FINAL REPORT

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NOVEL ANTIBODY CONJUGATES FOR ENHANCED TUMOR UPTAKE

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List of Publications

1. John E, Thakur ML, DeFulvio JD, et al. Rhenium-186 labeled monoclonal antibodies for radioimmunotherapy: Preparation and evaluation. J Nucl Med 34: 260-267, 1994.

2. John EK, Thakur ML, Wilder S. Technetium-99m labeled monoclonal antibodies: Influence of Tc-99m binding sites. J Nucl Med 35: 876-881. 1994.

3. LI J, Merton DA, Thakur ML, et al. Influence of biological response modifiers: Measurements of tumor blood flow and temperature. J of Immunotherapy 16: 175-180, 1994.

4. John E, Wilder S, and Thakur ML. Structural perturbations of monoclonal antibodies following radiolabeling: *In vitro* evaluation of different techniques. Nucl Med Comm 15: 24-28, 1994.

5. Li, J, Merton DA, Duggaraju R, and Thakur ML. Augmenting of tumor uptake of anti-melanoma antibody MEM136: Influence of interferon. Nucl Med Biol 23: 873-879, 1996.

6. Li J, Duggaraju R, Maish DR, and Thakur ML. Effect of inteferon-α-2b on the enhancement of tumour uptake of 99mTc-labeled monoclonal antibodies. Nucl Med Comm 17: 346-352, 1996.

7. Thakur ML, Li J, Donegan M, Pallela VR, Kolan H, Duggaraju R, Maish D, and Srivastava S. Improved antibody targeting with inteferon-α-2b conjugate. J of Immunotherapy 20: 194-201, 1997.

PROGRESS REPORT

a) Labeling MAbs with Tc-99m and Re-186:

The techniques of labeling MAbs (IgM, IgG, $F(ab')_2$ or F(ab') with Tc-99m was developed in our laboratory in 1989 (43, 44) and that with Re-186 in 1992 (45). The techniques are in daily use in our laboratory since then and are adapted to a convenient kit formulation. The metal ions are bound at MAb sulfhydryls generated by a controlled reduction of a pair of disulfide groups (46). At least two types of MAbs labeled with Tc-99m by this method have been administered into patients and excellent diagnostic results have been obtained (47, 48).

b) Human melanoma tumors and specific monoclonal antibodies:

Over the past two and a half years we have been successfully growing human melanoma tumors in athymic Balb/c nude mice. The cell line, WM-9, was obtained from Dr. D. Herlyn's laboratory at Wistar Institute in Philadelphia. Sufficient quantities of antihuman melanoma specific antibodies ME 31.3 (Wistar, IgG-1 and MEM-136 (Hybritech, IgG-2a) and their $F(ab')_2$ fragments are also available in our laboratory.

c) Evaluation of Biological Response Modifiers:

The use of BRM is a rapidly evolving field. Over the past four years, we have evaluated a number of BRMs in a quest for agents that may augment MAb tumor uptake. These included interferon-α; a pokeweed mitogen and Ukrain, an alkaloid separated from a plant *Chelideonium Majis* (49). In these preliminary studies, normal Balb/c mice were used and the BRMs were given i.p. one hour prior to the i.v. administration of tumor necrosis factor or an MAb (TNT-$F(ab')_2$) labeled with Tc-99m which served as an imaging agent. Animals were sacrificed at 1.5 hr or 4 hrs post-injection. The reprint of this work is appended but the highlights of the work are given here in Table I.

Table I

Tumor/muscle and absolute (% ad. dose/g) tumor uptake of Tc-99m-TNF-α in control and BRM receiving animals at 1.5 and 4 hrs p.i.

	Tumor/muscle		Absolute tumor uptake	
	1.5 hr	4 hr	1.5 hr	4 hr
Control	4.1±2.2a	7.4±2.2a	1.8 ±0.4	2.6±0.9
IFN	8.3±2.7b	9.4±0.8	2.5±0.7	2.7±0.15
Ukrain	6.2±2.1	6.1±3.3	3.2±0.5c	2.8±1.3
PKWD	5.1±1.1	5.5±0.9	2.5±0.2	2.3±0.4

[a] T/M ratios at 1.5 hr and 4 hr p.i. in control animals
[b] T/M ratios at 1.5 hr and 4 hr p.i. in control and IFN receiving animals, P=0.01
[c] Absolute tumor uptake at 1.5 hr p.i. in control and Ukrain receiving animals, P=0.006

These pilot data indicated that all BRMs augmented tumor uptake as early as 1.5 hrs post-injection and IFN gave the best results. These results formed the basis of further systematic studies in which we used IFN-α-2b as a BRM, athymic Balb/c nude mice bearing human melanoma as an animal model, and Tc-99m labeled ME 31.3 an anti-human melanoma antibody as an agent.

Our interest in IFN-α-2b stemmed from the extensive investigations which displayed a broad spectrum of their biological activities (50-58). These studies suggest that once bound to the cell membrane, the recombinant DNA produced, IFN-α-2b initiates a complex sequence of intracellular events that include the enduction of certain enzymes. It is considered that this process, at least in part, is responsible for the various cellular responses of IFN-α-2b. These include inhibition of virus replication in virus-infected cells, suppression of cell proliferation and such immunological activities as enhancement of the phagocytic activity of macrophages and augmentation of the cytotoxicity lymphocytes for target cells. These activities also lead to enhance blood flow within the region of action. Our immediate interest in IFN-α-2b is related to the latter. Only the highlights of our findings are presented here, and detailed manuscripts are appended.

b) Blood flow enhancement:

Studies were performed in anesthetized nude mice bearing human melanoma tumors. Blood flow was measured continuously 30 minutes prior to and for 90 minutes following i.m. administration of 20 k i.u. of IFN-α-2b, using the conditions at which best tumor uptake was observed. Animals receiving an equivalent volume of saline served as controls. Blood flow was measured by laser Doppler flowmetry and by color Doppler imaging techniques rather than the use of radioactive microspheres which would have entailed sacrificing animals before and after administration of IFN-α-2b. The Doppler techniques permitted studies in the same living animals and allowed us to differentiate the blood flow changes before and after administration of the agents. Increased blood flow as indicated by increased intensity, enhanced amplitude and higher cardiac cycle pulse rate can be seen in Fig. 1. Results shown in Table 2 combined with other experimental observations indicated that 30 minutes post i.m. administration 20 i.u. IFN-α-2b, there was a greater than 300% enhancement ($P < 0.01$) in tumor perfusion which persisted for a much longer period of time than in normal tissues.

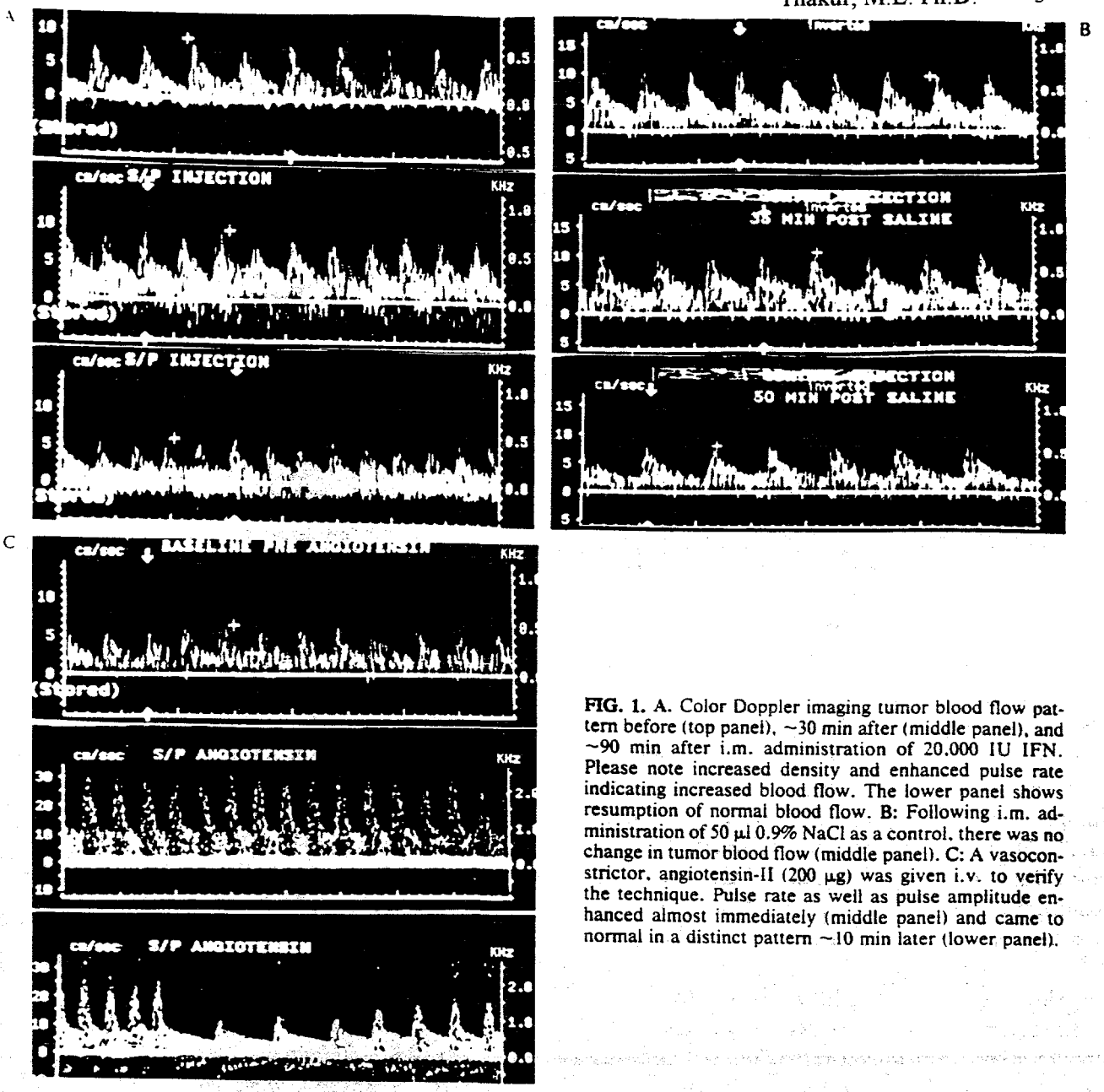


FIG. 1. A. Color Doppler imaging tumor blood flow pattern before (top panel), ~30 min after (middle panel), and ~90 min after i.m. administration of 20.000 IU IFN. Please note increased density and enhanced pulse rate indicating increased blood flow. The lower panel shows resumption of normal blood flow. B: Following i.m. administration of 50 μl 0.9% NaCl as a control, there was no change in tumor blood flow (middle panel). C: A vasoconstrictor, angiotensin-II (200 μg) was given i.v. to verify the technique. Pulse rate as well as pulse amplitude enhanced almost immediately (middle panel) and came to normal in a distinct pattern ~10 min later (lower panel).

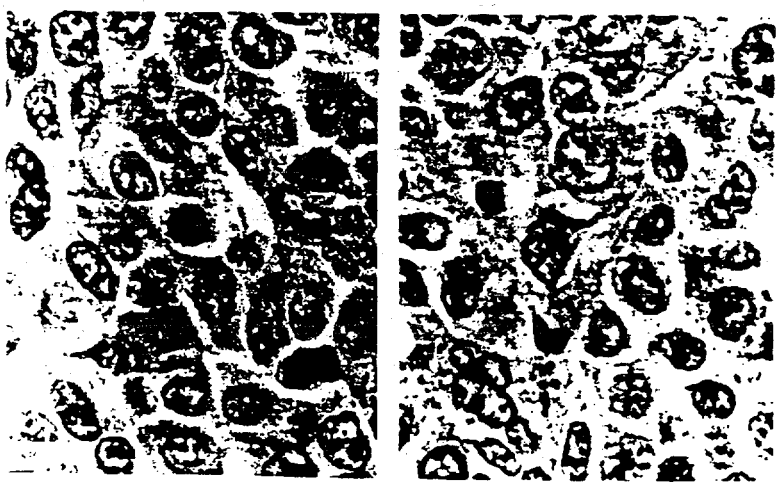


FIG. 2. A composite of two histological section of tumors from two separate animals. The one on the left is from an animal without receiving interferon (IFN) and the one on the right is from another mouse, 72 h after receiving 20.000 IU of IFN. No cellular changes can be seen.

Histological examinations of tumors obtained from animals given IFN-α-2b, 72 hours previously did not show changes in inflammatory cells and also indicated that the MAb tumor enhancement was blood flow mediated rather than due to cellular action exerted by IFN-α-2b (Fig. 2).

c) Augmentation of tumor uptake, influence of quantity of IFN-α-2b and route of its administration:

IFN-α-2b was administered to groups of five mice bearing human melanoma tumors. IFN-α-2b in quantities 10×10^3, 20×10^3, 50×10^3 or 100×10^3 i.u. were given to the animals either i.m. or i.v.. Thirty minutes later animals were given 50-200 μCi ME 31.3 in 0.9% NaCl through a lateral tail vein. Animals were sacrificed at 4 or 24 hrs later, tissues dissected and percent administered dose/g of tissue calculated. Animals receiving saline only served as controls.

Results given in Table 2 and Fig. 3 indicate that the highest tumor uptake, 360% of the control ($2.0\pm1.4\%$ vs $7.2\pm1.2\%$) was obtained at 24 hr post-administration of Tc-99m ME 31.3 (IgG1) in animals receiving 20×10^3 i.u. IFN-α-2b intramuscularly. Under identical conditions these results were reproducible in two additional groups of animals. In animals receiving 20×10^3 i.u. IFN-α-2b i.v. or larger quantities i.m. augmented tumor uptake also occurred at 24 hr post-injection but was not as high as with 20k i.m. dose of IFN. Unfortunately, however, in animals receiving 20 k IFN-α-2b, the blood levels as well as almost all other tissue activity levels were also higher at 24 hr post-injection than the control group. Increased blood activity levels were also noted in all 24 hr groups of animals receiving all concentrations of IFN-α-2b. Although the reasons for lower tumor uptake at higher IFN-α-2b doses, higher uptake in normal tissues at 20k i.m. injection, and higher blood levels at all IFN concentrations are not quite clear, antigen shedding from tumor cells and thereby decreasing the tumor uptake and increasing the blood levels (circulating antibody-antigen complex) have been cited as possible reasons by Murray et al (59) and by Greiner et al (60). The general high liver uptake of this MAb was also a matter of concern. The PAGE analysis in our laboratory of plasma samples of liver homogenates obtained from these tumor bearing animals, however, did not reveal a protein band of an apparent M.W. of such an antibody-antigen complex. It is conceivable that the quantities of such a complex may be too small for detection by this technique.

Pertinent to this proposal, however, is the observation made by the Epstein group (40, 41) that IL-2 given alone to the animals also increased blood radioactivity levels, but the pre-treatment with IL-2-MAb conjugate followed by the administration of I-125-F(ab')$_2$dramatically decreased the blood radioactivity as well as the normal tissue radioactivity levels. We anticipated similar results in animals treated with IFN-α-2b-MAb complex and given Tc-99m-F(ab')$_2$.

d) Influence of pretreating animals with IFN-α-2b-MAb conjugate:

In order to assess the influence of pretreating animals with MAb:IFN-α-2b conjugation in anticipation that it will reduce liver uptake and enhance tumor uptake we performed the following experiment. Five hundred μg of ME 31.3 was conjugated with IFN-α-2b by the method of Khawli et al (41). The conjugated fraction was separated on HPLC using (Waters Associates size exclusion) protein pak SW 300 column and 0.05 M phosphate buffer (pH 6.8) in 0.9% NaCl as an eluent.

The conjugated peak separated five minutes prior to the unreacted protein and several minutes prior to the elution of unreacted IFN-α-2b was pooled and concentrated using Centricon-30 molecular filtration device. Protein concentration was determined spectrophotometrically.

Fig.3

Tumor uptake of Tc-99m-ME31.3 in human melanoma bearing nude mice after i.m. administration of IFN

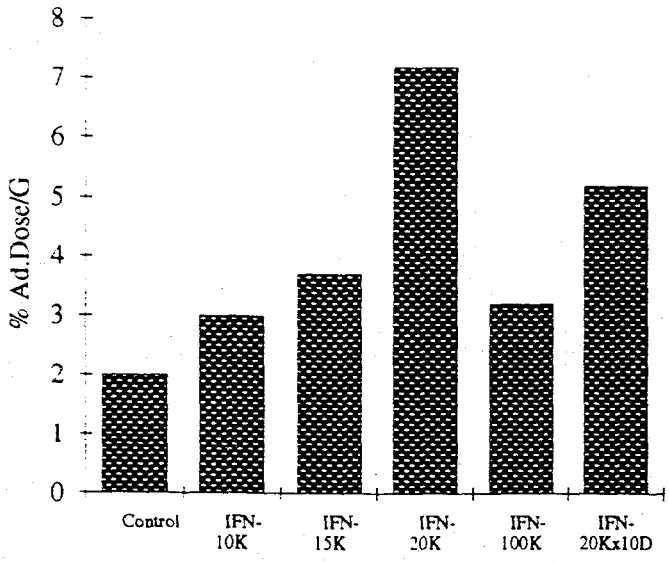
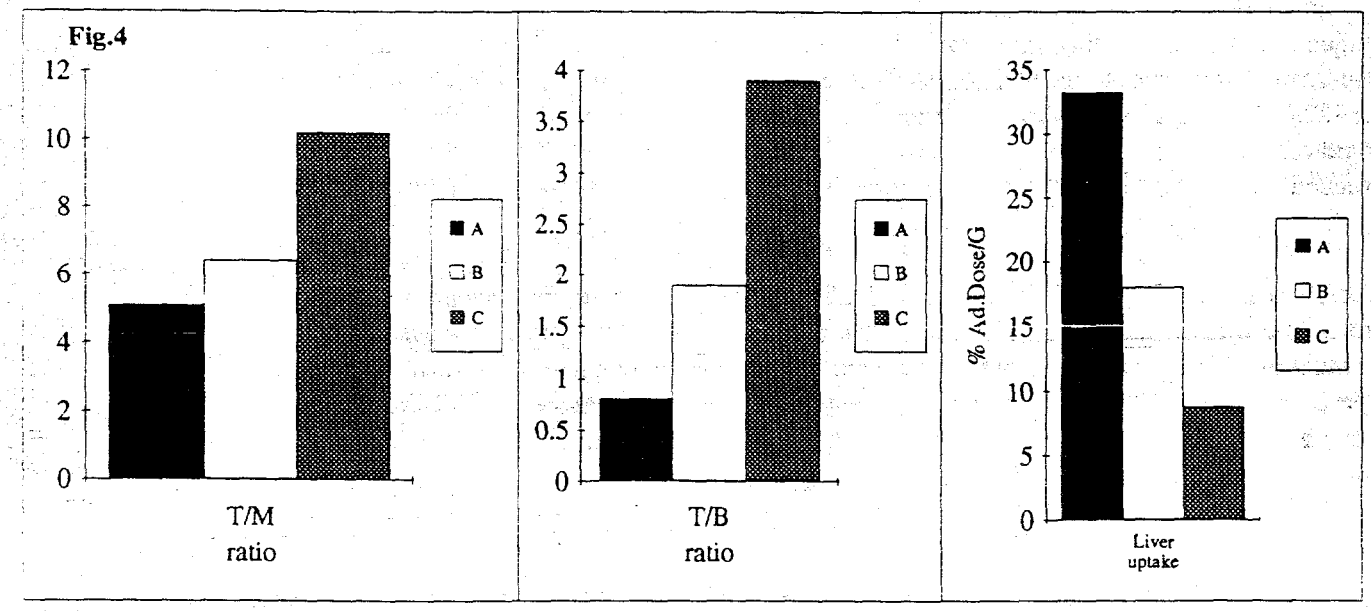


Fig.3 Figure shows that a single i.m. injection of 20K i.u.IFN-a-2b enhances tumor uptake by greater than 300% (control 2.0±1.4% vs. 7.2±1.2%, Table 3).

Fig.4. (Below) Shows 24 hr tumor/muscle(T/M) and tumor/blood(T/B) ratios and liver uptake in three separate groups of 5 animals each. Animals in exp.A were given Tc-99m-ME31.3 followed by a single i.m. injection of 20K IFN-a-2b, in exp.B were given Tc-99m-ME31.3-F(ab')2 followed by a single i.m.injection of 20K IFN-a-2b and exp.C,Tc-99m-ME31.3-F(ab')2 followed by a single i.v.injection ME31.3-IFN-a-2b conjugate. The improvements obtained due to the conjugate experiment are highly encouraging. Please note the improvement ratios in the inset table.



Exp.A: Tc99m-ME31.3 with IFN-20K i.m.
Exp.B: Tc99m-ME31.3-F(ab')2 with IFN-20K i.m.
Exp.C: Tc99m-ME31.3-F(ab')2 with ME31.3-IFN conjugate i.v.

	Exp.A	Exp.B	Exp.C
T/M ratio	5.1 ± 1.53	6.4 ± 3.02	10.2 ± 4.55
T/B ratio	0.8 ± 0.33	1.9 ± 0.74	3.9 ± 1.04
Liver uptake	32.1 ± 1.48	18.0 ± 2.22	8.8 ± 2.01

Groups of 5 mice each bearing human melanoma were then injected i.v. with 30 μg of the conjugate and 3 hours later with approximately 20 μg ME 31.3 $F(ab;)_2$ labeled with approximately 100 μCi Tc-99m. Animals were sacrificed 24 hours later, tissues were dissected and % ad. dose/g tissue calculated. As shown in Fig. 4, the experiment provided strikingly favorable results. As compared to the tumor, blood and liver uptake in animals treated with IFN-α-2b alone and followed by the injection of Tc-99m-ME 31.3 or Tc-99m-ME-31.3-$F(ab')_2$, the conjugate treated animals showed substantially increased tumor uptake and decreased blood and liver uptake. The conjugate experiment improved the tumor/blood ratios by 500%, tumor/muscle ratios by 200% and decreased the liver uptake to nearly 25%. In repeat experiments, these results were reproducible and are consistent with those of the Epstein group. These results form the basis of this proposal.

We believe that we can further improve these results in two ways. First, by using another human melanoma specific murine MAb MEM-136 which already has a much lower liver uptake, higher tumor uptake and lower blood uptake at 24 hours post-injection than ME 31.3 under identical conditions (see section e), and second by using in the IFN-MAb conjugate treated animals and Tc-99m-$F(ab')_2$ preparations in which Tc-99m atoms would be bound specifically to sulfhydryls and not to ε-amino groups non-specifically. Specifically bound Tc-99m is shown in our laboratory to enhance tumor uptake and decrease the liver uptake (see section f).

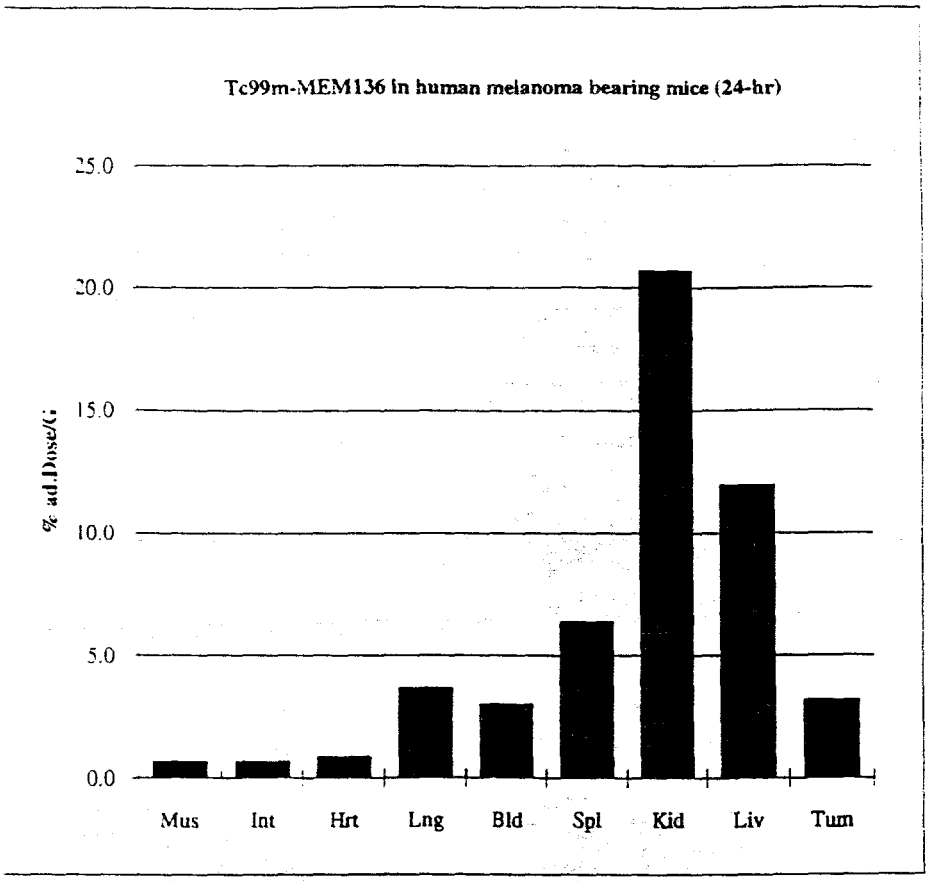
e) Tissue distribution of Tc-99m-MEM-136:

Lately we have obtained a large quantity of another human melanoma specific murine antibody, MEM-136, and its fragments $F(ab')_2$ (Hybritech, Inc.).This MAb recognizes an epitope on human melanoma associated proteoglycan and blocks melanoma cell interaction with basement membrane components *in vitro* (61). This antibody was labeled with Tc-99m and given to human melanoma bearing mice. Twenty-four hour tissue distributon (Fig. 5) showed that the liver uptake was only 12±3.7% as compared to that of 24.3±8.9% with ME 31.3 (Table 2, control) and the tumor uptake was also higher (3.2±1.2 vs 2.0±1.4%). The use of MEM-136 and its fragments in the IFN conjugation experiment is therefore warranted.

Another prominent anti-human melanoma antibody is 9.2.27 (IgG-2a) that recognizes 250kD melanoma-associated antigen. A small quantity of this was obtained from Dr.Reisfeld of Scripp Clinic. When it was labeled with Tc-99m and given to melanoma bearing mice, it had slightly higher tumor uptake than the MEM-136, about the same liver uptake but much higher blood uptake (9.9±1.3% vs 3.3±2.8%) than the Tc-99m-MEM-136 (Table 2). At the time of this writing, therefore, we are considering not to use this MAb for this application.

f) Influence of Tc-99m binding sites on tumor uptake and tissue distribution:

We have observed that a certain proportion of Tc-99m binds non-specifically, probably to the ε-amino groups when MAbs, intact or fragments are labeled with the radionuclide by the reduction or BFCA techniques (45). This portion of the radioactivity, in part, increases liver uptake and reduces tumor uptake of MAb. Contrary to this, when ε-amino groups are first blocked with fluoroscein isothiocynate (FITC) and Tc-99m is bound specifically only to the desired groups, the tumor uptake enhanced and liver uptake reduced. Tables 3 and 4 given below provide quantitative data and Fig. 6 the visual effect. Data given in Table 3 was obtained from MAb labeled with direct (AA reduction) method and in Table 4 from MAb labeled by a BFCA (N_2S_4) method. The % ad. dose/g tissue given in the middle row provide the numbers for comparison obtained with a corresponding regular preparation, in which none of the functional groups, specific or non-specific, were blocked.



Tissue	%Ad.D./G
Muscle	0.7 ± 0.38
Intestine	0.7 ± 0.16
Heart	0.9 ± 0.11
Lungs	3.7 ± 2.98
Blood	3.0 ± 0.51
Spleen	6.4 ± 3.20
Kidneys	20.6 ± 3.23
Liver	12.0 ± 3.76
Tumor	3.2 ± 1.21
T/B ratio	1.0 ± 0.36
T/M ratio	5.2 ± 3.13

Fig. 5 24 hr. tissue distribution of another anti-human melanoma antibody MEM-136 labeled with Tc-99m. Please note that as compared to that with Tc-99m-ME 31.3 (Table 2, control) under identical conditions, MEM-136 has lower liver uptake (12±3 vs. 24.3±8.9%), higher tumor uptake (3.2±1.2 vs. 2.0±1.4%) but higher blood uptake (9.9±1.3 vs. 3.3±2.8%).

Tc-99m-TNT-1

Specific **Regular** **Non-specific**

Fig. 6 Posterior images of 3 separate mice each bearing embryonal carcinoma in the right thigh, given Tc-99m labeled anti-histone antibody TNT-1 4 hrs. ago. Please note that the specific preparation (FITC) better delineates the tumor than the regular preparation, and that with a non-specific (SH groups blocked with iodoacetate) preparation tumor is not detectable. Table 3 also shows that the liver uptake with a specific preparation was less than that with the regular and non-specific preparation.

Table 2
24 hour biodistribution of ^{99m}Tc-ME 31.3 in human melanoma bearing nude mice after i.m. or i.v. administration of IFN.

Tissue	Control n = 10	IFN-10K-i.m. n = 5	IFN-20K-i.m. n = 15	IFN-100K-i.m. n = 5	IFN-20K-i.v. n 5
Muscle	0.6±0.2	0.8±0.1**	1.4±0.2**	0.9±0.1**	0.8±0.3*
Intestine	1.0±0.5	1.4±0.7	2.2±0.3**	2.0±0.8**	1.5±0.2**
Heart	1.1±0.5	1.5±0.6	2.7±0.5**	2.3±0.2**	1.7±0.3**
Lungs	2.6±1.2	3.5±1.3	6.3±1.0**	4.8±0.4**	4.7±2.2**
Blood	3.3±2.6	6.0±2.4*	9.6±2.0**	8.9±1.3**	5.7±1.2*
Spleen	8.8±3.9	6.7±1.8	9.2±1.0	14.5±2.2**	12.7±1.7*
Kidneys	11.7±2.6	13.5±5.3	19.7±4.3**	17.0±1.8**	14.1±1.9*
Liver	24.3±8.9	19.5±3.3	32.1±4.4**	45.8±3.2**	49.3±5.3**
Tumor	2.0±1.4	3.0±0.5*	7.2±1.2**	3.2±0.7*	3.5±1.8

Note:

* Significant ($p \leq 0.05$)
** Significant ($p \leq 0.01$)

Table 3

Tissues (% ID/g) in tumor-bearing mice 4 hrs after i.v. administration of Tc-99m (AA)-TNT-1 (n=5)

Organ	99mTc-TNT-1-FITC (Specific)	99mTc-TNT-1 (Regular)	99mTc-TNT-1 (Non-specific)
Muscle	0.37±0.03	0.33±0.06	0.28±0.07
Intestine	1.45±0.45	1.43±0.39	0.85±0.22
Urine	60.4±52.5	46.6±25.4	41.0±5.4
Heart	1.47±0.33	2.19±1.3	0.75±0.07
Lungs	2.51±0.49	2.83±0.65	0.75±0.20
Blood	6.37±0.80	4.26±1.4	1.87±0.89
Spleen	13.2±3.5	32.5±3.3	16.6±2.4
Kidneys	13.3±2.6	14.2±1.2	7.96±0.81
Liver	15.3±2.0	23.5±4.4	25.8±5.4
Tumor	2.10±0.55	1.9±0.76	0.92±0.19

Table 4

Tissues (%ID/g) in tumor-bearing mice 4 hrs after i.v. administration of 99mTc (N_2S_4)-TNT-1 (n=5)

Tissues	Specific	Regular (control)	Non-specific
Muscle	0.6±0.22	0.42±0.02	0.27±0.07
Intestine	2.5±0.46	2.85±0.74	0.84±0.03
Urine	70.7±37	70.0±26.7	38.4±5.7
Heart	2.27±0.24	1.94±0.14	0.54±0.07
Lungs	5.4±0.75	3.79±0.89	1.5±0.24
Blood	10.6±1.49	7.9±1.3	1.65±0.21
Spleen	25.9±1.74	26.6±4.7	27.9±4.8
Kidneys	10.2±3.3	11.7±1.9	8.83±1.47
Liver	21.9±1.6	23.3±1.6	26.0±3.7
Tumor	3.1±0.4	2.8±0.47	0.9±0.27

In this proposal, in additional groups of mice we will examine influence of $F(ab')_2$ labeled with Tc-99m only to the specific binding sites. It is anticipated that such preparations given to animals pretreated with the IFN-α-2b-antibody conjugate will achieve more favorable results than those obtained in pretreated animals receiving the regular preparations of Tc-99m-$F(ab')_2$. Needless to say, such results will be of additional importance in therapeutic applications of Re-186-$F(ab')_2$.