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Immunophotodiagnosis of colon carcinomas in patients injected with fluoresceinated chimeric antibodies against carcinoembryonic antigen

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ABSTRACT **Based on previous experiments in nude mice, showing that fluoresceinated monoclonal antibodies against carcinoembryonic antigen localized specifically in human carcinoma xenografts and could be detected by laser-induced fluorescence, we performed a feasibility study to determine whether this immunophotodiagnosis method could be applied in the clinic. Six patients, with known primary colorectal carcinoma, received an i.v. injection of 4.5 or 9 mg of mouse–human chimeric anti-carcinoembryonic antigen monoclonal antibody coupled with 0.10–0.28 mg of fluorescein (molar ratio 1/10 to 1/14). The monoclonal antibody was also labeled with 0.2–0.4 mCi of ^{125}I (1 Ci = 37 GBq). Photodetection of the tumor was done *ex vivo* on surgically resected tissues for the six patients and *in vivo* by fluorescence rectosigmoidoscopy for the sixth patient. Upon laser irradiation, clearly detectable heterogeneous green fluorescence from the dye–antibody conjugate was visually observed on all six tumors; almost no such fluorescence was detectable on normal mucosa. The yellowish tissue autofluorescence, which was emitted from both tumor and normal mucosa, could be subtracted by real-time image processing. Radioactivity measurements confirmed the specificity of tumor localization by the conjugate; tissue concentrations of up to 0.059% injected dose per g of tumor and 10 times less (0.006%) per g of normal mucosa were found. The overall results demonstrate the feasibility of tumor immunophotodiagnosis at the clinical level.**

Despite major progress in understanding the process of malignant transformation and the technical improvement in conventional cancer treatments—such as surgery, radiotherapy, and chemotherapy—the average survival time of patients with the most common types of cancer has not significantly changed over the last decades.

One way to improve the prognosis of cancer patients is to detect and destroy small tumors earlier. After systemic injection of certain dyes, laser-induced fluorescence endoscopy was shown to be helpful for the photodiagnosis of early bronchial carcinoma (1–5). Such a diagnostic procedure, although limited to accessible tumors (directly or endoscopically), can be applied to many tumors, including carcinomas of the respiratory, gastrointestinal, and urogenital systems as well as ocular and skin cancers (3).

A major limitation of tumor photodiagnosis and therapy, however, is the lack of selectivity for cancerous tissues of the presently available dyes. To overcome this problem, coupling the dyes to antibodies directed against tumor-associated antigens has been proposed. Dyes, such as porphyrins or chlorins, have been coupled to monoclonal antibodies (mAbs), but these conjugates were studied primarily *in vitro* (6–8), and the few experimental immunophototherapy studies did not yield highly significant results (9). The obvious advantage of using mAbs as vectors for tumor localization of dyes is the ability of a mAb to bind specifically to an antigen that is more abundant in tumor than in normal tissue. Furthermore, this technique allows selection of the dye on the basis of its photophysical and spectral properties, independently of its weak tumor-localizing properties.

We chose human–mouse chimeric mAb directed against carcinoembryonic antigen (CEA) (10) because anti-CEA antibodies have given the best experimental and clinical results for colorectal carcinoma localization (11, 12) and chimeric mAbs were less immunogenic in patients than their murine counterpart (13). We selected fluorescein as the dye, primarily for its favorable photophysical properties, as shown in the innumerable *in vitro* applications of mAb–fluorescein conjugates and secondly because it can be injected in large doses into patients without side effects (14).

We have previously shown that anti-CEA mAb–fluorescein conjugates injected i.v. in nude mice bearing human colon carcinoma xenografts allow clear immunophotodetection of these tumors (15). The purpose of the present pilot clinical trial was to determine whether such type of tumor immunophotodiagnosis is feasible in patients.

MATERIALS AND METHODS

mAb. The mouse–human chimeric anti-CEA mAb (CGP44290) of human IgG4 κ isotype used was derived from one of our murine anti-CEA mAbs B7–25 (10, 16). This mAb has a high affinity for CEA (2×10^{10} M^{-1}) and was shown by immunoscintigraphy to localize well in carcinomas (17).

Preparation and Characterization of Conjugates. Five or 10 mg of chimeric anti-CEA mAb was labeled with 0.2 or 0.4 mCi of ^{125}I (1 Ci = 37 GBq) by the Iodo-Gen (Pierce) method (12). Separately, 1 or 2 mg of the same chimeric mAb was labeled with 0.2 or 0.4 mCi of ^{131}I.

Five milligrams of ^{125}I-labeled chimeric mAb was coupled to 0.2 mg of fluorescein isothyocyanate (isomer I; Sigma) diluted in pure dimethylformamide, as described (15). The conjugate was filtered through a Sephadex G200 column (Pharmacia), in pyrogen-free 0.15 M NaCl, to remove free fluorescein and any aggregated material. The fluorescein/mAb ratio was determined (15), and the conjugate was sterilized by filtration through a 0.22-μm Millipore filter.

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Abbreviations: CEA, carcinoembryonic antigen; mAb, monoclonal antibody.

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Immunoreactivity of each conjugate preparation was determined before injection and on a sample from the patient's serum (collected 2 hr after injection) by a direct binding assay (15, 16) to an excess of purified CEA coupled to CNBr-activated Sepharose (Pharmacia).

Patients and Antibody Perfusion. A series of six patients with known primary carcinoma of the colon or rectum were investigated. Each patient gave informed consent before inclusion in the study. Table 1 shows the clinical features of each patient and the amounts of antibody–fluorescein conjugates injected. Patients had their thyroids blocked by Lugol's 5% (vol/vol) iodine solution, and they received an antihistaminic drug and prednisolone before slow (30 min) i.v. injection of the antibody conjugate, as described (12).

Patients 2 to 6 received in the same perfusion 1 mg of the same chimeric mAb not conjugated with fluorescein and labeled with 0.2 mCi of ^{131}I.

Immunophotodetection of Tumors. Surgery was performed on the first five patients 24 hr after injection. The resected colon segment containing the tumor was analyzed *ex vivo* for fluorescence, under homogeneous Argon ion laser excitation light at 488 nm, with an intensity of 50 mW per cm^2 and a 200-μm-core optical fiber. For the sixth patient, a fluorescence rectosigmoidoscopy was performed *in vivo*, 24 hr after injection, followed by resection and *ex vivo* photodetection 24 hr later. The laser light was introduced by a quartz optical fiber that passed through the biopsy channel of the endoscope. At the distal end, the light emitted from the fiber passed through a special optical system for homogeneous illumination (5); in that case light intensity was $\approx$10 mW per cm^2.

In all patients, the fluorescence was first visualized through a Kodak Wratten filter (no. 12), which eliminates the reflection of the 488-nm excitation light but not the tissue autofluorescence. Color photographs (see Fig. 1) were taken through the same filter, with Kodak film (Ektachrom, 200 ASA) exposed for 15 sec (aperture of objective, F/5.6; laser excitation light intensity, 10 mW per cm^2).

Then, the fluorescence was analyzed by a specially designed photodetection apparatus (5) allowing real-time image processing and subtraction of most tissue autofluorescence, as described (2, 5). The subtracted image, which is essentially due to the mAb–fluorescein conjugate, was shown directly on a video screen (see Fig. 2).

Differential Radioactivity Measurement. Samples from different parts of the resected tumor and from the dissected normal bowel mucosa, serosa (remaining bowel wall after removal of mucosa), and normal fat, as well as from blood taken at surgery, were collected and weighed; the specific radioactivity of ^{125}I and of ^{131}I was measured in a dual-channel scintillation counter (16). Results were expressed as percentages of the injected dose of radioactivity per g of tissue (12, 16). The tumor-to-normal tissue ratios were calculated by dividing the percentage of injected dose per g of tumor by that of the different adjacent normal tissues.

The circulating plasma half-lives of the mAb–fluorescein conjugates and of the unconjugated mAb were derived from the specific radioactivity measured in serial blood samples taken at 2, 6, and 24 hr after injection.

RESULTS

***In Vitro* Characterization of Antibody Conjugates.** The conjugate preparations gave a single protein peak after filtration on Sephadex G200 with 0–3% of aggregates that were discarded. The mAb-to-fluorescein molar ratio in the selected immunoconjugate peak ranged from 1/10 to 1/14 (Table 1), which was found to be optimal in previous studies in nude mice (15).

Percentage of binding to insolubilized CEA of the conjugates before injection and that obtained from patient' serum 2 hr after injection were always high, ranging from 75.3% to 93.2%.

Immunophotodetection of Colorectal Carcinomas. None of the six patients injected with the immunoconjugate (Table 1) showed any adverse effect during or after the injection. Photodetection of tumors was performed *ex vivo* on surgically resected tissue for the first five patients and also *in vivo* by fluorescence rectosigmoidoscopy for the sixth patient.

Just after surgery, the resected colon segment was opened by a longitudinal incision and homogeneously irradiated with a laser light at 488 nm. Visual examination through a cut-off filter that eliminates the reflected excitation light showed a heterogeneously distributed green fluorescence in different parts of the tumor, which was not detectable on normal bowel mucosa. Fig. 1 shows color photographs of the tumor and adjacent normal tissue from patients 2, 3, and 6 illuminated by normal light (Fig. 1 *A*, *C*, and *E*) and by the laser light (*B*, *D*, and *F*). The selective and patchy green fluorescence of the tumor is clearly visible on the photographs, but a yellowish autofluorescence, observed on both tumor and normal bowel, interferes with optimal detection of the green fluorescence.

After visual observation and color photographs, all resected tumors and normal bowel segments were also analyzed by a photodetection apparatus allowing pixel-by-pixel subtraction of tissue nonspecific autofluorescence from specific fluorescence due to the antibody–fluorescein conjugate by real-time image processing (5). Fig. 2 illustrates this technique on the resected tumor and normal bowel mucosa from patient 3.

Patient 6 had her tumor directly examined by fluorescence rectosigmoidoscopy 24 hr after injection. Fig. 3 shows the video-screen image obtained during this *in vivo* immunophotodiagnosis procedure. The rectosigmoid carcinoma gave a well-delineated fluorescence signal after computerized subtraction of autofluorescence.

^{125}I-Labeled mAb Conjugate and ^{131}I-Labeled mAb Concentration in Tumor and Normal Tissues. To confirm the photodetection results, for all six patients the percentage of injected dose of ^{125}I-labeled mAb–fluorescein conjugate and that of ^{131}I-labeled unconjugated mAb simultaneously in-

Table 1. Clinical features and description of antibody–fluorescein conjugates for the six patients studied

Patient				Duke	Serum CEA,	Fluorescein-to-mAb	Injected mAb,	Injected
No.	Age	Sex	Primary tumor	stage	μg/liter*	molar ratios	mg	fluorescein, mg
1	64	M	Rectum	C	2.7	14	4.5	0.14
2	60	F	Rectum	A	3.6	11	4.5	0.11
3	71	F	Transverse colon	B	2.5	11	4.5	0.11
4	65	F	Rectum†	C	2.6	10	4.5	0.10
5	71	M	Rectum	B	0.9	14	9.0	0.28
6	59	F	Rectosigmoid	B	2.6	14	9.0	0.28

*Serum CEA values were measured by enzyme immunoassay, giving normal values from 0 to 2.5 μg/liter (16).
†Recurrence of rectum adenocarcinoma.

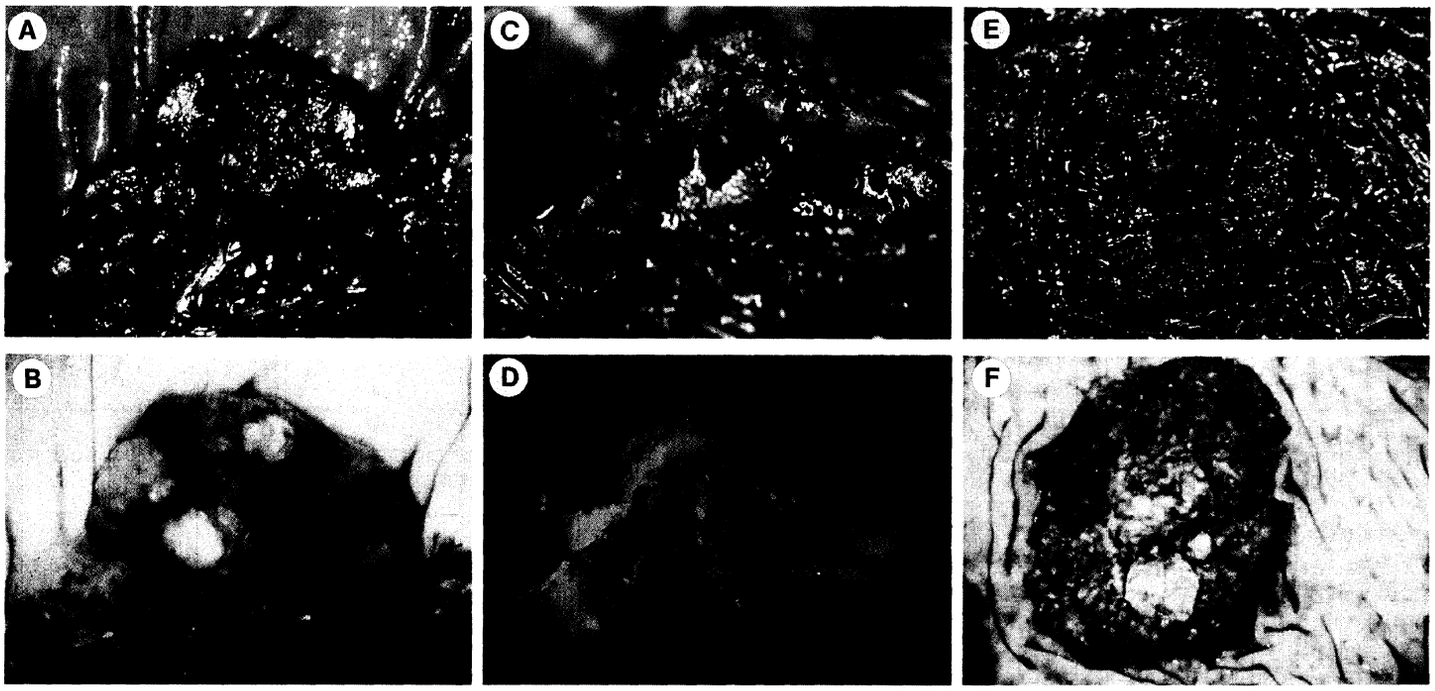

FIG. 1. Examples of laser-induced fluorescence, observed without subtraction of autofluorescence, in large-bowel carcinomas from patients 2, 3, and 6, who received an i.v. injection of chimeric anti-CEA mAb–fluorescein conjugate. (*A*, *C*, and *E*) Carcinoma from the rectum, transverse colon, and rectosigmoid, respectively, illuminated with normal light. (*B*, *D*, and *F*) Corresponding tumors and normal tissue after homogeneous laser excitation at 488 nm, photographed through a filter cutting only the exciting light. The green-dye fluorescence is heterogeneously distributed in the tumor tissue and almost undetectable in normal bowel mucosa. Size of the three tumors ranged from 3 to 4.5 cm in the largest diameter.

jected in patients 2–6 were determined by differential radioactivity measurement of several fragments of the tumor and of adjacent normal tissues, allowing comparison of the tumor-localizing capacity of the mAb–fluorescein conjugate with that of unconjugated mAb.

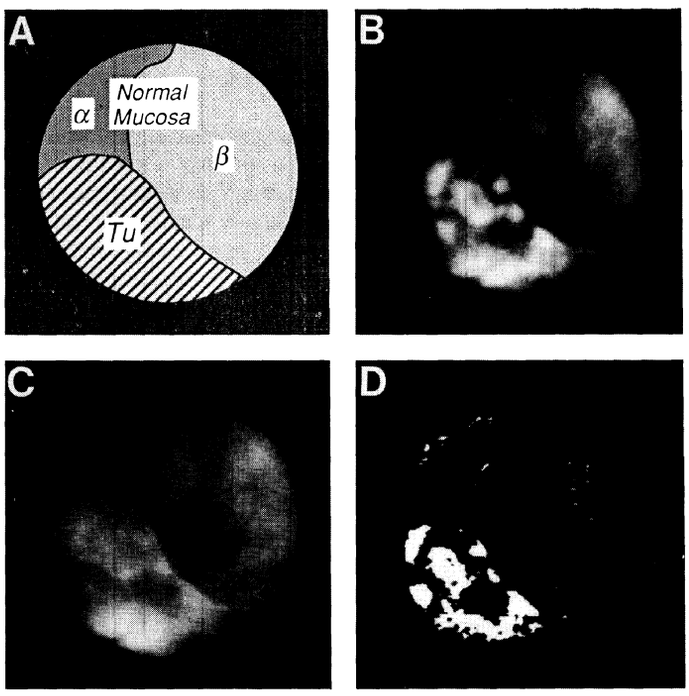

FIG. 2. Demonstration of immunophotodetection and subtraction of autofluorescence by real-time image processing (2, 5) for the tumor from patient 3. (*A*) Schematic representation of tumor area (Tu) and normal mucosa, separated in poorly illuminated (α) and highly illuminated (β) regions. (*B*) Fluorescent image in the spectral domain between 520 and 600 nm, detecting the fluorescein emission and part of autofluorescence. (*C*) Fluorescent image in the spectral domain between 600 and 700 nm, detecting autofluorescence. (*D*) Computerized subtraction of image in *C* from image in *B*.

Table 2 presents the results of the radioactivity measurements from different fragments of the tumors (mean values and ranges) and from normal tissues and blood samples, expressed as percentage of injected dose per g of tissue ($\times 10^3$). The radioactivity concentrations in the different tumor fragments and in normal tissues from patient 4 are further illustrated in Fig. 4. The heterogeneity of tumor localization, determined by radioactivity measurement, agrees with the patchy distribution of immunofluorescence seen by immunophotodetection (Figs. 1 and 2).

Table 3 shows the ratios of radioactivity concentration between tumor and normal tissues calculated for mAb–fluorescein conjugates and unconjugated mAb. The results indicate that mAb–fluorescein conjugates are almost as efficient as unconjugated mAbs in terms of specificity of *in vivo* tumor localization.

Analysis of Patient Serum Samples. The mean circulating half-life of ^{131}I-labeled chimeric anti-CEA antibody nonconjugated with fluorescein was 52 hr (range, 46–63 hr); for

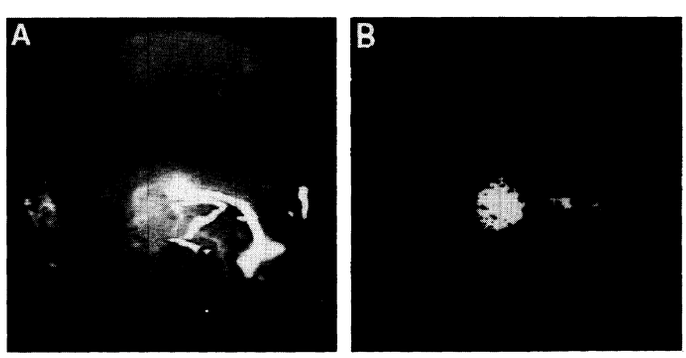

FIG. 3. Fluorescence rectosigmoidoscopy done in patient 6, 24 hr after i.v. injection of chimeric mAb–fluorescein conjugate. (*A*) Normal light endoscopic image of the rectosigmoid carcinoma. Note that the broad white line extending to the right of the tumor is an artifact from specular reflection. (*B*) Results from computerized subtraction of autofluorescence show immunophotodetection of the tumor by the same endoscope.

Table 2. Concentration of ^{125}I-labeled chimeric mAb–fluorescein conjugate and ^{131}I-unconjugated mAb in tumor, adjacent normal tissue, and blood, as determined by radioactivity measurement

	Concentration of ^{125}I-labeled conjugated or unconjugated mAb, (mean % injected dose/g of tissue) × 10^3									
	Tumor* (range)		Mucosa		Serosa		Fat		Blood	
Patient	mAb–fluorescein	mAb	mAb–fluorescein	mAb	mAb–fluorescein	mAb	mAb–fluorescein	mAb	mAb–fluorescein	mAb
1	6.7 (3.5–25.2)	ND	0.9	ND	ND	ND	0.3	ND	4.6	ND
2	25.1 (20.3–40.1)	54.6 (41.5–84.3)	3.5	6.3	1.8	2.3	0.7	1.2	8.4	14.0
3	11.6 (8.9–17.1)	22.4 (15.3–34.6)	2.2	3.4	2.1	2.9	0.8	1.2	9.2	12.6
4	38.4 (17.5–59.8)	64.4 (30.5–95.8)	6.5	10.3	2.6	3.3	2.2	3.2	12.9	18.1
5	4.4 (1.1–5.0)	13.7 (2.2–15.4)	0.8	2.1	0.4	2.1	0.3	0.5	5.5	11.7
6	11.1 (2.4–24.8)	38.7 (8.9–81.1)	0.7	3.3	0.5	2.1	0.3	0.8	4.9	11.8

ND, not determined.
*Concentrations were measured 24 hr after injection for patients 1–5 and 48 hr after injection for patient 6. The mean % injected dose/g was calculated by dividing total specific radioactivity measured in four to six tumor fragments by total weight of these fragments.

antibody fluorescein conjugates with a molar ratio of 1/10 or 1/11, the mean half-life was 36 hr (29–42 hr), whereas for the immunoconjugates with a molar ratio of 1/14, the mean half-life was 24 hr (20–31 hr).

The search for human anti-mouse IgG, anti-idiotype or anti-fluorescein antibodies in patient serum samples, collected 2 to 5 months after injection, by a solid-phase radioimmunoassay with a sensitivity of 0.1 μg per ml (to be described elsewhere) gave entirely negative results.

DISCUSSION

The results presented here show that an anti-tumor-associated antigen antibody labeled with a fluorescent dye and injected i.v. in patients can bring sufficient amounts of the dye into a carcinomatous lesion to make it detectable by laser-induced fluorescence. This demonstration may open the way to the development of clinical immunophotodiagnosis and, possibly, to more selective forms of phototherapy. Previously, injected mAbs localized in the patient's tumor have always been detected by γ-emitting isotopes coupled to the antibodies (12, 18). Now, after laser irradiation we can visualize the patchy distribution of the fluoresceinated antibody localized in the patient's tumor.

To bridge the gap between the fields of photodiagnosis and immunoscintigraphy, we have also labeled our mAb–fluorescein conjugates with a small amount of ^{125}I. This labeling allowed confirmation of the specificity of tumor localization of the mAb–fluorescein conjugates by precise radioactivity measurements in tumor and normal tissues.

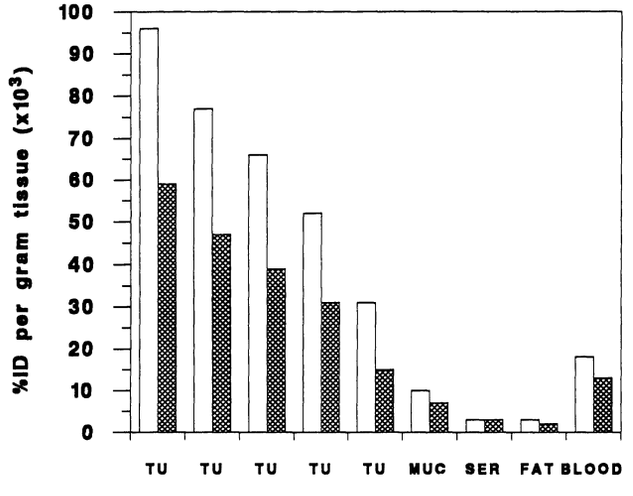

FIG. 4. Concentration of unconjugated chimeric anti-CEA mAb (open bars) and fluorescein-coupled chimeric mAb (shaded bars) in five resected tumor fragments (TU; highest-to-lowest concentration) and in adjacent normal mucosa (MUC), normal serosa (SER), fat, and blood from patient 4, obtained 24 hr after injection. The results expressed in percentage of injected dose (ID) per g of tissue (×10^3) (ordinate) were determined by differential radioactivity counting.

The reason for these favorable results are 3-fold. (*i*) We used a chimeric mAb (10) with high affinity for CEA, a tumor marker that has been shown for many years to be one of the best target antigens for mAb localization in tumors (11, 12, 18). (*ii*) We had previously demonstrated in the nude mice model that up to 10 molecules of fluorescein can be coupled to anti-CEA mAb without decreasing its capacity for localization in the target tumor (15). (*iii*) The fluorescein has optimal photophysical properties for diagnosis with a large absorption coefficient and a quantum yield of fluorescence emission of up to 85% (19). In addition, fluorescein excitation wavelength corresponds to the strong emission at 488 nm of a conventional Argon ion laser.

Importantly, the injection of up to 9 mg of mouse–human chimeric mAb–fluorescein conjugates was well tolerated by all patients and did not induce any detectable antibody response directed either against the chimeric mAb idiotype or framework region or against the fluorescein molecules coupled to the mAb.

It may appear surprising that fluoresceinated antibodies, which have been one of the most commonly used immunological reagents *in vitro* for identification of antigen on tissue sections and on cell surface, particularly for flow cytofluorimetry and cell sorting, have never been used for tumor diagnosis *in vivo*. Fluoresceinated anti-CEA antibodies have been sprayed directly on resected stomach tissue to detect carcinoma cells (20), but no study of *in vivo* tumor localization of i.v. injected fluoresceinated mAb was done.

Other photosensitizing dyes have been coupled to different mAbs for the purpose of phototherapy (6–9), but no *in vivo* biodistribution or tumor localization results have been reported. One reason for the absence of results from *in vivo* studies of dye–antibody conjugates is that some of the most commonly used photoactive dyes, such as hematoporphyrins or chlorins, have hydrophobic properties. Thus, when several molecules of these dyes are coupled—either directly to each antibody molecule or through a polypeptide or polyvinylalcohol intermediate (8, 21)—the circulating half-life of the antibody may be drastically reduced, precluding satisfactory tumor localization.

One particular advantage of fluorescein isothiocyanate is that it can be randomly coupled to the NH_2 residues of antibodies at a molecular ratio of 1/10 to 1/12, without excessively decreasing the circulating half-life and tumor-localization capacity of the anti-CEA mAb. This fact has been shown in nude mice by using the murine anti-CEA mAb 35 (15) and is confirmed here with the chimeric anti-CEA mAb CGP44290 (10). The circulating half-life of the mAb–

Table 3. Tumor-to-normal tissue ratios of radioactivity concentration for ^{125}I-labeled chimeric mAb–fluorescein conjugate and ^{131}I-labeled unconjugated chimeric mAb

	Tumor/mucosa		Tumor/serosa		Tumor/fat		Tumor/blood	
Patient	mAb–fluorescein	mAb	mAb–fluorescein	mAb	mAb–fluorescein	mAb	mAb–fluorescein	mAb
1	7.4 (3.9–28.0)	ND	ND	ND	22.3 (11.7–84.0)	ND	1.5 (0.8–5.5)	ND
2	7.2 (5.8–11.5)	8.7 (6.6–13.4)	13.9 (11.3–22.3)	23.7 (18.0–36.7)	35.9 (29.0–57.3)	45.5 (34.6–70.3)	3.0 (2.4–4.8)	3.9 (3.0–10.0)
3	5.3 (4.0–7.8)	6.6 (4.5–10.2)	5.5 (4.2–8.1)	7.7 (5.3–11.9)	14.5 (11.1–21.4)	18.7 (12.8–28.8)	1.3 (1.0–1.9)	1.8 (1.2–2.7)
4	5.9 (2.4–9.0)	6.3 (3.0–9.3)	14.8 (5.9–22.6)	19.5 (9.2–29.0)	17.5 (7.0–26.7)	20.1 (9.5–29.9)	3.0 (1.2–4.6)	3.6 (1.7–5.3)
5	4.4 (1.0–5.0)	6.5 (1.0–7.3)	5.5 (1.4–6.3)	6.5 (1.0–7.3)	14.7 (3.3–16.7)	27.4 (4.4–30.8)	0.8 (0.2–0.9)	1.2 (0.2–1.3)
6	11.1 (2.4–24.8)	11.6 (2.7–24.6)	15.9 (3.4–35.4)	18.2 (4.2–38.6)	37.0 (8.0–82.7)	47.9 (11.1–101.4)	2.3 (0.5–5.1)	3.2 (0.8–6.9)

Tumor-to-normal tissue ratios (mean and range) were obtained by dividing percentage of injected dose per g of tumor by percentage of injected dose per g of adjacent normal tissues. ND, not determined.

fluorescein conjugates was ≈70% of that of unconjugated mAb, for the conjugates with molar ratio of 1/10 and 48% for the conjugates with molar ratio of 1/14. Table 2 shows that the percentage of injected dose per g of tumor is ≈50% of that observed with unconjugated mAb for the fluorescein conjugates with a molar ratio of 10 or 11 and ≈30% for the conjugates with a molar ratio of 14.

Despite the relatively high fluorescein substitution of the injected mAb–fluorescein conjugate, the absolute amount of fluorescein injected remained very low–0.10–0.28 mg coupled to 4.5–9 mg of chimeric anti-CEA mAb. Knowing from radioactivity measurement that the average percentage of injected dose of immunoconjugate per g of the positive tumor fragments was ≈0.02% (Table 2), it can be calculated that 22 to 56 ng of fluorescein localized per g of tumor. This amount is definitely within the range of detection by laser-induced fluorescence, especially when the adjacent normal tissue contains 8–20 times less fluorescein. Interestingly, 22–56 ng of fluorescein per g of tumor (consisting of ≈10^9 cells) represents an average of 35,000–88,000 fluorescein molecules per tumor cell.

Now, the question remains: can the encouraging results from the present clinical feasibility study be extrapolated to the detection of much smaller superficial carcinomas, which represent a real diagnostic problem. The fact that radiolabeled mAbs have been shown to localize at a higher concentration in smaller tumors than in larger ones (16) suggests a positive answer to this question.

Among the different types of small carcinomas that may benefit from immunophotodiagnosis are not only the superficial bronchial carcinomas but also relapses of bladder carcinomas and incipient peritoneal carcinomatosis, which could be detected by peritoneoscopy. Our observation of fluorescent tumor tissue through glasses equipped with the appropriate optical filters suggests that immunophotodiagnosis could even be helpful during surgical laparoscopy for the identification of small nests of metastatic tumor cells. For deep-seated lesions, radiolabeled mAbs, detected either by tomoscintigraphy (12) or by direct application of a radiosensitive probe during surgery (22), will be more efficient. For superficial lesions, however, photodetection of disseminated tumors after injection of fluorescein–mAb conjugates may be a more precise method.

Ultimately, immunophototherapy should be the optimal complement and logical development of immunophotodiagnosis. For that purpose, a high priority should be given to the identification and testing of photoactive dyes with a high quantum yield of singlet oxygen production, which can be coupled to antibodies without altering their biodistribution and tumor-localizing properties.

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