Gamma Probe Assisted Ex Vivo Detection of Small Lymph Node Metastases Following the Administration of Indium-111-Labeled Monoclonal Antibodies to Colorectal Cancers

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This study evaluated the ability of ex vivo gamma-probe scanning to detect lymph node metastases in resected surgical specimens from primary colorectal carcinoma patients undergoing external scintigraphy following intravenous administration of 4.1–5.3 mCi of ^{111}In-labeled anti CEA monoclonal antibodies. The ex vivo probe counting technique led to a twofold to fourfold increase in the number of detectable lymph nodes with the majority measuring 2–5 mm in diameter. Results indicate a potentially useful role for ex vivo probe counting in detection and mapping small (2–5 mm) lymph nodes metastases.

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The number and location of cancer-positive lymph nodes found in mesenteric tissue following resection of primary colorectal carcinoma plays an important role in the staging and prognosis of patients. If the resected specimen contains no cancer-positive lymph nodes, survival rates of up to 80% have been described. On the other hand, when one to four lymph nodes are involved, 5-yr survival drops to 25%; if more than five lymph nodes are involved, 5-yr survival is a mere 10% (*1*).

In the resected specimen, lymph nodes are usually obtained for histological examination after recognition by the pathologist. Previous studies have demonstrated that while manual and visual examination of the mesentery allows detection of lymph nodes larger than 5 mm, those under 5 mm in diameter are not routinely recognized (*2*), which may contribute to gross underestimation of clinical staging. A more accurate means of staging patients, which relies on dissolving mesenteric fat and clearing the lymph node, has been previously described (*3,4*). A lymph node clearing technique consists of treating resected specimens by dissolving fat with cedar wood oil, which results in a translucent mesentery that, while preserving the original anatomical architecture, allows detection of smaller (2–5 mm) nodes. Using this technique, Herrera et al. identified metastases in lymph nodes measuring less than 5 mm and were able to upstage patients from Duke's B to C (*1*).

Martin et al. describe a more recent technique for the intraoperative localization of tumor foci (*5,6*). Using a hand-held gamma detection probe, Martin and his colleagues studied patients after administering radiolabeled monoclonal antibodies (Mabs) and identified minute tumor foci previously undetected by sight or manual examination (*5,6*). These and other studies (*7–10*) have demonstrated the unique ability of radiolabeled Mabs to detect small tumor foci and lymph node metastases. For the past year, we have used a gamma-detecting probe for localizing lymph node metastases in ex vivo specimens resected from patients with primary colorectal carcinoma to whom ^{111}In-labeled radiolabeled Mabs were previously administered.

MATERIALS AND METHODS

A prospective nonrandomized study included 13 patients, all males, ranging in age from 52 to 72 yr (mean age 68.3 ± 6.7 yr). All patients had a histologically confirmed diagnosis of primary colon or rectal cancer and were included because of their participation in ongoing imaging and tumor localization studies with radiolabeled Mabs. At the time of presentation, primary lesions were located at the following segments of the colon: rectum (three patients); sigmoid (four patients); recto-sigmoid (two patients); descending colon (one patient); transverse colon (one patient); and cecum/ascending colon (two patients).

After signing the informed consent required by our Institutional Human Safety and Ethics Committee, patients received one of two Mabs: anti-CEA Mab IVP ZCE 025 (Hybritech, Incorporated, San Diego, CA) or anti-CEA Mab CYT-372 (Cytogen Corporation, Princeton, NJ), both labeled with ^{111}In with doses ranging from 4 to 5.5 mCi. Five patients received the IVP ZCE 025 Mab, at doses of 1 or 2 mg, and eight patients received the CYT-372 Mab at doses ranging from 0.5 to 1 mg. Patients were routinely scanned with a nuclear gamma camera at days 1–3 (CYT-372) or between days 2–5 (IVP ZCE 025), and then under-

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went surgery with curative intent usually between 7 and 14 days following the administration of the antibody.

The lymph node clearing technique has been described previously (*3,4*), but can be briefly summarized as follows: The resected colon and attached mesocolon is sent from the operating room into the pathology lab. The mesocolon is rapidly dissected away from the main section of the colon, then washed thoroughly and fixed overnight in 10% neutral buffered formalin. On Day 2, the margins of the mesocolon are mapped with ink so as not to lose the relationship to the tumor or the rest of the colon. The specimen is then placed in a large container on a Plexiglas grid anchored solidly through plastic anchors for the duration of the study. Following three 95% ethyl alcohol fixations between Days 2 and 4, the specimen is placed in 100% ethyl alcohol for three more changes and then on Day 5, while the lymph nodes are still not visible, cedar wood oil is added to the container and the specimen is left overnight. On Day 6, the mesocolon, still anchored to the plexiglas base grid, is removed from the container and placed on a light box where the lymph nodes become visible. These lymph nodes are then picked one at a time and placed on a cover sheet, numbered and placed in numerical order on rectangular pieces of numbered cardboard and a diagram of each block is recorded and infiltrated with paraffin overnight. Lymph nodes are then examined for histology, and in selected cases, CEA distribution was assessed by immunoperoxidase staining of paraffin-embedded ex vivo lymph node sections using the avidinbiotin method.

Characteristics of Gamma-Counting Probes

The physical characteristics of the gamma-counting hand-held probe (NeoProbe™, Columbus Ohio) have been described in detail previously (*5,6*). In summary, the gamma-detecting probe consists of an 18 cm long stainless steel tube housing on one end, a 1-cm diameter cadmium telluride crystal, connected by the other end to a preamplifier unit which converts the gamma emission to both an audible signal and a digital readout. The intensity and frequency of the audible signal are directly proportional to the amount of radioactivity detected. Previous studies have shown a target-to-nontarget ratio of 2:1 to be the critical value from which the audible signal in tumor is different from that in normal tissue background (*6,11*).

The count rate capability of the probe for ^{111}In was assessed in separate experiments prior to this study (*12*). Briefly, serial dilutions of ^{111}In ranging from 10 to 90 μCi/ml were prepared, and two 100-μl aliquots from each dilution pipetted into a 75 mm × 12 mm test tube. Each tube was then positioned directly over the collimated probe and counted for 10 sec each. The average of both readings plotted against ^{111}In concentrations showed a decline in count rate efficacy at concentrations of 70 μCi/ml or more, and this was verified in a separate set of experiments where serial dilutions between 50 and 80 μCi/ml were made and counted as above. From these experiments, we demonstrated that the count rate capability of NeoProbe for ^{111}In is 21,000 counts/10 sec.

Ex Vivo Gamma Detector Probe Counting

On Day 2 following surgery (usually days 9–16 post-Mab administration), the anchored mesocolon was surveyed with the gamma probe. First, a transparent grid consisting of 28 squares, 1 in. in size each, is laid over the mesocolon and secured to the sides of the container with adhesive tape. Then, 3-sec readings with the settings adjusted for the ^{111}In peak are obtained over the mesocolon. Nine readings covering the periphery and center of each 1-in. grid are obtained in duplicate and the counts directly transcribed onto the transparent grid. This procedure is repeated a second time after the fat has been dissolved, usually on Day 6 or 7 postsurgery. Probe counts prior to and following fat-clearing are averaged and correlated with the presence or absence of lymph nodes which were initially identified manually by the pathologist prior to lymph node clearing and then with the final histological and immunohistopathological data.

RESULTS

A total of 628 lymph nodes were found in the 13 resected specimens. Ninety-three (14%) lymph nodes, at least 5 mm in diameter, were discovered by manual palpation. Five hundred and thirty-five lymph nodes (85%), 5 mm or less in diameter, were identified only after fat clearing. A total of 44 lymph nodes were positive for tumor in eight of the 13 patients. Table 1 lists the distribution of these lymph nodes per patient as well as the number of positive nodes related to size. Briefly, tumor was found in 19 lymph nodes 5 mm or larger, while 25 lymph nodes smaller than 5 mm were involved with tumor and were only discovered following the lymph node clearing technique.

Positive gamma probe counts (at least 2:1 tumor-to-normal tissue count ratios) were obtained from all tumor positive lymph nodes for a sensitivity of 100%. Probe counting rates varied between specimens examined from 250 count/3 sec to 2750 count/3 sec for tumor-positive lymph nodes. However, tumor-to-nontumor ratios of at least 2:1, and in most cases 5:1, were constantly obtained. As expected, a decline in count rates between the first and second probing session, due primarily to the physical decay of the radioisotope, was observed. This indicates that the clearing process and long incubation in cedar wood oil had no effect on antibody uptake by tumored lymph nodes. In

TABLE 1
Lymph Nodes Found by Manual Palpation and Lymph Node Clearing Treatment of Surgically Resected Specimens

Patient no.	No. of tumor-positive nodes/no. of nodes found in specimen		No. of positive nodes/total no. of nodes	Tumor staging
	≥5 mm	≤5 mm		
1	2/12	0/53	2/65	T_3
2	0/2	5/27	2/29	T_3
3	0/6	0/22	0/28	T_2
4	1/4	5/44	6/48	T_3
5	8/11	11/69*	19/80	T_3
6	0/6	0/85	0/91	T_3
7	1/4	1/9	2/10	T_2
8	1/3†	0/31	1/34	T_3
9	1/5	0/14	1/19	T_3
10	0/9	0/59	0/68	T_3
11	0/17	0/49	0/66	T_3
12	5/6	3/19	8/25	T_2
13	0/8	0/54	0/62	T_3
Total:	19/93	25/535	44/628	

*Vascular invasion detected by probe counting.
†Node found tumor-positive after step sectioning due to high probe counts.

TABLE 2
Number of Gamma Probe-Positive/Number of Tumor-Positive Lymph Nodes

Size of lymph nodes	Patient no. 1	2	4	5	7	8	9	12
≥5 mm	5/2*	0/0	1/1	8/8	1/1	1/1	1/1	5/5
Average volume (cc) = 0.162								
Average gamma probe counts (cpm/cc) = 1358								
≤5 mm	0/0	5/5	5/5	11/11	1/1	0/0	0/0	3/3
Average node volume = 0.012								
Average gamma probe counts (cpm/cc) = 4125								

*High gamma probe counts (tumor-to-background ratio of 2:1) were observed in three tumor-negative lymph nodes of which one was highly vascular and the other two contained histiocytes which stained positive for CEA by immunoperoxidase staining.

some patients, a tumor-to-nontumor ratio of 17:1 was obtained, particularly in small (≤5 mm) nodes that varied from 0.1 × 0.4 cm to 0.3 × 0.3 cm in size. As depicted in Table 1, gamma probing helped upstage one patient (#2) by detecting tumor in five positive nodes which measured <5 mm and were not suspected by manual examination. In another patient (#5), vascular invasion in the pericolic zone was detected due to high probe counts. In a third patient (#8), persistently high positive gamma probe counts in a node larger than 5 mm led to step sectioning of that node and subsequent detection of metastases.

The number of gamma probe-positive nodes, compared to the number of tumor-positive nodes is illustrated in Table 2. In one patient who had received IVP ZCE 025 Mab, persistently high gamma probe counts were recorded in three tumor-negative lymph nodes. One of these nodes was highly vascular and the other two contained hystiocytes that stained positive for CEA by immunoperoxidase staining.

Table 3 illustrates the influence of tumor-to-nontumor count ratios on sensitivity and specificity of ex vivo probe counting in detecting lymph node metastases. On the other hand, when a 5:1 tumor-to-nontumor ratio was considered positive for tumors, a slight decrease in sensitivity (93%), but an increase in specificity (100%) was observed. This did not have an effect on nodes that measured <5 mm, and the total combined sensitivity and specificity was improved.

External planar scintigrams of patient #12 obtained 72 hr after ^{111}In CYT-372 administration are illustrated in Figure 1. Positive Mab accumulation in a lesion in the right colon is identified with the suggestions of metastatic involvement in one lymph node in the periaortic region. As noted in Table 1, this patient had 8 of 25 tumor-positive lymph nodes. When considering a 2:1 tumor-to-nontumor ratio is positive for tumors, the sensitivity of ex vivo probing was 100% for nodes ≥5.5 mm and 93% for nodes <5.5 mm. However, the specificity was lower (87%) for nodes ≥5.5 mm than those smaller than 5.5 mm. Figures 2A and 2B illustrate an example of the clearing effect on the fat and better visualization of lymph nodes.

DISCUSSION

This study demonstrates the usefulness of the ex vivo probe-counting technique in the detection of metastatic involvement of mesocolic lymph nodes in patients with primary colorectal carcinoma and also shows that it is a useful technique in the detection and localization of lymph node metastases <5 mm.

The high sensitivity and specificity of this technique (overall sensitivity 93% and specificity 97%) suggest that ex vivo gamma probing may be used to assist the pathologist in better evaluation of resected samples for lymph node metastases. As indicated by the results of the study, ex vivo gamma-probe counting was highly positive in eight of eight patients with subsequently confirmed metastatic involvement of the pericolic lymph nodes. The technique shows that in a select group of patients, ex vivo probe counting was the only modality which allowed the detection and identification of metastases in small (<5 mm lymph nodes) and also increased the number of detectable lymph nodes per specimen in four patients (#4, #5, #7 and #12).

TABLE 3
Influence of Tumor: Background Ratios in Gamma Probe Assisted Detection Parameters (Sensitivity and Specificity) in Patients with Tumor-Positive Lymph Nodes

Tumor: normal tissue counts		Gamma probe counting efficacy parameters TP	FN	FP	TN	Sensitivity	Specificity
2:1	≥5.5mm	19	0	7	47	100%	87%
	≤5.5mm	22	3	0	266	93%	87%
5:1	≥5.5mm	16	3	0	47	84%	100%
	≤5.5mm	22	3	0	266	93%	97%
		2:1	5:1				
Combined:	Sensitivity	85.4%	100%				
	Specificity	99%	98%				

TP = Positive gamma probe/tumor-positive LN; FN = Negative gamma probe/tumor-positive LN; FP = Positive gamma probe/tumor-negative LN; and TN = Negative gamma probe/tumor-negative LN.

FIGURE 1. Anterior planar scans at Day 3 postinfusion of ^{111}In-anti-CEA Mab CYT-372 showing a large area of Mab accumulation in the right middle quadrant (arrow), consistent with the patient's primary adenocarcinoma in that region. One or perhaps two small tumor foci in mesenteric lymph nodes are also identified (small arrows).

By manual palpation and visual inspection of the mesocolon, only 19 of 93 nodes measuring 5 mm or greater in diameter were detected and were consistent with metastatic involvement. On the other hand, 25 of 535 lymph nodes measuring ≤5 mm were found to be involved with tumor after the lymph node clearing procedure. The relatively low yield of positive lymph nodes in specimens ≤5 mm is consistent with previous studies by Herrera-Ornelas et al. (*1,13*). The finding of an average of 41 lymph nodes per surgical specimen following the lymph node clearing technique compares favorably with results by Cawthorn and Herrera-Ornelas, and validates our technique for lymph node clearing. As indicated in prior studies, the identification of additional nodes per specimen places the patient in a higher category in the Duke's staging, thus adversely affecting long-term survival. As demonstrated in this study, the combination of the ex vivo probe counting and lymph node clearing increased the number of cancer-positive lymph nodes in four of the eight patients (50%), and may eventually put these patients in a different category for therapy. It is also important to mention that the ex vivo probe counting alone led to the identification of vascular invasion in the peripheral zone of the mesocolon in one patient and in another patient (#8), an isolated lymph node in the distant mesocolic region was suspected by persistently high probe counts while initial examination of this lymph node revealed no tumor. However, step sectioning showed a cluster of metastatic cells. This demonstrates that although a rare event in colonic cancer, skip metastases to principle lymph nodes can occur and should be suspected in the presence of high probe counts.

The study also demonstrates that there is a potential for false-positive readings using the probe counts when a tumor-to-nontumor ratio of 2:1 is considered suspicious for tumor. Thus, we found high counts (2:1) in highly vascular lymph nodes, lymph nodes closely matted together, blood vessels, calcified lymph nodes, lymph nodes larger than 1 cm, antigen-positive/tumor-negative nodes and hystiocytes staining positively for CEA. The presence of high counts in antigen-positive/tumor-negative or in hystiocytes staining positive for CEA is of unknown and uncertain significance.

This study also demonstrates the need to compare count rates within the same specimen if false results are to be avoided. Consistent with previous reports of radioimmunoguided surgery (RIGS)™, the tumor-to-nontumor ratio determines the presence or absence of tumors rather than the count rate in a given specimen. In this study, tumor-to-nontumor ratios of 2:1 to 5:1 were usually obtained despite the wide variability in count rates from patient to patient, but not within specimens. We attribute the wide variation (250 cc to 2750 cc/sec in tumor positive nodes) to several factors, including the amount of radioactivity injected (4–5.5 mCi), the time elapsed between antibody injection, surgery and subsequent analysis and probing of the specimen, as well as variability in the in vivo fate of these Mabs between patients. As mentioned earlier, two antibodies (CYT-372 and IgG_{2a} and IVP ZCE 025 and IgG_1) were used in this study, and our preliminary pharmacokinetic analysis indicates a shorter $T_{1/2}$ β of CYT-372 than IVP ZCE 025, although this difference was not statistically significant.

Selecting a higher (5:1 ratio of tumor-to-nontumor background) setting led to an increase in specificity, with a relative decrease in sensitivity, seen mostly in nodes ≥5 mm. This is not a bothersome finding, since most of these

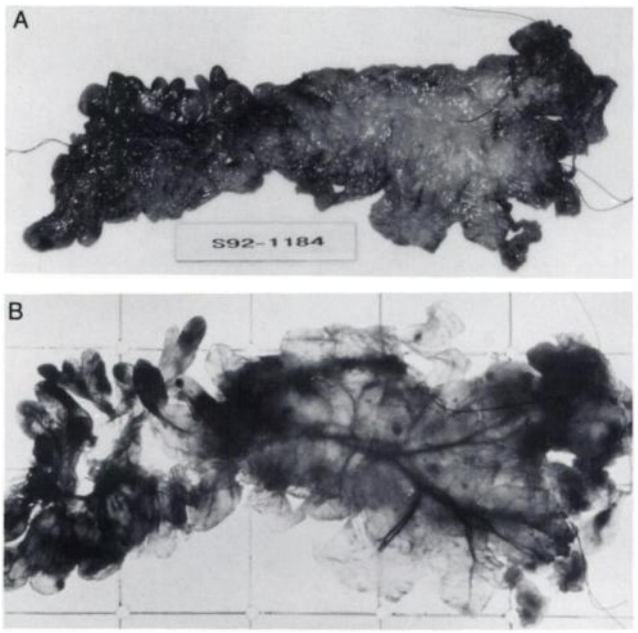

FIGURE 2. Resected mesentery prior (A) and after (B) lymph node clearing method. Lymph nodes are now clearly visualized.

nodes would be identified by the pathologist using manual palpation and visual examination. However, when a positive 5:1 ratio was selected, neither the sensitivity nor the specificity for lymph nodes ≤5 mm were affected and we thus recommend using a 5:1 tumor-to-nontumor ratio in further studies.

With a combined sensitivity and specificity of 93% and 97%, respectively, for this technique which was confirmed by lymph node clearing method, we believe that the ex vivo probe counting method is reliable and can accurately identify the presence of metastatic involvement in small (<5 mm) lymph nodes and may impact on patient management. Its use would obviate the need for performing the lymph node clearing procedure, which is time consuming (up to 1 wk) and relatively expensive (about $600–$800 per specimen) when the technologist's time and materials and supplies are taken into consideration. This becomes of particular importance when one considers the low incidence of lymph node metastases in glands ≤5 mm in diameter.

In conclusion, we found that the ex vivo probe counting technique to be particularly useful for the detection and accurate staging of lymph node metastases in patients with primary colon carcinoma and that it is particularly helpful in the lymph node metastases measuring 5 mm or less. The impact of this technology on the staging and long-term survival in patients with colon/rectal carcinoma is yet to be determined.

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