Radioimmunoscintigraphy of Human Colon Cancer Xenografts in Mice with Radioiodinated Monoclonal Antibody B72.3

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Monoclonal antibody B72.3 reacts with a tumor-associated antigen that is found on human breast and colon carcinomas in significantly higher concentration than in normal adult tissues. Intact B72.3 IgG was labeled with I-131 or I-125 and injected into athymic mice bearing xenografts of human colon cancer. Whole-body scintiphotos obtained with a pinhole collimator demonstrated tumor localization within 24 hr after intravenous administration, and the tumor-to-background ratio rose continuously for at least 14 days. Progressive antibody accumulation was observed in the tumor during the first 3 days, but no significant normal organ localization was observed at any time. No localization was seen in control tumors, a human melanoma xenograft that lacks the antigen recognized by B72.3. The pharmacokinetics of this antibody in tumor-bearing mice suggest that I-131 B72.3 may be useful for radioimmunotherapy as well as radioimmunoscintigraphy of colon cancer in man.

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Radiolabeled monoclonal antibodies have gained widespread acceptance as receptor-specific radiopharmaceuticals for clinical applications. Since the hybridoma method was first described by Kohler and Milstein (*1*), many different monoclonal antibodies and their fragments have been labeled with a variety of radionuclides for gamma imaging of tumors and other tissues in animals and man (*2*). Recent clinical therapeutic trials indicate the potential role of radiolabeled monoclonal antibodies for radioimmunotherapy of cancer in man (*3,4*). The present report describes the in vivo characteristics of a radioiodinated monoclonal antibody reactive with human colon carcinomas, studied by external scintigraphy and direct tissue measurements of athymic mice bearing xenografts of human colon cancer.

Monoclonal antibody B72.3 is a murine IgG_1 recently developed using a human breast-tumor metastasis as immunogen (*5*). This antibody reacts with a glycoprotein complex of high molecular weight (~220,000–400,000 dalton), which is expressed on the surface of 50% of breast tumors and 80% of colon tumors tested (*6,7*). B72.3 does not bind to the surface of any noncarcinoma cell tested, including human melanomas, sarcomas, leukemias, and a broad range of normal adult human cell types, both red and white blood cells, nor does it react with carcinoembryonic antigen (CEA) derived from a number of tumor sources (*5*). Although it is not present in normal subjects, an occasional patient with advanced colon cancer may have low levels of circulating antigen in blood (unpublished data).

LS-174T is a trypsinized variant of a human colon adenocarcinoma cell line cultured from a single patient (*8*) and was chosen as a tumor model for the study of B72.3 because it can readily be implanted into athymic mice and exhibits high reactivity with this antibody in vivo. On the other hand, A375 is a human melanoma cell line with similar growth characteristics that can also be readily implanted into athymic mice, but lacks recognition by B72.3 and serves here as a control tumor. Computerized imaging studies were performed with the gamma camera to evaluate the pharmacokinetics of radioiodinated B72.3 antibody in tumor-bearing athymic

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mice as a model for the radioimmunoscintigraphy and radioimmunotherapy of colon cancer in man.

MATERIALS AND METHODS

Antibody preparation. The monoclonal antibody B72.3 was generated by standard hybridoma methods described elsewhere (*9*), and the immunoreactivity has been reported (*5–7*). The antibody was purified from ascitic fluid obtained from pristine-primed BALB/c mice inoculated intraperitoneally with 10 million hybridoma cells. The resulting fluid was clarified by centrifugation at 10,000 *g* for 10 min. The supernatant was adjusted to pH 7.5 with 0. 1*M* Tris-HCl, and the immunoglobulin was precipitated by the addition of an equal volume of saturated ammonium sulfate. After 1 hr at 4°C, the antibody was pelleted by centrifugation at 10,000 *g* for 10 min, dissolved in 5 ml of 10 m*M* sodium phosphate buffer (pH 7.5), dialyzed against the same buffer, and applied to an ion-exchange column (DE52, 15 ml). The column was washed with 75 ml of 10 mM sodium phosphate buffer (pH 7.5), and the antibody was eluted with a salt gradient (150 ml) of 10 to 100 m*M* sodium phosphate, pH 7.5. Fractions (2 ml) were collected and A_{280} was determined. Solid-phase radioimmunoassays (RIAs), using an extract of a human mammary-tumor metastasis as antigen, were used to localize fractions containing antibody. The appropriate fractions were pooled and dialyzed against phosphate-buffered saline. The protein concentration was determined by the method of Lowry et al (*10*).

The intact IgG was labeled with either $Na^{125}I$ or $Na^{131}I$ using iodogen as described previously (*11*) with minor modifications. For the purposes of the present experiments, 40 μg of the antibody were adjusted to 0.1 *M* sodium phosphate buffer (pH 7.2), and 0.5 mCi of $Na^{125}I$ or an equal molar amount of $Na^{131}I$ was added. The iodination protocol yielded labeled IgG with a specific activity of approximately 15 μCi/μg, with up to 60% of the input radioiodine bound to the protein. The labeled antibodies were analyzed on discontinuous sodium dodecyl sulfate/polyacrylamide gels (*12,13*). The immunoreactivity of the radioiodinated B72.3 was measured in a solid-phase RIA and averaged 80%.

Tumor preparation. Female athymic mice (nu/nu) on a BALB/c background were obtained at approximately 4 wk of age. One week later they were inoculated subcutaneously in the flank with LS-174T human colon tumor cell line (1-to-4 million cells/animal) or with A375 human melanoma cell line (4 million cells/animal). The mice developed tumors ranging from 0.5 to 1.0 cm in diameter 14 days after inoculation. They were housed in a controlled environment with KI in their drinking water to minimize nonspecific uptake of unincorporated radioiodine.

Radioimaging. For the imaging studies, two groups of animals were injected by tail vein with either I-131- or I-125-labeled B72.3. Four animals bearing the LS-174T xenografts, and two animals with A375 tumors received 62 μCi (9.0 μg) of I-131 B72.3 in 0.1 ml phosphate-buffered saline. In a separate experiment, five animals with LS-174T tumors, and two with A375 tumors received 69 μCi (9.1 μg) of I-125 B72.3 in 0.1 ml phosphate-buffered saline. Avertin was given by intraperitoneal injection before each imaging procedure to ensure adequate anesthesia. Serial scintiphotos were obtained on subsequent days with a gamma camera equipped with a pinhole collimator (aperture = 0.25 in.) positioned at a standard distance above the dorsum of each animal. A 20% energy window was centered over the 364-keV photopeak of I-131 or over the low-energy gamma and x-rays of I-125 as appropriate for each study. Each image was acquired into a 64-by-64-pixel matrix using a digital computer and stored on disk for later analysis and display. Fifty thousand counts per image were acquired during the first 4 days after I-131 B72.3 administration, and 25,000 counts were collected thereafter due to losses from decay and biological clearance. After injection of I-125 B72.3, 100,000 counts per image were acquired during the first 11 days; thereafter 50,000 counts were collected. Images were interpolated to a 256-by-256-pixel matrix for display, but no background subtraction or computer smoothing was used.

Scintigraphic analysis. Computer-assisted region-of-interest (ROI) techniques were applied to each image to integrate the counts present in total body, tumor, and body background at serial time intervals. ROIs of the same size and shape were applied consistently to the LS-174T tumor and to a corresponding site on the contralateral side of the animal, and total body integrations were obtained. Computer smoothing and correction for field nonuniformity resulting from pinhole collimation were applied before analysis, and integrated counts were corrected for tracer decay and differences in counting times to yield corrected counts per minute (cpm).

Tissue measurements. Another group of animals bearing LS-174T xenografts was used for in vitro measurements. Thirty-eight animals were given 2.7 μCi (0.4 μg) of I-131 B72.3 by i.v. injection, then killed at suitable time points. Tumors and solid organs were removed and washed before counting, and whole blood was measured as drained.

RESULTS

Radioimaging. Athymic mice bearing LS-174T human colon carcinoma xenografts were given intravenous injections of either 62 μCi of I-131 B72.3 or 69 μCi of I-125 B72.3, and were imaged at daily intervals until the count rate became too low to avoid interference from spontaneous background counts due to prolonged

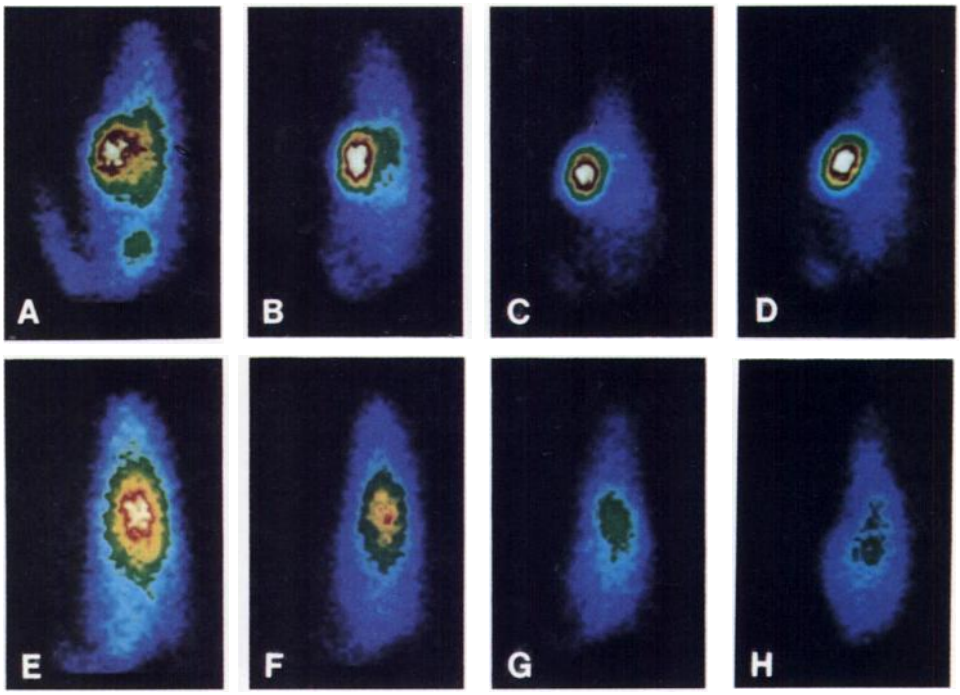

FIG. 1. Dorsal scintiphotos of athymic mice after i.v. injection with 62 μCi of I-131 B72.3. A through D: mouse bearing 0.8-cm human colon tumor on left flank is shown on Days 1, 2, 3, and 4. E through H: similar series for human melanoma of comparable size and location. Colon tumor becomes increasingly distinct as nonspecific background activity decreases, but no accumulation is detected in melanoma. Bladder activity near base of tail is visible on Day 1 (A and E) but not thereafter. Body background was comparable in two animals but appears higher in melanoma-bearing mouse due to longer counting times. Each image consists of 50,000 counts acquired into 64- by 64-pixel matrix, interpolated to a 256 × 256 matrix without background subtraction or computer smoothing. Color bar at right shows common scale used for all images, with highest value at top representing maximum pixel value in image sequence.

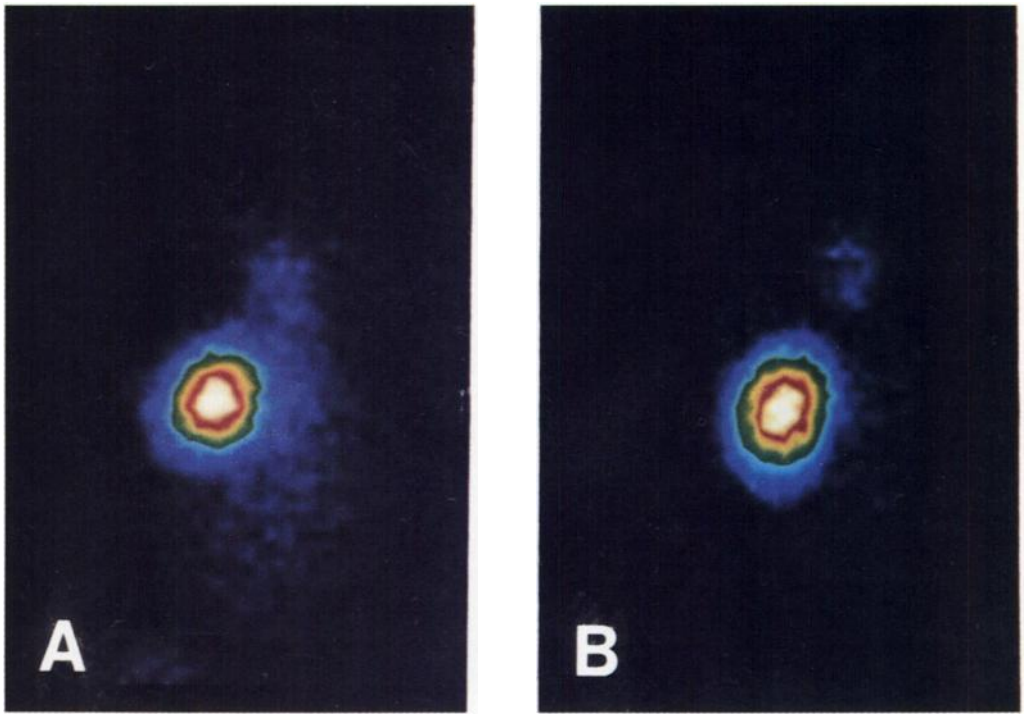

FIG. 2. Dorsal scintiphotos of athymic mouse after i.v. injection with 62 μCi of I-131 B72.3. Mouse of Fig. 1, A–D is shown here on Days 7 (A) and 14 (B). Each image consists of 25,000 counts acquired into 64 × 64 matrix, interpolated to 256 × 256 matrix without background subtraction or computer smoothing. Color bar at right shows common scale used for both images, with highest value at top representing maximum pixel value in image pair.

TABLE 1. SCINTIGRAPHIC MEASUREMENTS OF I-131 B72.3 IN TUMOR-BEARING MICE

	LS-174T*					A375
Days	Whole-body cpm	Tumor cpm	Percent in tumor	Background cpm	Tumor background	Whole-body cpm
1	13246	777	5.9	369	2.1	11787
2	9042	857	9.5	237	3.6	9737
3	7949	1064	13.4	158	6.7	8712
4	6254	838	13.4	123	6.8	8387
7	4860	1024	21.0	67	15.3	—
14	3609	745	20.6	29	25.7	—

* Four animals bearing LS-174T tumors and two animals bearing A375 tumors were studied; data shown are from single representative animal from each of two tumor groups.

imaging times (>20 min). Tumor localization was clearly visible 24 hr after administration of antibody (Fig. 1), and became increasingly clear throughout the study as body background subsided. Only tumor remained visible by Day 14 (Fig. 2). Oral KI blocked any detectable uptake of free radioiodine in thyroid tissue, and transient bladder activity was seen early in the study (Fig. 1). At no time during the study was any significant activity seen in lungs, liver, spleen, kidneys, or elsewhere other than tumor and bladder as noted above. The same pinhole collimator was used for both I-131 and I-125, and similar images were obtained with both radioisotopes.

Mice bearing A375 human melanoma xenografts similar in size to LS-174T tumors were also given either 62 μCi of I-131 B72.3 or 69 μCi of I-125 B72.3, and all were imaged in the manner described above. The tumors were not visible at any time after injection of antibody, and no specific organ localization was detected. Body background activity was seen in the early images (Fig. 1) but rapidly decreased over subsequent days.

Scintigraphic analysis. Table 1 lists computer-integrated cpm from ROIs over the total body, tumor, and representative background areas of the mice injected with I-131 B72.3. Whole-body radioactivity cleared with a biological half-time of ~5 days in animals bearing either tumor, but LS-174T tumor activity showed slow biological clearance, with progressive antibody accumulation during the first 3 days and minimal dissociation thereafter. The percentage of whole-body cpm in the tumor, and the tumor-to-background ratio, continually rose (Table 1), indicating improved imaging with time as seen by scintigraphy (Figs. 1 and 2). Similar results were obtained from mice injected with I-125 B72.3 (data

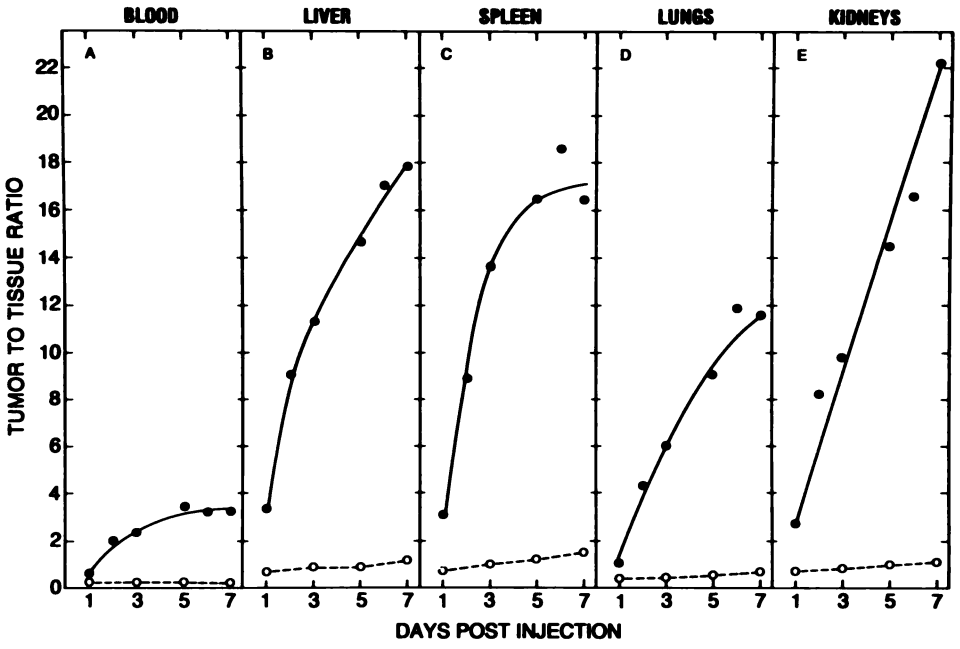

FIG. 3. Tumor-to-tissue ratios for LS-174T (solid lines) and A375 (dashed lines) xenografts in athymic mice after i.v. injection of 2.7 μCi of I-131 B72.3, shown as function of time relative to blood, liver, spleen, lungs, and kidneys.

TABLE 2. TISSUE UPTAKE OF I-131 B72.3 IN TUMOR-BEARING MICE

Tumor	Days	Percent injected dose per gram*					
		Tumor	Blood	Kidneys	Spleen	Lungs	Liver
LS-174T	1	9.24 (7.62–10.85)	15.72 (14.81–16.64)	3.39 (3.28–3.50)	3.06 (2.68–3.45)	8.34 (8.10–8.58)	2.72 (2.69–2.76)
	2	26.20 (22.03–30.50)	12.78 (11.31–14.24)	3.22 (2.51–4.01)	3.00 (2.84–3.13)	6.07 (5.48–6.64)	2.88 (2.74–3.00)
	3	21.75 (18.21–24.97)	9.45 (6.91–12.59)	2.21 (1.69–2.81)	1.59 (1.26–1.96)	5.40 (4.41–6.06)	1.91 (1.42–2.51)
	5	26.80 (20.77–32.83)	8.00 (5.39–10.68)	1.90 (1.32–2.47)	1.65 (1.19–2.10)	3.00 (2.19–3.84)	2.00 (1.55–2.25)
	6	23.86 (22.59–25.12)	5.19 (2.78–7.59)	1.07 (0.63–1.51)	0.98 0.61–1.35)	2.75 (1.27–4.22)	1.08 (0.69–1.47)
	7	18.50 (17.20–19.79)	3.48 (2.64–4.32)	0.86 (0.57–1.14)	1.16 (0.98–1.35)	1.62 (1.25–1.99)	1.06 (0.83–1.30)
A375	1	3.86 (3.01–4.71)	23.19 (20.41–25.97)	5.09 (4.78–5.40)	5.03 (4.77–5.28)	13.75 (8.07–19.41)	5.57 (5.46–5.62)
	3	3.20 (1.69–4.70)	16.14 (11.76–20.52)	3.46 (2.11–4.81)	2.96 (1.67–4.24)	6.16 (4.60–7.71)	3.26 (1.97–4.54)
	5	2.26 (1.90–2.61)	10.91 (8.03–13.79)	2.30 (1.61–2.98)	1.80 (1.42–2.17)	3.81 (2.93–4.68)	2.40 (1.75–3.05)
	7	2.73 (2.39–3.07)	11.20 (9.05–13.35)	2.37 (1.79–2.94)	1.70 (1.41–1.98)	4.02 (2.73–5.32)	2.26 (1.82–2.69)

* Total of 38 animals bearing either LS-174T or A375 tumor were studied; approximately four animals were sacrificed at each time point in each tumor group, and data are shown as means and ranges.

not shown), in which the decay-corrected radioactivity detected in the tumor by gamma imaging rose during the first 10 days before starting to fall, and provided good-quality images as late as 28 days after injection.

Tissue measurements. Necropsy studies of animals injected with I-131 B72.3 further confirm the results of gamma imaging. The rising tumor-to-tissue ratios demonstrate specific uptake in the LS-174T tumor, but the low ratios for the A375 tumor indicate minimal uptake (Fig. 3). The relatively low tumor-to-blood ratios suggest that most of the antibody not present on the LS-174T was circulating in the plasma immunoglobulin pool, and that organ and A375 activity were largely due to the blood pool. The percent injected dose per gram rose in the LS-174T tumor during the first 5 days, reaching a maximum value of 26.8%/g before starting to fall, (Table 2). The decline in percent dose per gram after Day 5 was due to tumor growth rather than loss of radioactivity from the tumor site. No preferential uptake of antibody was seen in the melanoma xenografts or in any organ of mice bearing either tumor. In other tissue measurement studies (data not shown), MOPC-21, an irrelevant monoclonal IgG_1 with no known specificity, showed no specific localization in LS-174T tumors in vivo. The distribution of the labeled MOPC-21 was similar to that of B72.3 in the A375 control tumors; therefore, the B72.3 uptake in the LS-174T tumor indicates immunospecificity.

DISCUSSION

Colorectal carcinoma is responsible for 20% of all cancer deaths in the United States with an annual fatality rate of 16 to 18 per 100,000 population (*14*). The survival rate has changed little with current therapy, largely due to late diagnosis (*15*). Each year nearly 120,000 new cases are discovered in this country (*16*), and of these, 35% will already have lymph-node involvement and another 25% will have distant metastases (*17*). The development of a RIA to measure blood levels of CEA by Gold and associates (*18*) strengthened hope that a circulating tumor-associated antigen might provide earlier detection of colorectal cancer and thus afford a more favorable response to available therapy, serving

as a screening test for high risk populations or as a marker with which to monitor the course of known disease. More recently, Goldenberg and DeLand used purified goat serum containing anti-CEA polyclonal antibodies to detect a variety of tumors in man (*19*). However, this study demonstrated nonspecific uptake, and computer subtraction of background radioactivity was necessary to aid in interpretation in areas of high blood pool activity. Mach and co-workers have been less optimistic about the clinical use of polyclonal anti-CEA (*20*), but they were able to achieve slightly higher sensitivity (50% compared with 41%) using monoclonal anti-CEA antibodies (*20,21*). Animal studies with polyclonal anti-CEA antibodies showed persistent nonspecific localization of radiotracer 6 days after injection (*22,23*). Although we have not made a direct comparison with anti-CEA antibodies in our laboratory, in this animal study it appears that B72.3 results in less nonspecific uptake (Figs. 1 and 2). CEA itself has been shown to vary considerably in structure (*24*), and furthermore, B72.3 can react with tumors that fail to express CEA (*6*). Heterogeneous anti-sera are by nature variable in immunoglobulin content and require extensive absorption to achieve specificity. Thus, hybridoma technology yielding monoclonal antibodies with defined specificity provides a more reproducible approach to tumor immunology.

Our results show that I-131 B72.3 monoclonal antibody appears to have the necessary qualities to be a useful clinical tool for the detection, staging, and management of colorectal carcinoma in man, and furthermore, that the characteristics of this antibody in vivo indicate its potential as a therapeutic agent in the treatment of advanced disease or as adjunctive therapy after primary resection. Significant tumor uptake is quickly achieved and persists, whereas the nontumor accumulation is lower and clears much more rapidly. Biopsied lesions can readily be examined for reactivity with B72.3 by standard immunoperoxidase techniques, even on fixed tissue samples. This antibody can be efficiently labeled with radioiodine without major loss of immunoreactivity, and diagnostic scintigrams could be obtained before resection to measure the extent of disease in cases of biopsy-proven reactivity. Patients whose lesions are not accessible to endoscopic biopsy could be imaged after surgery to assess the need for adjunctive therapy. Late recurrences may also be detectable when other diagnostic studies or clinical signs suggest progression of disease.

The dosimetric characteristics of the I-131-labeled antibody are favorable for therapeutic considerations, due to the high specific activity achievable, the strong avidity for binding sites in colorectal carcinoma tissue, the rapid uptake in tumor, and the lack of accumulation in nontarget tissues. Tumor dosimetry in the present study can be estimated from the percent administered dose taken up by tumor: if 26.8% of the dose collects per gram of tumor tissue, then 268 μCi/g would result per millicurie administered. Assuming a negligible biologic half-time as seen in this study, and if uptake is taken to be instantaneous, then the equilibrium dose constant for nonpenetrating radiation from I-131 (0.8065 g-rad/μCi-hr) yields a tumor dose of over 60,000 rad/mCi. It is strictly speculative to extrapolate to man, but simply on the basis of an approximate ratio in body mass of 3000:1 for man:mouse, the tumor radiation dose could be as high as 20 rad/mCi, or 2000 rad from a 100 mCi therapy dose, a practical quantity in the clinical situation, resulting in adequate tumor dosimetry if given repeatedly.

The behavior of a murine antibody in mice cannot be assumed to be the same as in man, and differences in tumor location, vascularity, and antigen expression will also occur. Furthermore, the development of human antimurine antibodies may pose a serious threat to the clinical use of murine monoclonal antibodies. Clinical trials in carefully selected patients will be necessary to demonstrate the pharmacokinetics of this antibody in man. Techniques to enhance tumor uptake and promote body clearance are under study. Fab or $F(ab')_2$ fragments may result in less nonspecific radiation exposure, minimal toxicity, and reduced likelihood of generating a human antibody response. Future methods for amplifying antigen expression in tumor and for producing human monoclonal antibodies with characteristics similar to B72.3 should eventually provide the nuclear physician with a formidable battery of agents with which to diagnose and treat cancer. In the meantime, B72.3 demonstrates the desirable qualities currently achievable for radioimmune techniques in nuclear medicine today. Clinical trials with B72.3 are currently in progress in our institution.

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