clearly demonstrated in Fig. 4, where the indocyanin-MAb signal is detectable even across the mouse skin (Fig. 4A) and is more intense than the fluorescein-MAb signal in mice from which the skin was removed (compare Fig. 4, B and C). In fact without removal of the skin the fluorescein-MAb signal was not detectable (data not shown).

In addition to the better tissue penetration of the excitation and emission light of indocyanin, another advantageous photophysical property of this dye is its extremely high absorption coefficient of more than 200,000.

The second important information gained from the use of this new immunoconjugate is that there was almost no nonspecific tissue autofluorescence during the excitation at 640 nm, which induced a specific fluorescence emission at 667 nm by the indocyanin localized in the tumor (Fig. 4, A and B), whereas the immunophotodetection of tumor with fluorescein-MAb conjugate gave a marked nonspecific tissue autofluorescence (Fig. 4C). Even when a resected tumor from a mouse given injections of indocyanin-MAb conjugate was placed in the middle of the open abdomen of the dead mouse, the tumor gave a specific red fluorescence without autofluorescence of the liver, stomach, and bowels (data not shown). This point is important for future clinical applications of indocyanin-MAb in immunophotodiagnostics.

At the immunological level, the indocyanin-MAb E48 conjugate at a molecular ratio of 2 gave the same high percentage (80%) of binding...
to an excess of SCC cells in vitro as unconjugated MAb (Table 1). In vivo, this indocyanin-MAb conjugate had almost the same circulating half-life and tumor localization capacity as the unconjugated MAb, while MAb conjugated with a higher number of indocyanin molecules showed a decrease in the circulating half-life and a smaller percentage of the injected dose localized by g of tumor (data not shown).

What was encouraging was the excellent tumor:normal tissue ratios observed with the indocyanin-MAb conjugate, with values of 5 for tumor: liver up to 34 for tumor: large bowel, which were as good as those obtained with 125I-labeled unconjugated MAb E48. Since radiolabeled MAb E48 was shown to give good tumor localization by immunoscintigraphy for head and neck SSC (21, 22), we think that the indocyanin-MAb E48 conjugate should be a reagent of great interest for laser-induced immunophotodiagnosis of small SCC by bronchoscopy.

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REFERENCES


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