Monoclonal Antibody Imaging of Human Melanoma

Radioimmunodetection by Subcutaneous or Systemic Injection

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Fab fragments of monoclonal antibodies (MoAb) to melanoma, radiolabeled with ^{131}I, were evaluated as diagnostic reagents to determine their ability to localize systemic—MoAb injected intravenously (IV)—or nodal metastatic disease—injected subcutaneously (SQ) at a site proximal to draining lymph nodes. Sixty-one scans were performed (40 IV, 21 SQ) in 59 patients who had injections of 0.2–50 mg of ^{131}I coupled (0.2–12 mCi) antibody. These included 48.7, which identifies a high molecular weight antigen (HMW), or 96.5, which identifies a transferrin like molecule, p97. ^{125}I coupled nonspecific Fab 1.4, reacting with murine leukemia virus, or the whole antibody BL3, reactive with a human B cell idiotypic determinant, was generally used in tandem with the patients injected SQ as a nonspecific control. All patients had immunohistochemical studies performed on biopsied lesions and demonstrated binding to the antibodies injected. Of the IV patients, 22/38 (58%) had (+) scans, 13 at SQ or nodal sites, four at visceral sites, and five at visceral and SQ sites. Patients with clinical stage II disease had SQ injection of MoAb, including 11 additional patients injected with the whole antibody 9.2.27 (anti-HMW) labeled with ^{111}In (6 patients) or ^{131}I (5 patients). Nodal dissection was performed 2–4 days later. All ^{111}In coupled antibodies demonstrated excellent nodal delineation without specific identification of tumor deposits. Of the 21 patients injected SQ with MoAb, 17 had confirmed tumor in nodes. Of patients injected with Fab fragments, 4/8 (50%) had specific uptake of MoAb, although only two were successfully imaged. Increased uptake of antimelanoma antibodies was observed in some patients in lymph nodes not containing tumor and was possibly related to antigen shedding. Clearance of labeled antibody from the injection site occurred with a half life of 16–50 hours. Toxicity was limited to local discomfort at the site of SQ injection. Melanoma metastases can be identified with IV or SQ injection of radiolabeled antibodies. These reagents may be useful in the diagnosis or therapy of human melanoma. Further evaluation will be required before they could be considered clinically useful.

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THE ABILITY TO IDENTIFY tumor deposits using monoclonal antibodies has been a goal since the development of hybridoma technology.[1] Large amounts of specific, highly purified monoclonal antibodies reactive with melanoma cell surface associated antigens are now available and have been used in both diagnostic and therapeutic approaches to this tumor.[2–6] In addition to intravenous administration, we have evaluated both whole antibodies and Fab fragments of antimelanoma antibodies as diagnostic reagents injected subcutaneously (SQ) to determine if tumor deposits could be identified in regional draining lymph nodes. Since the role of nodal dissection in patients with clinical stage I disease remains controversial,[7,8] attempts to localize lymphatic drainage and spread are still being carried out.[9] Based on studies conducted in rodents,[10,11] we evaluated the lymphatic route of administration of monoclonal antibodies in patients with known stage II disease to determine if the theoretically greater sensitivity, higher target to background ratio, faster localization, and lower toxicity could be achieved. We have contrasted our results using subcutaneous injection sites in 21 patients with 40 scans done in patients receiving Fab fragments of antimelanoma antibodies as to toxicity, clearance, and identification of tumor deposits.

Materials and Methods

Patients

All melanoma patients considered for these evaluations were treated on two clinical research committee approved protocols utilizing (1) antibodies for diagnostic and possible therapeutic intravenous use and (2) antibodies for subcutaneous injection, coupled with surgical dissection of stage II disease. All patients signed an informed consent indicating the possible risks and the investigative nature of these studies. To be included, patients had to have clinical evidence of stage II or III melanoma, no known allergy to iodine or mouse antibodies, and, in patients with stage II disease, were required to have no history of radiotherapy or prior dissection of the regional lymph nodes. All patients received complete blood counts, platelet counts, liver function and renal function studies, and thyroid function tests. Radiologic evaluation of cerebral, pulmonary, and hepatic disease as well as measurement of evaluable lymph nodes and local tumor nodules was performed. Plasma was stored prior to and at intervals after imaging for detection of human antimouse antibodies. Immunohistochemical testing of a biopsy of the tumor or its metastases was carried out on frozen tissue if available. When tumor was not available prior to injection (stage II patients), tumor obtained at the time of nodal dissection was submitted for confirmation of antigen positivity.

Monoclonal Antibodies and Radioactive Labeling

The MoAb 9.2.27 is a murine antibody (IgG2a,k) described by Morgan and coworkers.[12] It recognizes a high molecular weight (240 Kd chondroitin sulfate/proteoglycan) cell surface antigen present on 90% of human melanoma. The 9.2.27 MoAb was obtained by immunizing mice with soluble extracts from human melanoma cells. Hybridomas were developed by fusion of Balb/C splenocytes with the murine myeloma p3-x63-Ag-8. Immunoglobulin was purified from hybridoma ascites using a double precipitation procedure with Na_2SO_4. The antibody was tested as recommended by the Office of Biologics, U.S. Food and Drug Administration (FDA).

A modification of the bifunctional chelating method of Krejcarek was utilized to conjugate diethylenetriaminepentaacetic acid (DTPA) to 9.2.27.[13] The antibody was received in kit form (Hybritech, Inc., La Jolla, CA), consisting of 1 mg DTPA-conjugated 9.2.27 MoAb in 1% human serum albumin (HSA). Labeling was performed by incubating approximately 5 mCi ^{111}In with 1 mg of DTPA-conjugated 9.2.27. Excess DTPA was then added to scavenge any free ^{111}In. The antibody was administered under an Investigational New Drug application approved by the FDA.

The antibody 48.7 is a murine[14] monoclonal antibody (IgG_1) that also identifies the high molecular weight antigen on human melanoma. This antibody was prepared by fusing SP2/0 mouse myeloma cells with spleen cells from mice immunized with a cultured melanoma line. Weak staining of some blood vessels was noted, but other normal cells including skin melanocytes and other tumor types were unstained using peroxidase immunohistochemical stains. The antibody 96.5 is a murine[15,16] antibody that identifies p97, a protein associated with human melanoma, and is present in only very small amounts in solublized normal tissues (<10 ng/mg). The antibody 1.4 is an IgG_1 that recognizes a murine leukemia viral antigen, GP70, and is negative for all human cells tested (Brown, unpublished). The whole antibody BL-3, raised against a human B cell lymphoma, identifies unique idiotypic determinants on that patient's cells not found on cells from other individuals (K. Foon, unpublished) and was used as a negative control. Immunohistochemical studies were performed as previously reported.[14] All tumors were graded as no staining (0), 25% staining (1+), 50% of cells stained (2+), 75% of cells stained (3+), and very strong (100% of cells) staining (4+).

Fab fragments were obtained by first incubating the antibody with papain (150 μg) in 4 ml of phosphate buffered saline (pH 7.2) containing 10 mM cysteine and 2 mM EDTA under nitrogen at 37 C for 3 hours.

Iodoacetamide was added to a final concentration of 50 mM and the digest filtered on Sephadex G-25 (Pharmacia, Piscataway, NJ) equilibrated with saline. Fc fragments were removed by passage through a 5 ml column of protein A coupled to Sepharose CL-4B (Pharmacia, Piscataway, NJ). Iodination was carried out by the method of Ferens et al.[17] The Fab fragments were incubated with Na ^{125}I or Na ^{131}I (DuPont/NEN Medical Products, N. Bilerica, MA) and 23 μg of chloramine T per mg of Fab fragments, in phosphate buffered saline for 10 minutes at 0 C. The reaction was stopped by adding 81 μg of sodium thiosufate per mg protein and the labeled protein separated on a column of Sephadex G-25.

Radiopharmaceutical Purity and Quality Control

Immunoreactivity was determined using the H1777 or Fem XII melanoma cell lines, which express both p97 and HMW antigens. Cells (2×10^6) were suspended in 100 μl of the test sample containing 5 ng of the radiolabeled protein. Phosphate buffered saline was added, the mixture was centrifuged, and cells were separated and counted for radioactivity. Results are expressed as the percentage of total added radioactivity bound to cells. The percentage of counts protein bound was determined by trichlorocetic acid precipitation of protein. ^{131}I preparations were analyzed for free iodine using paper chromatography (Whatman I) and an 85% methanol in water

solvent. ^{111}In preparations were analyzed for ^{111}In DTPA using instant thin layer chromatography in silica gel with methanol:water (1:1) with 5% ammonium acetate as a solvent. Pyrogen and sterility testing was routinely carried out.

Antibody Administration and Sample Collection

Patients receiving intravenous administration of antibody had it infused slowly over 60 minutes through a peripheral vein. No adverse reactions during or subsequent to the infusion were noted. Some of the patients receiving antibody SQ were injected in 12 separate locations around the primary excision site and a comparable number symmetrically placed on the contralateral side. Other patients were injected in the four web spaces of each hand or foot. Equal quantities of specific and nonspecific antibody proteins (independent of the specific activity) were always injected. Problems associated with subcutaneous injection were limited to local discomfort and occasional erythema (Fig. 1).

Following administration, blood samples were collected at 5, 30, 60, 120, and 240 minutes and at daily intervals until surgery was performed. Daily urine collections were performed up to 48 hours after infusion. Excreted urinary counts were determined on collections obtained inclusive of all urine at 2, 24, and 48 hours using aliquots and then multiplying counts by total volume for that time period. Whole body retention was measured with a 2 × 2 inch sodium iodide crystal positioned 7.1 meters from the patient in a fixed geometric position. The immediate postinfusion value was taken as 100%.

Surgical samples were obtained from nodal dissections and examined by a pathologist (G.B.). Separate lymph nodes and other tissues were isolated by dissection, weighed, counted in a gamma counter (along with standards prepared from injected radiolabeled antibody), and submitted for histologic and immunohistochemical staining. Decay correction and spillover correction for ^{131}I counts in the ^{125}I channel were routinely performed.

Assay for Human Antimouse Antibody (HAMA)

A test sample of the patients' serum (10 μl) was incubated with 10 μl of ^{125}I-labeled mouse IgG (10^4 cpm) for 30 minutes at 20 C. *Staphylococcus aureus* (10 mg) was added in 1 ml of 20 mM Tris-HCl buffer, pH 8.0, containing 100 mM NaCl, 1 mM EDTA, and 0.5% Nonidet P40 (TNEN) (Bethesda Research Laboratories, Bethesda, MD). After a further 5-minute incubation, the bacteria were separated by centrifugation and washed three times with 10 ml of TNEN and counted for ^{125}I. A comparison was made between the test sample and a control serum that did not contain human antimouse antibodies (HAMA). A positive test was considered to be a value twice the control.

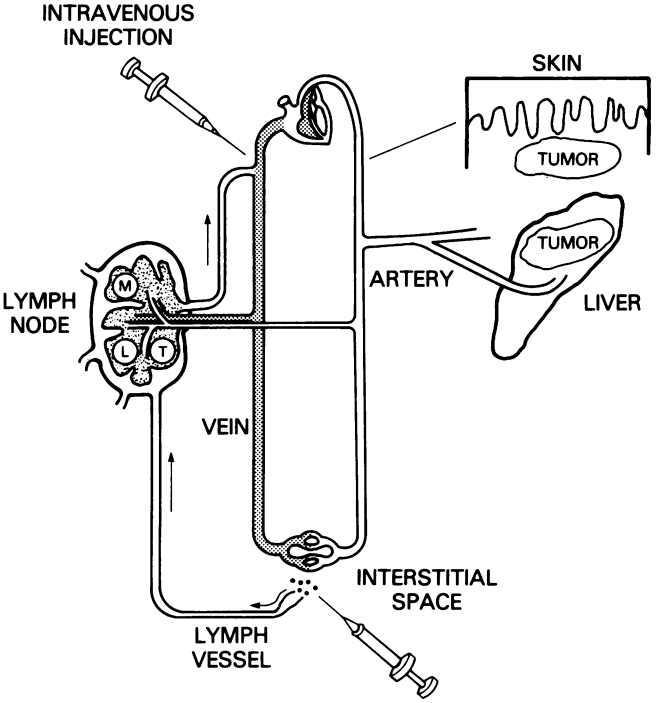

FIG. 1. Routes of administration of antibody in these studies. Intravenous injection was carried out to identify tumor deposits in systemic sites including skin, liver, lung, lymph nodes, *etc.* Subcutaneous injection using lower doses of radiolabeled antibody was used to identify nodal disease. Some delivery of antibody into the vein is expected to occur from efferent lymphatics draining eventually into the thoracic duct.

Patient Imaging

The patients were imaged within 2 hours of antibody administration and daily up to the day before operation. A large field of view gamma camera with a high energy collimator was used. A 20% window over the 364 kev gamma ray of ^{131}I was used to obtain anterior and posterior whole body images as well as multiple spot views (5–10 minutes). In addition to analog images, digital images were recorded with a Hewlett Packard scintigraphic computer (Rockville, MD). To calculate serial changes, images were analyzed with manually drawn regions of interest over the major organs and tumor. Values were expressed as counts per minute (CPM) corrected for isotope decay. For patients injected with Indium-111 labeled antibodies, images were obtained using a 20% window over the 172 and 247 kev gamma ray.

Results

Patients Studied and Reagents Used

The antibodies used in these studies are listed in Table 1. Patients studied by intravenous injection received only antimelanoma antibody but no irrelevant control. These intravenous studies were done in an attempt to identify

TABLE 1. *Antibodies Used in Diagnostic Imaging*

Designation	Subclass	Reactivity
48.7 Fab	IgG_1	High molecular weight (HMW) antigen on melanoma (>200 kD); on 70–90% of melanomas
96.5 Fab	IgG_{2a}	Recognizes p97, a transferrin like molecule on 70–90% of melanomas: low crossreactivity with liver
9.2.27 whole antibody	IgG_{2a}	Recognizes different epitope on same antigen as 48.7 (HMW)
1.4 Fab	IgG_1	Control antibody recognizes murine leukemia virus, negative on human cells
BL-3 whole antibody	IgG_1	Control antibody, raised against a human B cell lymphoma; anti-idiotype antibody

patients with tumor that might be amenable to therapy with the antibody. If visualized, the patients were considered for treatment with larger doses of radiolabeled (100–200 mCi/10 mg) antibody. The patients studied are listed in Table 2 along with information regarding their specific evaluation. The last seven patients listed (#34–40) had no diagnostic studies but were scanned while receiving therapy. Patients receiving subcutaneous specific and nonspecific antibody are listed in Table 3. Some of these patients were coadministered cold antibody (patient 56) or unlabeled human immunoglobulin preparations (patients 53 to 55) to decrease uptake into negative nodes. This was unsuccessful.

For patients with available tumor, immunohistochemical staining of tumor tissue was carried out with the an-

TABLE 2. *Patients Studied with Intravenous Injection of Monoclonal Antibodies to Melanoma*

Patient Scan Number	Age/Sex	Sites of Disease	Antibody Used	Mg Dose Ab	mCi Dose I-131	Image	Notes
1	46/M	Skin, nodes	48.7	10	5.6	− (NS)	Largest lesion 5 × 4 cm, others 1–2 cm
2	52/F	Skin, nodes	48.7	10	10.0	− (NS)	Largest lesion 3.5 × 5 cm
3	46/M	Skin, lung	48.7	10	6.4	+ (SS, SP)	Largest lesion 2 × 3 cm in skin, 6 cm in lung
4	68/M	Skin	48.7	10	8.9	+ (SS)	Largest lesion 3 × 7 cm in skin, smaller 2 × 3 cm not seen
5	52/M	Skin	48.7	10	8.9	+ (SS)	Largest lesion 5 × 6.5 cm in skin seen; 5 lesions < 1 cm not visualized
6	40/F	Nodes	48.7	10	8.7	− (NS)	Pelvic periaortic nodes not visualized
7	81/F	Skin, nodes	48.7	7.3	7.8	+ (SS)	Largest lesions 2.5 × 2.5, 1.5 × 1 visualized; of other 12 lesions <2.5 cm, only 1 seen
8	69/F	Skin, nodes	48.7	10	8.1	+ (SS)	1/8 lesions visualized
9	65/M	Lung	48.7	10	8.1	+ (SP)	Multiple lesions; treated
10	70/M	Skin	48.7	7.7	8.8	+ (SS)	4/22 lesions visualized
11	59/M	Skin	48.7	7.8	7.8	+ (SS)	3/4 lesions visualized
12	38/F	Skin	48.7	7.4	8.2	− (NS)	Multiple small lesions around head and neck not visualized
13	38/M	Skin	48.7	8.3	9.2	+ (AS)	7/7 lesions seen
14	34/M	Malignant ascites	48.7	7.1	6.6	+ (AG)	Marked uptake in peritoneal cavity
15	47/M	Skin, lung	48.7	8.2	7.7	+ (SS)	Diffuse uptake lungs
16	47/M	Liver	96.5	7.8	13.3	− (NH)	
17	65/M	Lung, spleen	96.5	7.8	13.2	− (NP, NL)	Extensive disease
18	77/M	Lung, nodes	96.5	10.0	10.0	+ (AP, SS)	
19	61/M	Lung, skin	96.5	50.0	10.0	+ (AP, AS)	Multiple lesions, subsequently treated × 3 with 100 mCi I-131-Ab
20	34/M	Lung, skin, spleen	48.7	50.0	5.0	− (NP, NS, NL)	7 subcutaneous lesions 1–2 cm, lung 2 × 2 cm, spleen 2 × 1/2 cm
21	36/M	Skin, lung	48.7	42.0	10.0	+ (SP, AS)	

TABLE 2. (*Continued*)

Patient Scan Number	Age/Sex	Sites of Disease	Antibody Used	Mg Dose Ab	mCi Dose I-131	Image	Notes
22	52/M	Liver, lung, brain, skin	48.7	42.0	10.0	+ (AS, AP, NH, NL)	
23	45/M	Skin, liver, bone	48.7	44.5	7.8	+ (SS, NH, NB)	1/3 lesions > 1 cm seen, 0/11 lesions <0.5 cm
24	34/F	Liver, skin	96.5	24.0	9.9	− (NS, NH)	
25	69/F	Skin, nodes	96.5	45.6	7.1	− (SS)	Same as patient #8, 0/20 lesions
26	62/F	Lung, skin, GI	96.5	9.1	10.0	+ (NP, NG, SS)	
27	35/M	Skin, lung	96.5	9.1	10.0	− (NS)	Visualized after 3rd dose of antibody on therapy, 200 mCi × 2
28	41/F	Lung, brain	96.5	11.5	9.6	+ (SS)	Treated with 200 mCi × 3
29	62/M	Lung	96.5	11.5	9.6	− (NP)	
30	30/F	Lung	96.5	7.8	10.0	− (NP)	Later treated with IL-2
31	34/F	Lung	96.5	7.8	10.0	− (NS)	False positive at site of recent operation left thigh
32	49/M	Brain, skin	96.5	9.0	10.0	− (NS)	Multiple cutaneous lesions
33	49/M	Brain, skin	96.5	44.8	10.0	− (NS)	Same as patient #32
Therapy scans only							
34	23/F	Skin, lung	96.5	10.0	100	− (NP, NS)	
35	30/F	Skin	96.5	10.0	100	+ (SS)	Largest lesion 5.5 × 5.5 cm
36	64/M	Lung, liver, GI, skin, heart, kidney	96.5	10.0	150	− (NP, NH, NG, NS)	
37	66/M	Liver	96.5	10.0	150	+ (AH)	
38	62/F	Lung, GI	96.5	10.0	150	− (NG, NP)	
39	41/M	Lung, skin	96.5	10.0	200	+ (SP, SS)	
40	35/M	Skin	96.5	10.0	200		

S = some; A = all; N = none; S = skin and nodes; P = pulmonary; H = liver; B = bone; C = brain (cerebrum); GI = gastrointestinal; L = spleen.

tibody used and demonstrated to be 2+ or greater with sole exception. Some patients had determinations with other antibodies as well. The results of these assays are presented in Table 4. Most tumors demonstrated significant binding of each of the antibodies evaluated.

No apparent toxicity was noted in any patient studied with either subcutaneous or systemic injection of antibody. No patient receiving subcutaneous or imaging (nontherapeutic) doses of antibodies developed HAMA. Some patients with repetitive therapeutic doses of antibody developed positive HAMA.

Immunoreactivity and Radiopharmaceutical Purity

All antimelanoma monoclonal antibodies injected were evaluated for their ability to bind to a cultured melanoma target prior to injection (Table 5) and following radiolabeling. Fab 96.5 was judged to have excellent binding characteristics, with a mean of approximately 60% of the antibody binding to cells (for preparations used both systemically and SQ). Similar results were obtained for the whole antibody 9.2.27. Although 48.7 is thought to recognize a different epitope of the same antigen as 9.2.27, its radiolabeled Fab fragment routinely gave poor results in the cell binding assays with <25% of the antibody binding in each preparation. Because of these difficulties, further subcutaneous studies were conducted with 96.5 or 9.2.27.

Systemic Imaging

Fab fragments were used for systemic imaging rather than whole antibody because they are less immunogenic and more closely approximate the ideal radiopharmaceutical. Nonspecific binding to Fc receptors on monocytes and polymorphonuclear leucocytes is eliminated and

TABLE 3. *Patients Studied with Subcutaneous Injection of Monoclonal Antibodies to Melanoma*

Patient Scan Number	Age/Sex	Site of Injection	Dissection	Antibody	Isotopes	Specific Ab μg* Dose	Specific Ab μCi* Dose	Non-specific Ab μg* Dose	Non-specific Ab μCi* Dose	No (+) Nodes	Image	Localizing to (+) Nodes/Comments
41	58/F	Ant. tibia	L. groin	48.7/1.4	I-131/I-125	200	200	200	133	1/9	+	+ (ratio 1.5)
42	50/M	Back	L. neck	48.7/1.4	I-131/I-125	200	210	200	86	0/9	−	NR
43	62/F	Back	L. axilla	96.5/1.4	I-131/I-125	200	200	200	172	1/17	−	± (ratio 1.6)
44	33/M	Back	R. axilla	96.5/1.4	I-131/I-125	220	157	220	200	8/21	−	+ (ratio 1.2–4.1)
45	62/M	Back	L. groin	96.5/1.4	I-131/I-125	200	200	200	200	0/19	−	NR
46	42/F	Back	R. groin	96.5/1.4	I-131/I-125	620	220	620	61	3/11	−	−
47	33/M	Back	L. axilla	96.5/1.4	I-131/I-125	220	200	220	71	1/20	−	−
48	59/F	Hands/webs	R. neck, parotid	96.5/1.4	I-131/I-125	100	200	100	76	14/35	−	−
49	37/M	Hands/webs	R. axilla	9.2.27/1.4	In-111/I-125	100	250	100	84	9/35	−	−
50	71/M	Feet/webs	R. groin	9.2.27/ND	In-111	100	242	ND	ND	0/9	−	NR
51	38/M	Hands/webs	R. axilla	9.2.27/1.4	In-111/I-125	94	250	94	67	1/21	−	−
52	65/M	Feet/webs	L. groin	9.2.27/ND	In-111	100	250	ND	ND	5/13	−	−
53	38/F	Hands/webs	R. axilla	9.2.27/ND	In-111	100	250	ND	ND	1/32	−	− Human IgG (90 mg)
54	24/M	Hands/webs	L. axilla	9.2.27/ND	In-111	1000	250	ND	ND	9/21	−	− Human IgG (37 mg)
55	32/M	Hands/webs	L. axilla	9.2.27/ND	I-131	100	200	ND	ND	1/17	−	− Human IgG (64 mg)
56	24/M	Back	L. neck/ parotid	9.2.27/ND	I-131	40	200	ND	ND	2/23	−	− Tenfold unlabeled 9.2.27
57	32/M	Feet/webs	L. groin	9.2.27/BL3	I-131/I-125	97	253	93	250	4/14	−	−
58	64/M	Head	R. neck	9.2.27/BL3	I-131/I-125	100	313	82.5	200	1/17	−	−
59	32/M	Feet/webs	L. groin	9.2.27/BL3	I-131/I-125	53	173	52	173	0/8	−	NR
60	40/F	Feet/webs	L. groin	96.5/1.4	I-131/I-125	1800	72	1400	66	15/16	+	+ (ratio 1.35–8.73)
61	61/M	Feet/webs	R. groin	96.5/1.4	I-131/I-125	2000	200	2000	200	2/17	−	−

ND = not done.
* Per side.
NR = not relevant (all nodes negative).

clearance is rapid. The mean clearance of the Fab fragments 48.7 and 96.5 is shown in Figure 2. In patients receiving Fab 48.7, less than 30% of the injected dose was present in the "whole body" at 48 hours with less than 3% present in the plasma. Similar findings were noted for the Fab 96.5.

Representative scans are shown in Figures 3 and 4. In Figure 3, scans from a patient injected intravenously (IV) with Fab 48.7 at 2 and 48 hours following injection are shown. As was true in other patients, disease greater than 1–2 cm in diameter could be visualized, but disease smaller than this size was rarely imaged. Serial scans on patient #19, imaged and subsequently treated with ^{131}I-96.5 Fab are shown in Figure 4. The 2-hour imaging study shows blood pool, bladder excretion of iodine, and early definition of some cutaneous lesions. A 24-hour scan shows cutaneous sites more clearly as well as some uptake of free iodine in the thyroid, stomach, and scrotum in addition to bladder excretion. This patient received three therapy doses of 100 mCi of ^{131}I Fab, and scans shown are at 6 days following the second and third treatments. Multiple cutaneous deposits and pulmonary lesions imaged very well. Uptake in the liver may have reflected the known weak staining of this tissue by this antibody or perhaps may represent uptake of antibody by cells of the reticuloendothelial system.

The results of systemic imaging in 40 studies conducted in 38 patients using these Fab fragments are summarized in Figure 5. Disease was imaged in skin and nodal sites in 13 patients, at visceral sites in four patients, and at skin and visceral sites in five patients. This is comparable to

TABLE 4. *Immunohistochemistry of Tumor from Patients Studied*

Antibody	96.5	HMW	GD3
0	0	1	0
1+	2	1	1
2+	17	20	16
3+	25	21	9
4+	1	3	0
Total	45	46	26

0, no cells stained; 1+, 25% cells staining; 2+, 50% cells staining; 3+, 75% cells staining; 4+, 100% cells staining. Nonspecific staining of 9.2.27 on some tissues without tumor gave high backgrounds.
HMW = high molecular weight antigen (48.7 or 9.2.27).

TABLE 5. *Immunoreactivity of Injected Antibodies and Radiopharmaceutical Purity*

Route	Antibody	No. of Studies	mCi, Mean (Range)	mg, Mean (Range)	Cell Binding Assay Mean (Range)	% Protein Bound Mean (Range)
IV	96.5	20	59.2 (9.6–200)	14 (7.8–50)	59.8% (31.6–97.6)	87.5 (77.1–95.8)
IV	48.7	20	8.0 (5.6–10.0)	17.9 (7.1–50)	17.1% (9.2–25.2)	86.6 (68.1–95.2)
SQ	96.5	8	0.41 (0.14–0.8)	1.3 (0.2–4.0)	63.1% (57.0–70.8)	92.1 (89–96.4)
SQ	48.7	2	0.41 (0.40–0.42)	0.4 (0.4)	20.1% (16.2–24.2)	89 (89)
SQ	9.2.27	11	0.48 (0.34–0.62)	0.25 (0.08–1.0)	59.8% (48.9–74.2)	95.8 (94–98.1)

our previous experience.[2,3] The major limitation in the use of these reagents was the lack of imaging of small (<1 cm) lesions.

Subcutaneous Injection and Nodal Imaging

A total of 21 studies were conducted over a 2-year period. The initial studies were conducted using Fab fragments because of their favorable characteristics (noted above). The first patient had a large 4 × 3 cm node in the left groin on presentation. This patient had injections of Fab 48.7 into the subcutaneous tissues overlying both the right and left anterior tibia. Clearance from the injection sites was rapid, with less than 15% remaining at 48 hours and less than 3% at 7 days (Fig. 6). A maximum of approximately 10% of the injected dose was found in the plasma at 20 hours, with subsequent clearance and less than 1% at 7 days. Similar clearance from other sites using this and other Fab fragments in other patients was noted. Excretion of radiolabel into the urine was also followed

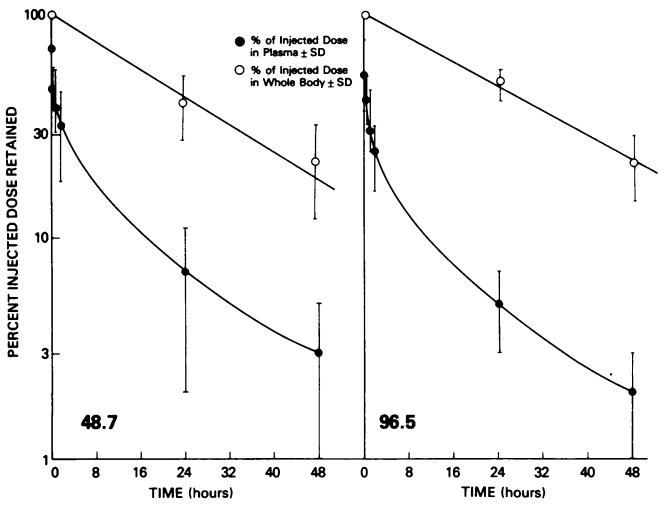

FIG. 2. Mean clearance of ^{131}I-antimelanoma Fab fragments injected intravenously in 24 patients. Rapid clearance and lack of nonspecific binding *via* Fc receptors was the major advantage of this approach.

(Table 6) in patients injected SQ. Most of the activity excreted in the urine in the first 2 hours was free of protein (nonprotein bound) and represented rapid clearance of the unconjugated radionuclide. Most of the counts excreted in the following 24–48 hours were associated with protein and probably represented unprocessed antibody or breakdown products. Clearance from the whole body and plasma is listed in Table 7.

Sequential scans in the first patient injected with ^{131}I-48.7 Fab are shown in Figure 7. Views of the anterior pelvis at 24 and 48 hours are shown. Activity in the bladder representing ^{131}I excretion is seen, and persistent counts are also apparent in the left groin at the site of localized 4 × 3 cm inguinal lymph node metastases. A similar study in a patient receiving ^{131}I-96.5 Fab is shown in Figure 8. This patient (#60) was injected into the web spaces of the feet as a convenient route to enter the lymphatics and more proximal lymph nodes. Views of the pelvis and abdomen at 24 hours were obtained. Excretion into the bladder and uptake into (left) inguinal nodes are noted. This patient had previously undergone a right inguinal nodal dissection. The specificity of antibody uptake was confirmed by *in vitro* counting of positive and negative tissues (Fig. 9). The patient, site of injection, and specific antibody (48.7 or 96.5) used are shown in Figure 9 with the ratio of each resected node or other tissue. All patients received an equal amount of ^{125}I labeled Fab 1.4, which reacts with a murine leukemia virus antigen and does not bind to human tissues. A ratio of counts from each tissue for specific and nonspecific antibody was determined, and all counts were converted for decay and downscatter (^{131}I counts into the ^{125}I channel). Patient #41, whose scan is demonstrated in Figure 7, had ratios in isolated portions of her single positive node of 1.2–1.6 with ratios in negative nodes of less than unity. The only other patient (#42) injected with Fab 48.7 had no tumor identified in nodes.

Patients injected with ^{131}I-96.5 Fab had ratios of greater than one in tumor containing nodes when compared to nonspecific antibody in virtually all cases. Most nonnodal tissue had ratios less than or equal to one. Increased ratios

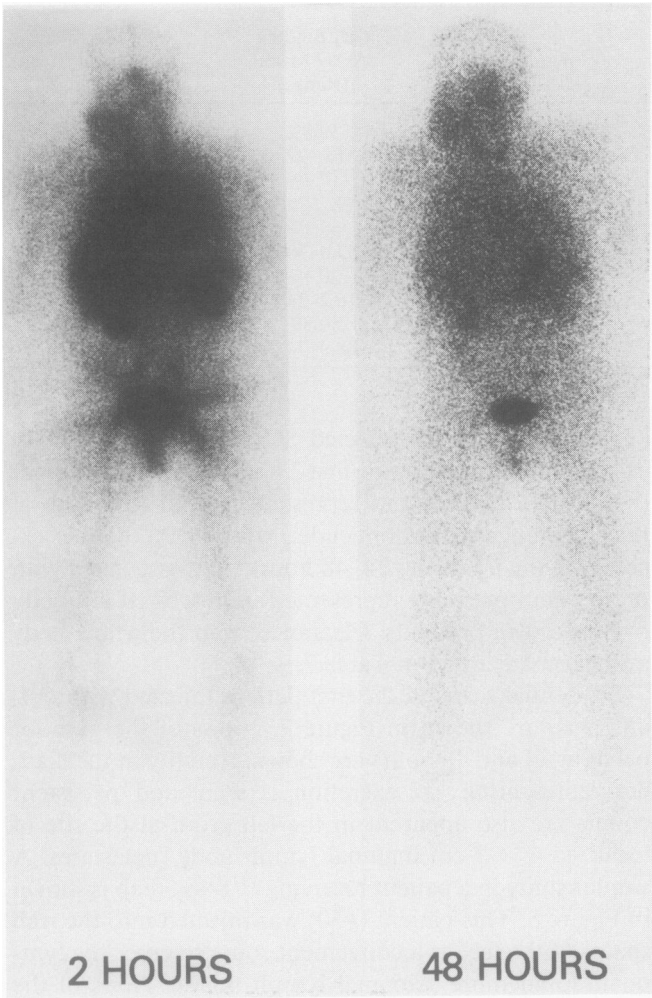

FIG. 3. Serial scans in a patient imaged with IV ^{131}I-Fab 48.7. A large mass present in the right neck is apparent in addition to activity in the blood pool at 2 hours in patient number 3. At 48 hours this activity in the neck persists and left sided pulmonary disease is apparent. Other sites of activity (thyroid, stomach, and bladder) are related to free iodine uptake following dehalogenation of the protein.

in nodes without apparent tumor were noted frequently. Explanation for this could include tumor present in planes other than those obtained for pathologic examination (thought to be unlikely) or shed antigen picked up by these nodes. Mean ratios in positive node(s) were significantly higher than those in negative node(s) in patients #41, 43, 44, 46, and 60. Only patients #41 and 60 were thought to have imaged.

Because of the modest results seen in the first nine patients with radioactive iodine Fab fragments, we evaluated a whole antibody, 9.2.27, which had been approved for use with ^{111}Indium. ^{111}In has more favorable imaging characteristics and dosimetry when compared to ^{131}I and a relatively short half life (72 hours). Injection in the hands in a patient with right axillary nodal disease (Fig. 10, left)

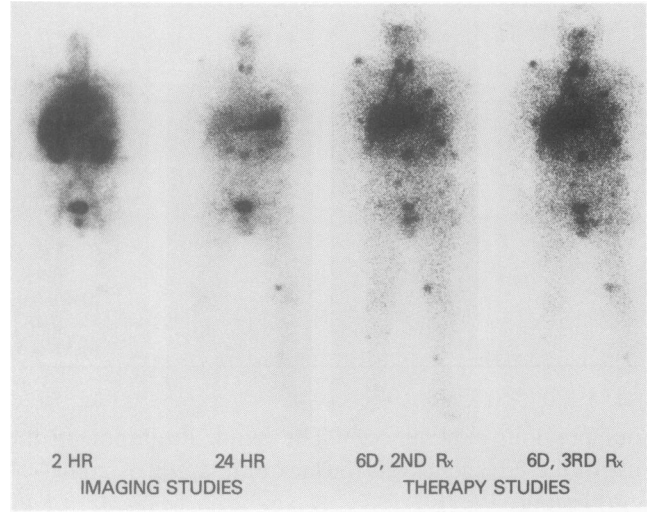

FIG. 4. Serial scans on a patient imaged and treated with ^{131}I-96.5 Fab, IV. Imaging studies using 50 mg of antibody labeled with 6.4 mCi of ^{131}I (patient #19) are shown at 2 hours and 24 hours. Therapy was conducted at weekly intervals with 100 mCi of radiolabeled antibody.

led to uptake at 24 hours in the spleen, liver, epitrochlear nodes, and supraclavicular nodes as well as minimal activity in the axial skeleton, probably secondary to free indium. Similar delineation of superficial inguinal, iliac, and para-aortic nodes was demonstrated in a patient receiving injections in the web space of the feet (Fig. 10, right). Epitrochlear, axillary, cervical, supraclavicular, and mediastinal nodes were imaged in a patient injected in both hands (Fig. 11). Tumor in nodes was not delineated with antigen specificity using this antibody. We were concerned that the high nonspecific background was related

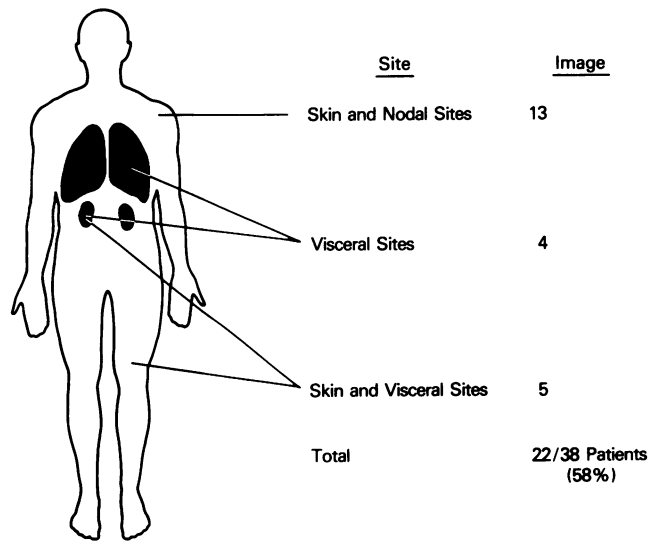

FIG. 5. Results of systemic imaging in 40 studies using radiolabeled antimelanoma Fab fragments. Disease was imaged in 22/38 patients or 58% of those attempted.

to Fc binding of the whole antibody. Hence we injected either cold antibody or human immunoglobulin concurrently with the ^{111}In labeled antibody to block this binding. These attempts were unsuccessful. Results with this antibody in 11 patients are summarized in Figure 12. The radioactivity in each node or other tissue is expressed as % injected dose/gram of tissue $\times 10^{-4}$. As expected, most nonlymphoid tissue had few counts. Subsequent detailed immunohistochemical studies demonstrated crossreactive binding of 9.2.27 at low levels to normal nodal tissue, and we believe this explains the abberant results with this antibody.

The results of all surgical procedures and subcutaneous nodal imaging are summarized in Figure 13. No patients with cervical disease imaged or had evidence of specific uptake in nodes. In contrast, 4/14 patients with axillary or inguinal disease demonstrated specific uptake in their cancerous lymph nodes. Two were imaged externally.

Discussion

Melanoma is an unusual cutaneous neoplasm that continues to kill almost 6000 individuals per year.[18] Treatment for clinical stage I disease clearly involves local excision, but the role of prophylactic nodal dissection remains controversial. Two prospective randomized trials, one by the World Health Organization[7] and the other by the Mayo Clinic,[19] have shown that therapeutic dissection at the time of clinical nodal positivity gives results comparable to those for prophylactic nodal dissection, sparing 80% of patients a potentially morbid procedure. Others have argued that long-term survival is improved by early dissection in selected subsets of patients.[8,20,21] A means to identify those patients with occult metastatic disease, and consequently those most likely to benefit from operation, would be helpful. Our study was exploratory in nature, attempting to determine if patients with known stage II disease could be imaged with radiolabeled monoclonal antibodies. Three separate antibodies, two as Fab fragments and one as a whole antibody, were used. Repetitive preparation of radiolabeled antibodies with adequate maintenance of immunoreactivity and radiopharmaceutical purity was demonstrated. We clearly demonstrated that the 9.2.27 whole antibody was capable of imaging nodes after subcutaneous injection, but that probable crossreactivity with normal nodal tissue precluded its further use in this setting.

In eight evaluable patients receiving Fab fragments (two others had no additional tumor in lymph nodes at dissection), four were thought to have evidence of specific antibody localization in positive lymph nodes by *in vitro* counting. Only two of these imaged. The clearance of these Fab fragments was rapid, and thus they served as ideal radiopharmaceuticals. In preclinical studies in guinea pigs, it appeared that 10 μCi of activity injected into these animals using an antitumor antibody gave good imaging results.[11] Given that a guinea pig weighs approximately 0.5 kg, a comparable dose in humans would be 1400 μCi. We used only 200 μCi of activity since we were limited by the long half life of ^{131}I and the approximate 30 rads delivered to local subcutaneous tissues with this dose. More recently, we have demonstrated that doses of up to 10 μg could be given to mice and were only partially saturating, using antibodies reactive with normal histocompatability antigens.[23] Higher doses (up to 35 mg) might be necessary to maximize imaging in humans. Future studies with subcutaneous injection of monoclonal antibodies will use larger doses of antibody (2 mg–10 mg), compared with the 0.2–2 mg dosage used in these studies. Reagents with more favorable imaging characteristics, such as ^{99}Tc and ^{111}In, are being evaluated. The fact that some tumors did image and most other tumors demonstrated selective uptake in nodes in this setting indicates

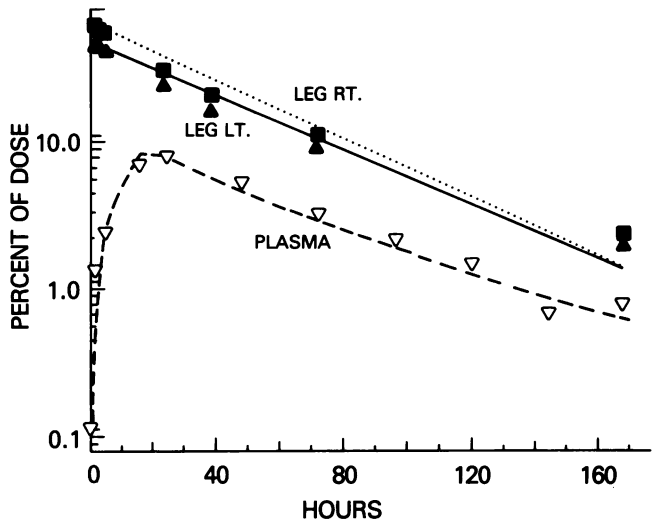

FIG. 6. Plasma and injection site clearance of ^{131}I-48.7 antimelanoma Fab following subcutaneous administration. This patient was injected around the site of a previously excised primary overlying the left tibia. A comparable site on the left leg was injected and plasma and injection site clearance followed.

TABLE 6. *Urinary Excretion of Radiolabel Following Subcutaneous Injection of Antibodies*

Antibody	No. of Patients	% Injected Dose (Range) Time Following Injection 0–2 Hours	2–24 Hours	24–48 Hours
96.5 Fab	6	2 (1–4)	25 (19–31)	23 (10–34)
48.7 Fab	2	1.7 (1.7)	7.9 (7.9)	18 (12–24)
9.2.27*	11	2.3 (0.2–8)	12.2 (1–34)	8.2 (1–21)
1.4 Fab	11	1.8 (0.4–6)	24 (8–60)	22 (12–35)
BL3*	3	1.1 (0.3–2)	23 (18–28)	14 (7–21)

* Whole antibody.

TABLE 7. *Clearance Data Following Subcutaneous or Intravenous Antibody Injection*

Route	Ab	N	Whole Body, % Dose (Range)		Plasma, % Dose (Range)	
			24 Hours	48 Hours	24 Hours	48 Hours
IV	96.5 (imaging doses only)	11	50.2 (31–96)	20.1 (11–34)	4.7 (1.7–10.0)	1.7 (1.0–3.0)
IV	48.7	20	43.3 (23–75)	25.2 (11–64)	8.3 (3.0–20)	3.4 (1.6–9.0)
SQ	96.5	8	ND	ND	4.8 (4.0–6.8)	2.5 (2.1–3.4)
SQ	48.7	2	ND	ND	6.7 (6.2–7.2)	4.6 (4.5–4.7)
SQ	9.2.27	10	ND	ND	10.6 (2.9–26)	10.9 (4.9–24)
SQ	1.4	12	ND	ND	8.9 (6.4–19)	6.8 (2.3–13)
SQ	BL-3	3	ND	ND	36.5 (25.1–57.3)	33 (20–46)

IV = intravenously; SQ = subcutaneously.

that further refinements might make this a feasible technique.

Previous efforts in lymphoscintigraphy have used either colloidal gold[24] or Tc-antimony sulfate[9] to identify drainage patterns. More recently, radiolabeled polyclonal antisera to carcinoembryonic antigen[25] to identify breast cancer or against ferritin to identify Hodgkin's disease[26] have been used. The development of monoclonal antibodies to specific tumor antigens would seem to eliminate the crossreactivity associated with polyclonal sera and the problem associated with labels on irrelevant immunoglobulin molecules. Unfortunately, many of these monoclonal reagents crossreact with a small proportion of normal tissues. The ideal imaging agent may consist of a pool of monoclonal antibodies recognizing different antigens or different epitopes present on the same antigen in a tumor. Evidence for the use of pooled monoclonals has been obtained *in vitro* using combinations of five separate monoclonal antibodies recognizing different antigenic determinants on melanoma.[27] Variable expression of antigens related to the state of differentiation[28] or to heterogenous tumor populations[29] arising from a single tumor in a patient may require a defined "cocktail" polyclonal antisera containing combinations of monoclonal anti-

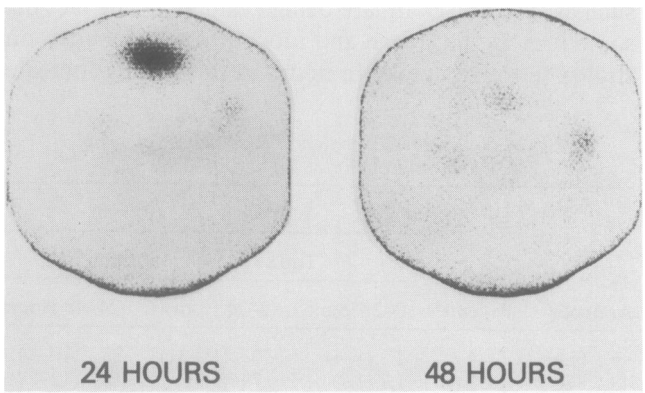

FIG. 7. Anterior pelvis. Sequential scans in a patient receiving ^{131}I-48.7 Fab subcutaneously. Activity in the bladder representing excreted antibody as well as localized disease in the left groin at a site of a 4 × 3 isolated tumor deposit is demonstrated.

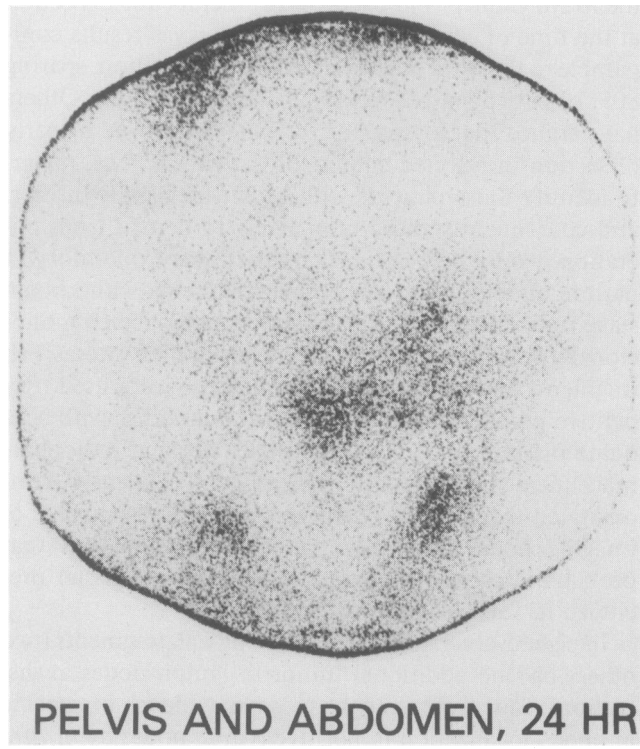

FIG. 8. Scan in a patient receiving ^{131}I-96.5 Fab subcutaneously. Specific uptake in left inguinal nodes as well as activity excreted into the bladder is noted.

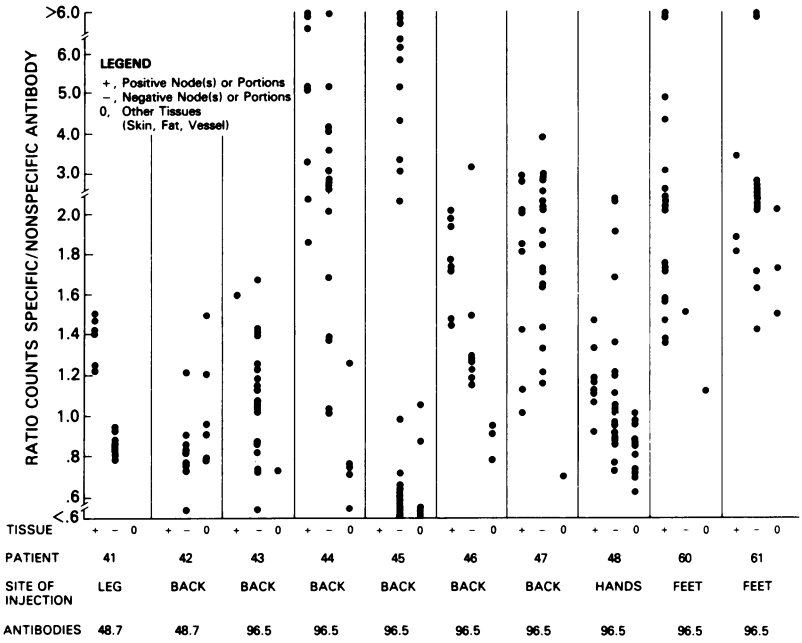

FIG. 9. Tissues from patients injected with subcutaneous Fab fragments. Each of the nodes as well as other tissues including skin, fat, and blood vessel was counted for both specific (^{131}I) and nonspecific (^{125}I) labeled antibodies and a ratio constructed. A ratio of greater than one would indicate some selective uptake. Two patients (#42 and 45) had nodes imaged using external scans.

bodies. We have recently used Indium-111 to image nodes in patients with T cell lymphomas when coupled to an anti-T cell (T101) antibody[30] and injected SQ. The ability to image nodes looking for filling defects using this or other reagents has been considered.

Previous studies of radiolabeled antibody to p97 *in vitro* have shown a 10–200 times higher binding to tumor cells than to normal tissues. *In vivo,* we have demonstrated imaging of tumor in 20/33 patients with metastatic melanoma.[2,3] Antigen specific localization with specific:nonspecific ratios (in biopsies taken from patients) of 3.7 (48 hours) and 3.4 (72 hours) was found. This is comparable to the ratios seen in our subcutaneous studies in this report, and the proportion of patients imaging using systemic injection of these antibodies reported here is also comparable to our previous experience. Uptake of specific antibody with these antibodies was strongly correlated with tumor antigen (p97) concentration, and blood clearance was significantly more rapid than for nonspecific Fab. In a recent series of reports,[4,5] ^{111}In labeled whole antibody (96.5) has imaged approximately 80% of established lesions at the optimum dose.

Our future efforts will include evaluations of the role of increased doses of antibodies used for imaging and

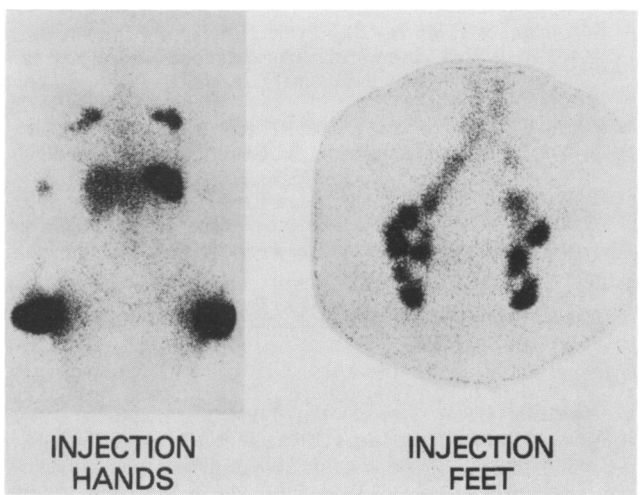

FIG. 10. Scans in patients injected with the whole ^{111}In-9.2.27 monoclonal antibody subcutaneously. The patient on the left was injected in the web spaces of the hands and the one on the right in the feet. Excellent delineation of nodes without specific identification of tumor is noted.

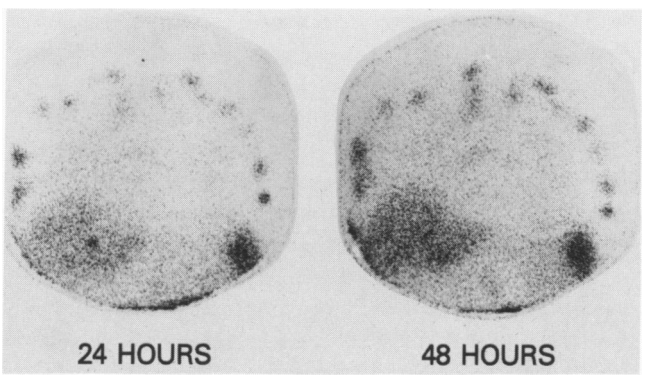

FIG. 11. Serial scans in a patient imaged with ^{111}In-9.2.27 monoclonal antibody injected subcutaneously. Excellent delineation of epitrochlear, axillary, cervical, and mediastinal nodes is noted. A small persistent hot spot in the mid portion of the liver had no radiologic (CT scan) or ultrasound correlate, and recent repeat studies at 1 year demonstrate no apparent disease in that organ.

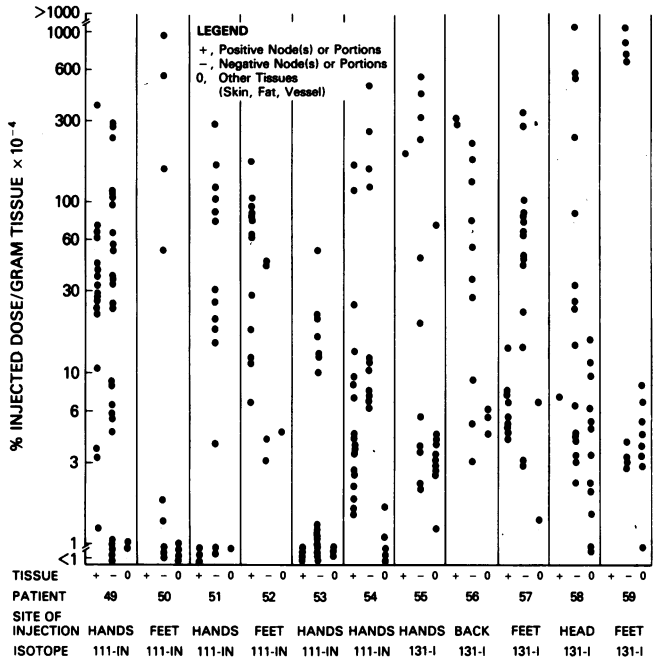

FIG. 12. Tissues from patients injected with subcutaneous 9.2.27 whole antibody. High counts were demonstrated in both negative and positive nodes whether antibody was labeled with ^{111}In or ^{131}I. Staining of cross-reactive antigens in normal lymphoid tissue was thought likely to explain these results.

therapy as well as a new antibody, 2B2, which recognizes a disialoganglioside present on the surface of melanoma cells and neuroectodermal cells.[31] Recent studies using the antibody, R24, which recognizes the same antigen, resulted in tumor regressions in 3/8 patients.[6] Based on previous estimates of biodistribution, we estimate that for every 100 mCi of ^{131}I-Fab given, the tumor would receive

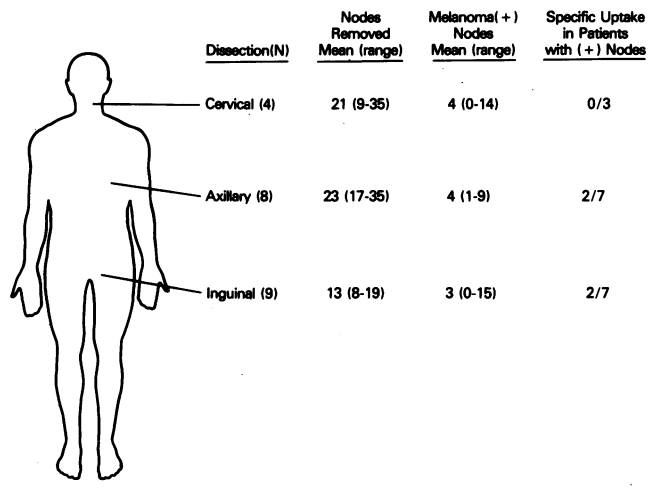

FIG. 13. Results of surgical procedures and subcutaneous nodal imaging in 21 studies using radiolabeled antimelanoma antibodies. Every patient injected with subcutaneous antibody had a nodal dissection 2–3 days following injection of both antimelanoma and control antibodies. Two patients were successfully imaged.

1040 rads (liver, 325 rads, and bone marrow, 30 rads). Further efforts to develop these reagents for therapeutic applications using gamma emitting radionuclides such as ^{131}I or alpha emitting reagents such as ^{90}Y or ^{211}Bi are being carried out. Other plans include attempts to increase antibody access to tumor sites using agents thought to increase capillary permeability such as interleukin-2 (IL-2). We have recently demonstrated that IL-2 administration causes significant melanoma regressions alone[32] or in conjunction with transferred lymphokine activated killer cells.[33] The coadministration of immunologic reagents such as these and monoclonal antibodies may lead to greater numbers of tumor responses. The use of antibody heteroconjugates with antitumor antibodies coupled to receptor molecules on lymphocyte surfaces such as CD3 (T3) and CD16 (Fc receptor) are being evaluated.[34] It is likely that these monoclonal reagents will prove clinically useful in the diagnosis and therapy of melanoma.

Acknowledgments

To Steven A. Rosenberg, M.D., Ph.D., for support of these studies, to Ray Farkes, Pharm. D., and Josephine Englert, Pharm. D., Richard Fejka, M.S., and Mark Rotman, Pharm. D., for preparation of radiolabeled antibodies, to Margaret Lora and Patrick Maloney for serum and urine analyses and determinations of immunoreactivity of radiolabeled antibodies, to Louise Lorario and Warren Rumble for whole body counting, and to Ms. Mary Ann Bodnar for careful preparation of this manuscript.

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Discussion

DR. RICHARD E. WILSON (Boston, Massachusetts): I think it would be inappropriate not to discuss such a dramatic presentation concerning research that is going to be so important for all of us.

I would like to ask the author a question about specificity for individual patients. As I understand it, this is a monoclonal antibody raised against an antigen (a common melanoma antigen), and in the systemic diagram I think that 58% of the patients they studied did show specific uptake. In the patients who did not have uptake, in whom they took out nodes, did they try to raise monoclonals against that tissue? If so, did they test it back in those patients to see if they could increase the specificity or the uptake in people who did not respond to the common melanoma antibody but did show additional uptake when a more specific antibody could be raised?

One of the real questions, I think, is how good are common antigen-raised antibodies as compared to specific antibodies in individual patients?

DR. JEROME J. DECOSSE (New York, New York): The authors have demonstrated a phenomenology. Have they quantitated predictiveness, sensitivity, specificity?

As I read the abstract, it would appear that of ten patients examined only one demonstrated imaging. Is this correct? Could you tell us about the clinical utility of this method?

DR. MICHAEL E. LOTZE (Closing discussion): First, answering Dr. Wilson's question: The problem of specificity, of course, is a major problem in any immunotherapeutic or immunodiagnostic modality. One of the advantages of using common melanoma antigen antibodies is that one can use the same reagent for many different patients. The problem with trying to raise specific reagents in each patient is the time, effort, and cost associated with trying to raise them. We have not attempted to do that but are trying to undertake similar approaches and generate cellular reagents and specific antitumor T cells, primarily for therapeutic purposes.

In terms of the value of the specific *versus* nonspecific antibodies, we are very concerned about many previous studies that have failed to use these nonspecific antibodies. We felt that it was important to determine whether the imaging of tumors occurred because of specific localization of the antitumor antibody or was just passive and had nothing to do with that antibody. Further trials using these monoclonals will require efforts to develop better polyspecific antibodies that could be used in individual patients. We are hopeful that better reagents can be identified.

Dr. DeCosse asked the central question for all of these efforts, which has to do with what is the clinical utility of these antibodies. Our hope initially was to use the systemically administered antibody and treat patients with very highly labeled I-131 or other radioisotopes and antibodies so that specific localization could be obtained. Individual tumor deposits would be irradiated and normal tissue would be spared. So far, we have treated about half a dozen patients in this manner and have not seen any responses. I believe that this is related to the antibodies we have. Again, we are hoping for better antibodies to be developed.

How useful is it in trying to image nodal disease? Again, our hopes were to be able to preclude nodal dissection in the 70–80% of patients who have clinical stage 1 disease, without occult nodal metastatic disease. It appears, at least in patients who have known stage 2 disease, that only two out of ten patients had positive scans. We are hoping that higher doses of antibody will allow us to image tumor in more patients.