Radioimmunoguided Surgery Using Iodine-125-Labeled Biotinylated Monoclonal Antibodies and Cold Avidin

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One of the limitations of intraoperative tumor detection with radiolabeled monoclonal antibody (Mab), by means of a gamma-detecting probe (GDP), is the long time interval needed between Mab injection and surgery to obtain low blood-pool activity. Such an interval can be shortened considerably, exploiting the high affinity between avidin and biotin. **Methods:** Twenty patients with colorectal cancer were injected with 1 mg of biotinylated ^{125}I monoclonal antibodies followed, 48 hr later, by a chase of cold avidin. During surgery, the GDP was used to detect radioactive emissions from the tumor and normal tissue. Tumor tissue samples were analyzed in vitro by immunohistochemical tests for the presence of tumor antigens and in vivo antibody localization. **Results:** At the time of surgery (average 7 days postinjection), the mean value of circulating radioactivity was 6% ± 3% of the injected dose. Of 20 patients studied, tumors were localized in 13 cases (65%). Subclinical tumors were detected in 3 patients (15%). **Conclusion:** The use of ^{125}I-labeled biotinylated Mabs followed by avidin as a chase enhances the applicability and effectiveness of radioimmunoguided surgery technology and will allow the use of radioisotopes with a shorter half-life than ^{125}I.

Key Words: monoclonal antibody; RIGS; avidin-biotin

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Radioimmunoguided surgery (RIGS) is a method that enables the surgeon to delineate the extent of a neoplasm and to identify lymph node metastases on the basis of their capacity to bind radiolabeled antitumor monoclonal antibodies (^{125}I-Mab) administered intravenously several days before surgery (*1,2*). The radiotracer is then revealed intraoperatively by a hand-held gamma-detecting probe (GDP). As the latter can be placed directly over the source of gamma emission during the surgical procedure, low radioactivity levels can be recorded (*2,3*). RIGS is efficient, reliable and of clinical relevance, allowing otherwise undiagnosed small, deep-lying neoplastic lesions to be detected (*1–5*). A major limitation of this method is the long time interval that elapses between the administration of the radiotracer and surgery, i.e., the time required to achieve a low blood-pool background level (*6*). This flexibility of time depends on the particular antibody used. However, with currently available antibodies, a mean time of 24 days is needed to obtain a tumor-to-normal tissue ratio high enough for accurate GDP detection (*7*). This inconvenience limits the clinical applicability and restricts the choice of radioisotopes (because these must have long half-lives) and of Mabs (that must have an unusually long residence time on the tumor and a relatively fast blood clearance).

Because the major source of background activity is blood, it would be advantageous to remove the antibody from the circulation, i.e., to increase its clearance to a time when the tumor-bound antibody has reached its maximal value (i.e., 24–36 hr) (*8,9*) to improve the tumor-to-background signal ratio. Several methods have been proposed to achieve this goal, including the use of fragments (with faster clearance) or the use of "chase" strategies. The chase consists of a ligand able to bind circulating antibodies that form complexes that are efficiently removed by the liver, resulting in increased blood clearance (*10,11*).

Avidin efficiently removes biotinylated antibodies from the blood in animal model systems (*12*) and in human patients (*13*). The affinity of avidin to biotin is extremely high; the dissociation constant of the avidin-biotin complex is on the order of 10^{-15} *M*, and the complexes are removed by the liver.

A method is described here in which RIGS is performed by the administration of biotinylated ^{125}I-Mabs followed by nonradioactive avidin as a chase. With this approach, the time needed between labeled antibody injection and RIGS can be reduced to 1 wk or even less. With regard to the intraoperative use of GDP, the authors evaluated the statistical suitability of the cutoff value that is currently used to detect lesions during surgery (*1,2*).

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TABLE 1
Summary of Patient Data

Patient No.	Mab	Tumor	Gap Mab-Surg	% ID Blood	T/BKG Probe	T/BKG Well	p Value	IHC
1	FO23C5	p Right colon	8 Days	8	0.5	1.3	NC	–
2		p Rectum	9 Days	8	1.8	NE	0.04	+
3		p Transverse colon	7 Days	4	5.8	8.0	<0.001	+
4		p Rectum	6 Days*	1	1.8	8.0	0.03	+
5		p Rectum	4 Days*	8	2.7	41.0	<0.001	+
6		p Transverse colon	4 Days*	NE	1.1	0.5	NS	–
7		p Transverse colon	8 Days	NE	2.4	4.0	0.005	+
8		p Rectum	8 Days	NE	1.3	1.4	NS	+
9		r Rectum	7 Days*	NE	1.9	3.2	0.008	+
10		p Rectum	6 Days*	2	1.8	3.7	<0.001	–
11		p Right colon	3 Days*	2	1.6	10.0	0.01	+
12		p Right colon	5 Days*	NE	2.0	2.8	0.02	+
13		p Rectum	8 Days	10	0.3	1.5	NC	+
14		r Rectum	9 Days	4	1.0	1.4	NS	+
15	B72.3	p Left colon	5 Days*	8	2.4	15.0	0.004	+
16		r Right colon	6 Days*	6	3.5	7.6	<0.001	+
17		r Transverse colon	13 Days	NE	1.9	NE	0.04	+
18		p Rectum	6 Days*	9	1.0	NE	NS	–
19		p Rectum	6 Days*	8	6.0	8.2	<0.001	+
20		r Rectum	8 Days	NE	1.5	NE	NS	–
Mean ± s.d.			7 ± 2	6 ± 3				positive 75% negative 25%

p = primary cancer; r = recurrence; NS = not significant; NC = not computed because T < BKG; % ID = percent of injected dose in blood, measured on blood sample at the day of surgery; NE = not executed; Gap Mab-Surg = gap between Mab injection and surgery; IHC = immunohistochemistry.
*Two avidin injections.

MATERIALS AND METHODS

Monoclonal Antibodies and Avidin

Mab B72.3 belongs to the immunoglobulin (Ig) G1 subclass and defines the tumor-associated antigen TAG-72, a high molecular weight (220–500 kD) tumor-associated glycoprotein found on human carcinoma cells. TAG-72 also occurs on several epithelium-derived cancers, including colon carcinoma, invasive ductal carcinoma, nonsmall cell lung cancer, ovarian carcinoma and many others. It has been widely tested in animal studies and clinical trials (*14*).

Mab FO23C5, also an IgG1, reacts with a protein epitope of the carcinoembryonic antigen (CEA) molecule. Its use in immunoscintigraphy has been reported (*15*).

The Mabs, purified and biotinylated, were labeled (Iodogen method) with ^{125}I and supplied by Sorin Biomedica (Saluggia, Italy). The specific activity was 37 MBq (1 mCi) of ^{125}I per milligram of protein.

Biotinylation and radiolabeling of antibodies has been described elsewhere (*4,13*). The degree of biotinylation was 5 ± 1 biotin per antibody determined, after protein digestion, spectrophotometrically as described (*16*). At this grade of biotinylation and specific activity, the retained immunoreactivity of the antibodies was more than 90%, as tested in a standard enzyme-linked immunosorbent assay system, as previously described (*13*).

Clinical-grade, pure hen egg avidin (tetravalent) was obtained from Società Prodotti Antibiotici (Milan, Italy).

GDP

A commercial system was used (model 1000, Neoprobe, Columbus, OH). The gamma detector consists of an 18 × 2-cm stainless steel tube with an angled tip for better maneuverability. The tip of the probe contains a cadmium tellurite crystal and a preamplifier. The signals detected by the probe are translated into digital readout and into acoustic signals. The intensity and frequency of the auditory signal is directly proportional to the level of radioactivity detected.

Patients

Twenty randomly selected patients with primary or recurrent colorectal cancer (Table 1) were enrolled in the study after informed written consent was obtained. Exclusion criteria were known iodine allergy, peritoneal carcinomatosis or extraabdominal disease, detected by conventional staging methods. Fifteen patients had primary tumors, and five had recurrent tumors. The location of the primary tumors were three, right colon; three, tranverse colon; one, left colon; and eight, rectum. The patients with recurrent tumors had pelvic recurrence (four patients) and pancreatic metastases (one patient). In the cases of primary tumors, the diagnosis was made by barium enema and/or colon-

scopy and confirmed by biopsy. Secondary tumors were detected by CT and/or ultrasonography.

Administration of Reagents

All patients were treated with a saturated solution of potassium iodide for thyroid blocking (10 drops, twice a day from 2 days before Mab injection until surgery).

Then 1 mg (37 MBq) of ^{125}I-radiolabeled biotinylated Mab (B72.3 in 6 patients and FO23C5 in 14 patients) was injected intravenously. Anti-CEA Mab was preferentially used in early stages of primary tumors. Forty-eight hours after the administration of antibodies, 1 mg of cold avidin in 3 ml of saline solution was administered. For reasons related to the patients' needs or to departmental organization, when the intervention had to be carried out while the activity over the heart measured with the GDP was still above 10 cps, a second dose of avidin was administered to reduce further the amount of radioactivity present in the blood. Thus, in 10 patients, a second injection of 3 mg of cold avidin was administered intravenously in 100 ml of saline solution.

Blood samples and external probe counts (on heart, thyroid, spleen, liver and abdominal wall) were obtained daily from the Mab injection to the day of surgery and at 1, 5, 10, 30, 60, 120 and 180 min after each avidin injection. A portion (0.2 ml) of each whole blood sample was assayed for ^{125}I content using a calibrated well counter. The decrease in circulating activity caused by the avidin injection was calculated using the counts per minute per milliliter measured on the blood samples.

With the use of the blood sample obtained on the day of surgery, a percentage of injected dose (%ID) in the total blood volume was calculated, counting the sample along with the standard of the injectate and normalizing the value for the circulating blood volume.

Patients underwent surgery when the activity over the heart measured with the GDP was below 10 cps.

Intraoperative Use of GDP

The intraoperative detection was carried out by two surgeons, one of whom was always present during the procedure. During surgery, the GDP was used to detect radioactive emissions from the tumor and normal tissue (normal colon and area surrounding sites of increased tracer uptake). The GDP was passed slowly over the evident primary, metastatic or recurrent tumor location. Then, the entire abdomen was scanned with the probe with particular attention to the tissue adjacent to the tumor; the main lymphatic drainage areas, such as the lymph nodes of mesenteric vessels; the periaortic zone; and the periportal area. The GDP scanning lasted for approximally 15 to 20 min.

The signal detected by the probe was translated into an acoustic signal. Each reading lasted for 2 sec and was repeated in triplicate and recorded for off-line analysis. The threshold (a change in sound pitch) was arbitrarily set at a tumor-to-normal tissue count ratio over 1.5 (*2*). All foci of increased uptake and all clinically suspected tumor locations (with or without increased probe counts) underwent biopsy or, if possible, were resected.

Biopsies from tumor and normal colon were counted in a well scintillation.

Statistical Analysis of GDP Counts

From triplicate readings for normal tissue (BKG) and tumor (T), the average and standard error were calculated for each subject. Two different cutoff methods were considered: (1) the difference T − BKG was considered positive when T was greater than BKG and the p value obtained by two sample t-tests was lower than 0.05 level (*17*) and (2) the T/BKG was computed, with the ratio considered positive when it was more than 1.5, according to the setting of the acoustic signal.

Pathologic and Immunohistochemical Tests

Histologic sections of each tumor were tested for the presence of the relevant antigens by immunoperoxidase staining. Slices were incubated with normal serum to block nonspecific antibody binding and with 3% H_2O_2 in acetone solution to block endogenous peroxidase before incubation with B72.3 and/or FO23C5. After washing for 5 min in buffered wash solution, biotinylated antimouse immunoglobulin second antibodies were applied at room temperature for 30 min. The washed slices were incubated with streptavidin-peroxidase complex at room temperature for 30 min in a humidity chamber. After washing in buffered wash solution, the color reaction was developed by incubating in 3-amino-9-ethylcarbazole for 15 min. The sections were washed in tap water, counterstained with hematoxylin for 60 sec and mounted in aqueous mounting medium.

A further section was incubated with streptavidin-peroxidase complex alone at room temperature for 30 min in a humidity chamber to detect the presence of biotinylated Mabs on resected tumor sections. After washing, the section was stained with 3-amino-9-ethylcarbazole at room temperature for 15 min and washed with tap water. Counterstained sections with hematoxylin were mounted in aqueous medium (*7*).

RESULTS

No toxicity related to antibodies or avidin administrations was observed in any patient. Table 1 reports the data relative to individual patients. With the use of ^{125}I-biotinylated Mabs followed by avidin, blood radioactivity decreased in each patient by about 80% over the 3 hr after the first avidin injection (a representative curve is shown in Fig. 1A). In particular, the circulating activity measured on the blood samples declined rapidly within the first 10 min postinjection of the chase, reaching the minimum value of 16.6% ± 2.7% of the activity present in the blood prior to the administration of avidin, at 1 hr (Table 2).

In some patients, an increase of 1% to 2% of radioactivity was seen in the samples taken at 120 and 180 min. Most of the radioactivity was trapped in the liver and spleen, probably as avidin-antibody complexes (*6,12–16,18,19*), as revealed by high GDP counts over these organs. Surgery was performed when the external probe counts over the heart were below 10 cps. This rate was achieved in a mean time of 7 days (range 3–13 days). The interval for each patient between Mab administration and surgery is reported in Table 1. Patients who received two injections of avidin (an example is reported in Fig. 1B) are marked with an asterisk. It is interesting to note that, in the patients operated on within 5 days, the T/BKG ratios were higher.

Table 2 shows the reduction of circulating activity expressed as the percent of the mean ± s.d. obtained from blood samples after each injection of avidin. With avidin as a chase to clear circulating biotinylated Mabs, patients were operated on in a shorter time (as little as 3 days) with only 2% ID in the blood (see Patient 11, Table 1).

The percentage of the injected dose in the blood on the

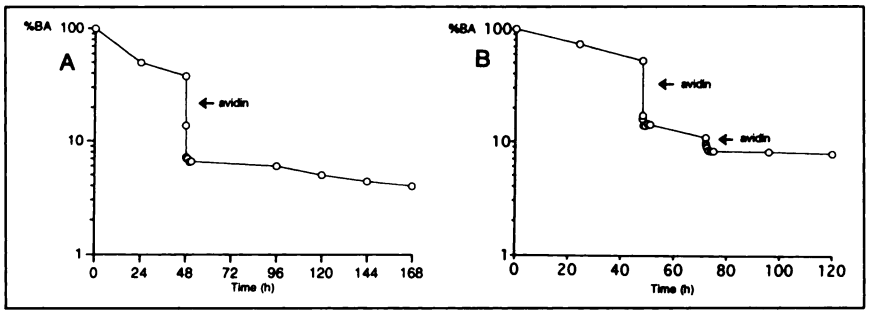

FIGURE 1. (A) Representative ^{125}I-biotinylated Mab blood clearance in Patient 3. On the y axis, the percentage of blood activity (%BA) obtained from blood samples is reported. The counts per minute per milliliter of the blood sample drawn 5 to 10 min after the administration of labeled Mab was considered to be 100%. A single injection of 1 mg of avidin reduced the circulating activity by 85% over 3 hr after the avidin injection. (B) Example of ^{125}I-biotinylated Mab blood clearance in Patient 4. To speed up the circulating activity clearance further of ^{125}I Biotinylated Mabs, a second injection of avidin was required.

day of surgery was 6% ± 3% (Table 1). Intraoperative radioimmunodetection (by the acoustic threshold set over 1.5 T/BKG ratio) was positive in 13 of 20 (65%) patients (9 of 14 tumors using the anti-CEA Mab FO23C5 and 4 of 6 using Mab B72.3). Of 15 patients with primary cancer, 8 had Stage II disease (no lymph nodes involved) and 7 had Stage III-IV disease (lymph node involved or distant metastases). The overall sensitivity of RIGS for Stage II cancer was 3 of 8 (37.5%), whereas for more advanced cancer, it was 7 of 7 (100%). Recurrent cancer was identified in 3 of 5 (65%) patients. The RIGS system detected metastatic lymph node (juxtaregional and iliac) in 3 of 6 cases. One of 20 patient had liver lesions, which were identified clinically only because of the high counting rate in normal liver.

The median T/BKG ratio obtained from the probe read out transcriptions in the 13 RIGS-positive patients was 2.4 (range 1.6–6.0). The tumor-to-normal colon ratio obtained from biopsy data was 8.0 (range 2.8–41.0). All the tissue samples removed by biopsy from normal colon were negative for the presence of tumor cells.

The individual cutoff based on the significance of the difference between tumor and background resulted in 100% concordance with the T/BKG ratio cutoff set over 1.5 (Table 1). However, because of the small number of cases, these results should be considered indicative.

Moreover, both methods allowed the surgeon to identify subclinical deposits of tumor that were not evident, either preoperatively or intraoperatively, in two cases of primary cancers (Patients 19 and 7, Table 1, Stages III-IV), which suggested an extension of the lymphadenectomy, and in one case of local recurrence (Patient 9, Table 1), which guided the pelvic dissection of the tumor, which was infiltrating the bladder and had metastasized to the right seminal vescicle.

On the basis of immunohistochemical analysis (Table 1), the patients were classified as true positive (12 patients), true negative (4 patients), false negative (3 patients) and false positive (1 patient). The presence of ^{125}I-biotinylated antibodies at the tumor level was also demonstrated by direct immunostaining of resected tumor sample by revealing in vivo localized biotinylated antibodies with streptavidin-peroxidase (*18*). Figure 2 is an example that shows the in vivo targeting of biotinylated Mab FO23C5 at a tumor site.

TABLE 2
Reduction of Circulating Activity (% Mean ± s.d., n = 8) as Functioning of Avidin Injection (Chase Effect) Measured on Blood Samples

	Time (min)	Mean (%)	s.d.
1st avidin	0	100.0	0.0
	5	24.0	5.7
	10	18.1	8.3
	30	17.7	8.0
	60	16.6	2.7
	120	18.3	3.1
	180	19.0	3.5
2nd avidin	0	18.3	9.2
	5	9.6	1.7
	10	8.6	2.6
	30	9.4	3.0
	60	10.6	4.0
	120	9.1	4.0
	180	7.9	3.8

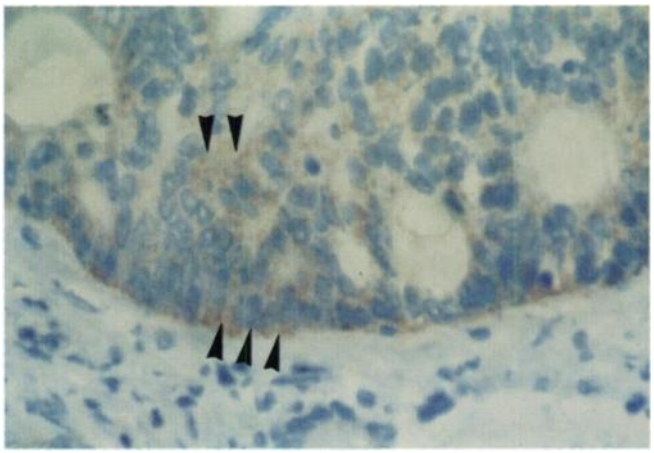

FIGURE 2. Direct immunostaining using streptavidin-peroxidase complex alone. Biotinylated Mab (FO23C5) on the tumor-resected section are revealed by streptavidin-peroxidase conjugate, which targets the biotin present on the tumor-bound antibodies showing the in vivo antibody distribution at tumor site (arrows).

DISCUSSION

One of the major drawbacks of RIGS is the long period (up to 5 wk) that elapses between labeled Mab injection and surgery (*20*). The high blood level counts reported in previous studies are related to many aspects, such as the dose of the antibody, competition for binding sites, radiolabeling techniques, metabolism of the radiolabel from the antibody, clearance of the radionuclides, shed antigen and the presence of human antimouse antibody (HAMA) (*21*). Moreover, certain Mabs clear more quickly than others both from tumor and other tissues (*22*). Nieroda et al. (*6*), using 5 mCi of ^{125}I per milligram of B72.3, carried out surgery 6 to 24 days after the day of injection. On the other hand, Martin and Carey (*7*) carried out surgery 21 to 28 days after injection of 2 mCi of ^{125}I-Mab B72.3. A small dose of ^{125}I (1–2 mCi) was used in the study of La Valle et al. (*23*). However, the patients were scheduled for surgery 3 to 4 wk after the radiolabeled antibody was given.

The use of ^{125}I-biotinylated Mabs followed by cold avidin described here was safe and provided faster clearance of the radiolabeled Mabs, thus allowing surgery to be performed within 1 wk. The major limitation of this procedure is the deposition of ^{125}I-biotinylated Mab-avidin complexes in the liver, which reduces the surgeon's ability to detect tumor in this organ and periportal nodes. As reported in the work of Cohen et al. (*5*), "traditional" RIGS was able to identify liver and periportal node occult disease in 37% of patients studied, which indeed represents a result of clinical relevance. However, ultrasound probes give good results in the case of the liver (*24*) and could be used to overcome the "avidin" RIGS limitation of high liver uptake.

The HAMA and human anti-avidin response (HAAR) were not tested in these patients because of a setback that occurred in the laboratory. Nevertheless, in other protocols, of more than 80 patients studied with biotinylated Mabs (including CEA, B72.3 and other mouse IgGs), none developed HAMA after having received 1 to 2 mg of antibody. The HAAR response was present in 20% of cases following administration of 5 to 6 mg of avidin.

In this study, the statistical significance of the currently used arbitrary 1.5 cutoff method was also assessed. In the detection of lesions, this method was equivalent to a method based on computing the statistical significance of the differences of tumor and background counts. The two methods were concordant in the range of counts experimentally detected in this study. For higher counts, the method based on the significance of the difference should be less conservative than the T/BKG ratio method, considering the Poisson distribution of the counts (*25*).

In a previous study, the authors used the ^{125}I-labeled Mab B72.3 and found a clear trend toward a higher sensitivity in more advanced cancer compared with less advanced tumor (*20*). Despite the fact that CEA is largely expressed by primary colon cancer, anti-CEA Mab FO23C5 has never been used in RIGS because it does not remain on the tumor cell surface longer than a few days (*26,27*). The long retention of Mab B72.3 in tumor (*28*) makes it more suitable for conventional RIGS (*22*).

In the present study, with biotinylated Mabs and avidin as a chase, Mab FO23C5 localized 7 of 12 primary tumors; 3 of the remaining 5 were CEA negative by immunohistochemical testing. Mab B72.3 localized 2 of 3 advanced primary tumors (the third resulted from a tumor that was TAG 72 negative by immunohistochemical analysis). In summary, in the 15 primary tumors studied, RIGS was positive in 9 of 11 (82%) antigen-positive cases. Patient 10's results were considered false positive on an immunohistochemical basis that revealed a low positivity for CEA with less than 20% of positive cells. Nevertheless, the direct method, using only strepto-peroxidase, revealed the in vivo presence of ^{125}I-biotinylated Mabs at the tumor site. This case is really a borderline case that was considered to be antigen negative in compliance with the authors' previous study (*4*) because only 20% of the cells expressed the antigen.

The data obtained by counting the biopsy (tumor and normal colon) in a well counter were higher than the ratio obtained in vivo with the probe, and they were always in concordance with such data. However, as far as the clinical utility of RIGS is concerned, in vivo target-to-nontarget ratios are really important. To increase tumor targeting and the sensitivity of detection, a larger amount or a cocktail of antibodies could be used. However, increasing the dose of Mabs might result in a higher blood-pool background (*8*). The efficient removal of radioactive Mabs from the blood herein described would allow the use of Mabs mixtures at high dose. Moreover, to achieve better tumor-to-background ratios, avidin-induced fast clearance could be started 24 to 36 hr postinjection when the Mab concentration is highest at tumor level (*8,9,27*). On average, surgery was performed 7 days after avidin was administered, but the operation can be carried out even earlier; 2 days usually suffice for optimal clearance after avidin injection. The fact that surgery was performed at different intervals was due to organizational reasons rather than a problem in the reduction of blood BKG. These modifications may further increase the applicability and effectiveness of the RIGS method.

This study also shows the possibility for the surgeon to assess instrumentally the radicality of the operation and modify to some extent the surgical procedure too. Sardi et al. (*3*) reported that the system altered the surgical approach in 18% of their patients with recurrent colorectal cancer, and this also occurred in 26% of cases reported by Sickle-Santanello et al. (*1*). In the authors' own experience with conventional RIGS (*20*), the operation was modified in two of five patients with recurrent tumors using ^{125}I-Mab B72.3. In a recent Italian multicenter study (*29*), the surgical approach was modified in the 27% of cases and, using the second-generation Mab CC49, Arnold et al. (*30*) reported a 50% incidence of surgical modification.

In the present work, RIGS was able to identify addi-

tional subclinical deposits of tumor, which were not evident either preoperatively or intraoperatively, in two cases of primary cancers and in one case of local recurrence. In these cases, the metastatic tissue was out of the areas that are normally resected "en bloc" with the tumor for standard oncologic radicality.

These results are encouraging in terms of clinical usefulness. The reduced time interval between antibody injection and surgery opens the way to the use of isotopes, such as ^{111}In or ^{99m}Tc, which may also allow the surgeon to scan the patient preoperatively by means of external body scintigraphy (*31*).

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