Inhibition of Autoradiolysis of Radiolabeled Monoclonal Antibodies by Cryopreservation

Richard L. Wahl, Joseph Wissing, Renato del Rosario, and Kenneth R. Zasadny

University of Michigan Medical Center, Department of Internal Medicine, Division of Nuclear Medicine, Ann-Arbor, Michigan

Autoradiolysis of therapeutic doses of monoclonal antibodies can occur rapidly, limits their shelf life and makes on-site radiolabeling a near-necessity. We evaluated freezing of three different ^{131}I-labeled murine monoclonal antibodies at −70°C, immediately following radiolabeling, as a method of diminishing autoradiolysis, and of preserving immunoreactivity. Freezing greatly limits the ability of radiation-induced free radicals to diffuse in solution and thus produce radiolytic damage. By freezing at −70°C autoradiolytic damage of immunoreactivity of three different ^{131}I monoclonal antibodies could be largely eliminated, in contrast to the 80–90% losses in immunoreactivity seen with storage at 4°C for a period of 1 to 12 days. Reduced in vitro deiodination rates are also seen for frozen antibodies. Limited studies with ^{125}I-labeled antibodies indicate autoradiolysis does occur, though at a slower rate per mCi than for ^{131}I, and that this process is also retarded by freezing. Freezing may be valuable while quality control procedures are performed following radiolabeling as well as if temporary storage or shipment of radioantibodies prior to patient dosing is undertaken. While the approach should be validated for each antibody studied, freezing of therapeutic doses of monoclonal antibodies appears to be a simple and effective approach to the problem of autoradiolysis.

J Nucl Med 1990; 31:84–89

Radiolabeled monoclonal antibodies are increasingly being studied as diagnostic and therapeutic agents (*1*). The specific delivery of therapeutic doses of radioactivity to tumors by antibodies can result in tumor shrinkage (*2,3*). Unfortunately, the radioactivity attached to the antibody potentially can also irradiate the antibody molecule in addition to the tumor and can lead to radiolysis with a loss of immunoreactivity (*4*). While radiolysis may not be a problem with the low radioactivity doses used for antibody imaging, increasing doses of antibody-associated radioactivity are being given for therapy. Thus, the possibility of radiolysis occurring in the interval from radiolabeling to administration to the patient is real, particularly during the time when quality control procedures such as assays of immunoreactivity and for endotoxin are performed prior to antibody administration to patients. In addition, as antibody therapy grows in utility, it is possible that regional facilities may label the antibodies for transport to other centers for administration, thus increasing the time from radiolabeling to injection and thereby increasing the risk of autoradiolysis. Kishore and colleagues have shown that external beam irradiation of slightly over 300 Gy will result in a 50% drop in immunoreactivity of a solution of anti-melanoma Fab antibody fragments (*4*). This radiation dose could be self-delivered to antibody in a solution by roughly 80 mCi iodine-131 (^{131}I) in 24 hr so the levels are quite easily achievable in the preparation of compounds for radioimmunotherapy (*4*).

It is believe that most radiation damage occurring in aqueous solutions subjected to external irradiation is mediated by mobile free-radicals (*5*). The situation in a vial containing radiolabeled monoclonal antibodies is similar, but additionally involves the fact that the radioactive decay is occurring in immediate contiguity with the antibody molecule due to the attachment of the radiolabel to the antibody molecule. It has been demonstrated that the addition of human albumin to a 2–5% w/v solution level to the vial containing radioantibody or hormones will slow radiolysis (*4,6*). This approach, while straightforward, does not totally prevent radiolysis and has the potential disadvantage that it does involve the addition of a human blood product derivative to the antibody dose. This manipulation in this era of concern regarding human blood products may be an undesirable addition.

A potential alternate method for diminishing the effects of irradiation on biologic systems is through freezing, which limits the diffusion of these short-lived free radicals (*5*). While it has traditionally been immunological dogma that the freezing of antibodies diminishes immunoreactivity, especially if there are multiple cycles of freezing and thawing, our experience with several murine monoclonal antibodies has been that a single freeze and thaw cycle generally has little adverse effect (Wahl RL: unpublished data). For these reasons we explored the efficacy of freezing as a means to avoid

Received Jan. 3, 1989; revision accepted Aug. 23, 1989.

For reprints contact: Richard L. Wahl, MD, University of Michigan Medical Center, Div. of Nuclear Medicine, 1500 East Medical Center Dr., Ann Arbor, MI 48109-0028.

or reduce the autoradiolysis occurring following antibody labeling with radioiodine.

METHODS

Three monoclonal antibodies were evaluated in this study: 5G6.4, a murine IgG2a monoclonal reactive with ovarian and many other carcinomas (*7*), 763.24T, a murine IgG1 reactive with a high molecular weight antigen present on the vast majority of melanomas, and 225.28S a murine IgG2a, reactive with a different epitope of the high molecular weight melanoma antigen recognized by 763.24T (*8,9*). All antibodies were grown as ascites in pristane-primed nude mice and were purified by either staphylococcal protein A (225.28S and 5G6.4) or DEAE chromatography 763.24T (*10,11*). Purity was assessed by S.D.S. polyacrylamide gels performed in the presence or absence of B-mercaptoethanol (*12*).

Radiolabeling of monoclonal antibodies was by the iodogen method (*13*). Iodine-131 and iodine-125 (^{125}I) were used for the initial studies, but since ^{125}I caused less radiolysis/mCi than ^{131}I (vide infra), ^{131}I (I.C.N.) was used for most subsequent experiments. Typical, low-level radiolabeling reaction conditions involved 10–15 mCi input ^{131}I being reacted with 500–1000 μg of purified antibody, with an iodogen/antibody protein ratio of roughly 1 μg/5 μg. Free iodine following radiolabeling was removed by anion exchange chromatography BioRad AgX-18. Higher level labeling of 5G6.4 involved an input radioactivity of approximately 76.5–80 mCi ^{131}I reacted with 5 mg of antibody protein. High level ^{125}I labeling was performed with 125 mCi of input ^{125}I and 5 mg of 5G6.4.

Immediately following radiolabeling, aliquots of the antibody preparations were divided for storage at either 4° centigrade or at −70° centigrade. The solutions stored were 0.5–1.0 cc in volume (though for the ^{125}I storage, a 2.0 ml volume was used). Total radioactivity was determined/vial through the use of an ionization chamber dose calibrator (Capintec). Separate aliquots were stored and then analyzed at varying time points including 2, 4, and for low-level labels, at 11–12 days postlabeling. Cell binding assays were conducted using viable human melanoma (HTB-63) for 763.24T or 225.285 or ovarian carcinoma (HTB-77 IP3) for 5G6.4, target cells. These were performed in conditions of antigen excess by addition of 10 or 100 ng of the antibody to ~5–10 × 10^6 target cells in a test tube at a volume of 0.5–1 cc. Incubation for 1–2 hr at 4°C was followed by washing × 2 and counting of the cell pellet in a gamma counter. No cells added and excess unlabeled antibody (100 μg) during incubation (blocked) con-

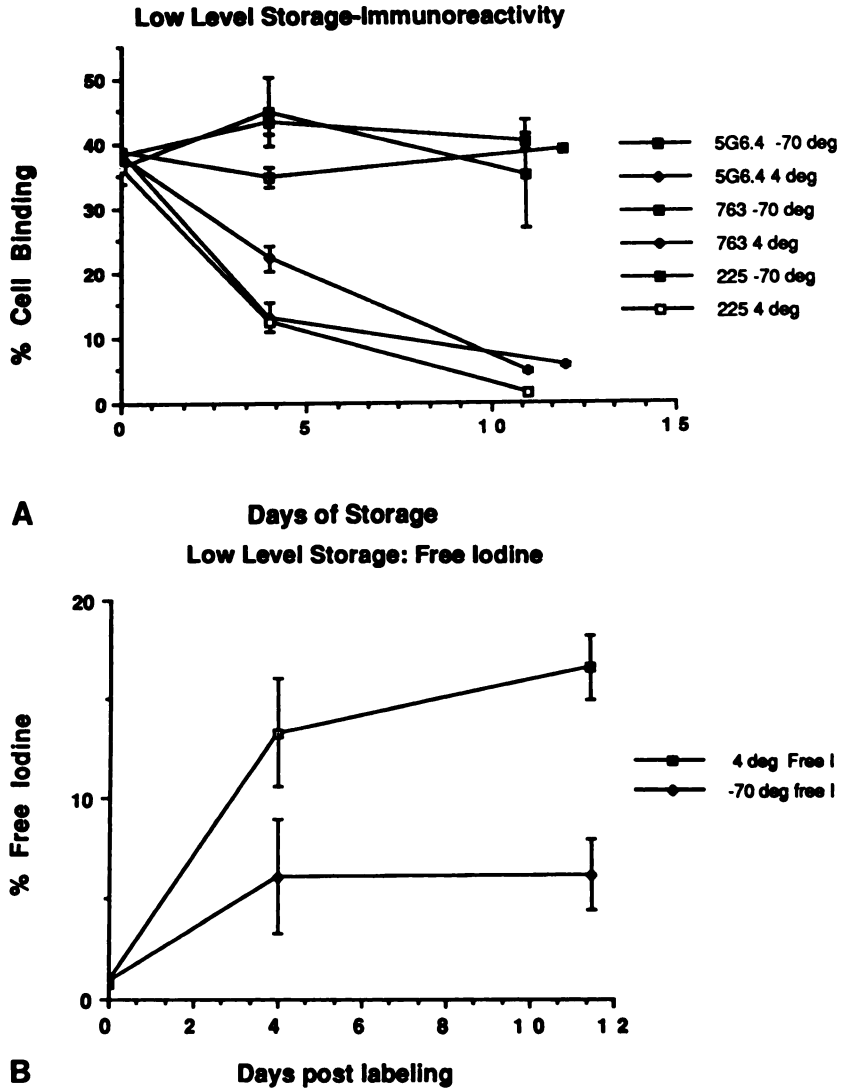

FIGURE 1
A: Mean ± s.e.m. of immunoreactivity assays following storage at 4°C or −70°C of ^{131}I 5G6.4, 763.24 T, or 225.28 S for varying intervals following radiolabeling. The estimated radiation dose/vial for 5G6.4 is: 4 days, 4°C, = 1,185 Gy; 4 days, −70°C = 1,307 Gy; 12 days, 4°C = 3,041 Gy; 12 days, −70° = 2,621 Gy. The estimated ^{131}I radiation dose for 225.28S is: 4 days, 4°C = 2,194 Gy; 4 days, −70°C = 2,100 Gy; 11 days, 4°C = 5,350 Gy; 11 days, −70°C = 5,236 Gy. The estimated ^{131}I radiation dose for 763.24T is: 4 days, 4°C = 2,012 Gy; 4 days, −70°C = 2,100 Gy; 11 days, 4°C = 4,625 Gy; 11 days, −70°C = 4,753 Gy. All −70°C storage immunoreactivity values are significantly superior to those obtained with storage at 4°C [$p < (0.025 - 0.0005)$]. B: Graphic display of thin layer chromatographic data mem ± s.e.m. of ^{131}I liberation from ^{131}I-labeled antibody preparations during storage under conditions described for Figure 1A. Free iodine liberation is significantly less at −70°C than at 4°C for early (4 day) and late (11–12 day) time points ($p < 0.025$ and $p < 0.0005$, respectively).

trols were also included. The immunoreactive fraction is:

$$= 100 \times \frac{\text{Total counts bound-background}}{\text{Total counts added-background}} - \frac{\text{Blocked counts bound-background}}{\text{Blocked counts added-background}}.$$

Thin layer chromatography (TLC) was performed using a Gelman ITLC-SG (silica gel) system, with 50% ethanol and 50% saline as the solvent system. Radiochromatograms were counted and analyzed using an automated scanner (BioScan). Free iodine migrates with the solvent front and antibody-bound radioactivity remains at or near the origin. The percentage of free/total iodine activity was computer-determined and recorded. The cell binding assays and TLC studies were performed immediately post-radiolabeling and immediately after removal of aliquots of radioactivity from storage at the selected time points. Sizing HPLC was performed on aliquots from the high-level ^{131}I and ^{125}I studies using a Pharmacia FPLC system with a flow rate of 0.5 cc/min and a Superose 12 size-exclusion column. No vials were frozen or thawed more than once. Radiation dose/vial was determined using the MIRD formalism for spheres of .5–2 cc volume. For a 1 cc sphere for ^{131}I, it was estimated that 97.8% of the radiation dose was due to electrons with the remainder from photons. The ^{125}I self dose for a 1 cc sphere assumes 78.4% of the self dose to be due to electrons with a 21.6% photon contribution. The values of Δ_i and the absorbed fraction data for photons from ^{131}I and ^{125}I were derived from published MIRD data (*14,15*).

RESULTS

Low-Level Storage

Studies of immunoreactivity of the 763.24T, 225.28S, and 5G6.4 antibodies following radiolabeling and storage are shown graphically in Figure 1A. In these studies, a clear decrease in immunoreactivity from baseline was seen at 4 days postlabeling, and in particular at 11–12 days postlabeling if storage at 4°C was undertaken. This loss of immunoreactivity was essentially abrogated when storage was conducted at −70°C. These decrements in immunoreactivity occurred with just 1.75 mCi to 3.68 mCi added/vial which over 4 days represents an approximate radiation dose of 1185–2194 Gy and by 11–12 days of 2621–5500 Gy (*4*). There is also significantly less free iodine in the antibody aliquots stored at −70°C than in those stored at 4°C, though the differences are somewhat less marked than those in immunoreactivity, though still significant (Fig. 1B).

Higher level labeling. In the first study, 60 mCi of ^{131}I 5G6.4 was divided into two nearly equal aliquots of 1 cc each. One aliquot (31 mCi) was stored at −70°C and one (27 mCi) at 4°C for 24 hr. Cell binding and ITLC assay results from before and after storage are shown in Table 1. There is a major drop in immunoreactivity in just 24 hr of storage at 4°C, while at −70°C there is a more modest decrement in immunoreactivity. The radiation dose in this 24-hr time period is estimated at 2592–2976 Gy. In a second study, storage was carried to 48 hr. In this study, 5G6.4 with a specific activity of 15.3 μCi/μg was divided into aliquots of 19.8 and 20.5 mCi/ml for storage at 4°C and −70°C, respectively, for radiation doses of 3645 to 3774 Gy. All binding data are shown in Table 1 with Radio FPLC shown in Figures 2A and B. Note the major loss in immunoreactivity with the 4°C storage, but the excellent preservation of immunoreactivity in the −70°C storage conditions. FPLC, Figure 2A, shows the appearance of low to intermediate molecular weight (autoradiolytic breakdown) species when storage at 4°C was undertaken, but little change in FPLC profile if −70°C storage was performed. To evaluate the effect of the ^{125}I label on

TABLE 1
5G6.4 Higher Level Storage Experiments

		Baseline	24 hr at 4° C*	24 hr at −70° C†
Experiment 1 (^{131}I)	Immunoreactivity	54.1%	28.0%	40.3%
	free iodine	1.6%	14.5%	4.3%
		Baseline	**48 hr at 4° C‡**	**48 hr at −70° C§**
Experiment 2 (^{131}I)	Immunoreactivity	55.9 ± 1.2%	5.6 ± .9%	58.2 ± 2.1%
		Baseline	**48 hr at 4° C¶**	**48 hr at −70° C¶**
Experiment 3 (^{125}I)	Immunoreactivity	65.1 ± 2.3%	48.3 ± 4.6%	65.5 ± 5.2%

* 27 mCi ^{131}I 5G6.4, specific activity 12 μCi/μg stored—~2,592 Gy Self-irradiation in 24 hr.
† 31 mCi ^{131}I 5G6.4, specific activity 12 μCi/μg stored—~2,976 Gy Self-irradiation in 24 hr.
‡ 19.8 mCi ^{131}I 5G6.4, specific activity 15.3 μCi/μg stored—~3,645 Gy Self-irradiation in 48 hr.
§ 20.5 mCi ^{131}I 5G6.4, specific activity 15.3 μCi/μg stored—~3,774 Gy Self-irradiation in 48 hr.
¶ 44 mCi ^{125}I 5G6.4, specific activity 25.0 μCi/μg stored—~567 Gy of Self-irradiation in 48 hr.

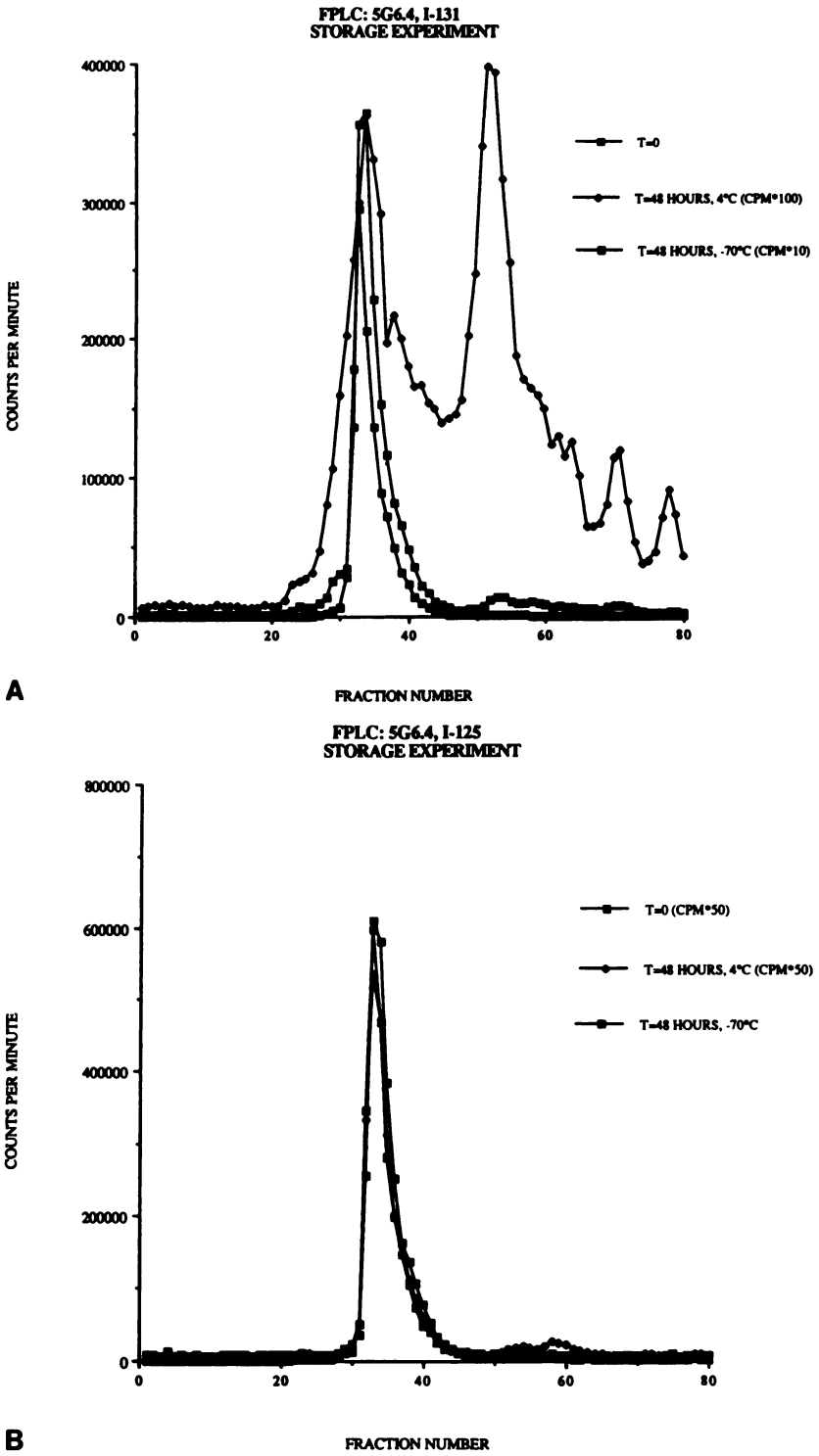

FIGURE 2
A: Sizing FPLC of ^{131}I 5G6.4 antibody from experiment 2 (high level label). Note the single peak of the native 5G6. antibody (t = 0 hr). At 48 hr with storage at −70°C, there is little change in the FPLC profile. By contrast, with storage at 4°C, a marked broadening of the FPLC elution profile is seen with a small quantity of a high molecular weight species and considerable amounts of lower molecular weight species (fx 40-55), (which are higher in weight than free Iodine which elutes at fx 58). B: FLPC profiles from high level ^{125}I labeling of 5G6.4 and storage. Note that the tracing t = 0 hr and t = 48 hr at −70°C are virtually identical, while for the storage at 4°C, there is mild liberation of free iodine (fx 58). Overall, the ^{125}I preparation shows much less decomposition than the ^{131}I/mCi.

immunoreactivity with storage, 5G6.4 was iodinated with ^{125}I to a specific activity of 25 μCi/μg. Forty-four millicuries were stored at 4°C, while an additional 44 mCi were stored at −70°C in 2 ml. The calculated ^{125}I irradiation self dose is but 567 Gy. The more modest loss in immunoreactivity at 4°C versus comparable mCi ^{131}I preparations (Table 1) as well as the superiority of the storage at −70°C versus 4°C is apparent for ^{125}I. FPLC tracings of the various ^{125}I preparations demonstrate the more modest quantity of breakdown products (mainly free iodine) seen at 4°C and the lack of such breakdown at −70°C (Fig. 2B). The more minimal appearance of ^{125}I breakdown products at 4°C is particularly apparent when contrasted with the FPLC profile of the ^{131}I preparations of comparable mCi added, as is shown in Figure 2A. Note that the MIRD calculated dose for ^{125}I is much lower than for a similar quantity of ^{131}I.

DISCUSSION

This report illustrates that decrements in immunoreactivity of antibodies and increases in the liberation of free iodine and lower molecular weight protein breakdown products occur over a relatively short-time period when low therapeutic levels of ^{131}I are attached to monoclonal antibodies. Similar phenomena also occur over a longer storage time course when lower ^{131}I doses are used. The degree of radiolysis is impressive given the relatively low amount of radioactivity used. The HPLC studies clearly demonstrate that the products of autoradiolysis are of low molecular weight and that their formation is retarded by freezing. By contrast, while radiolysis does occur with ^{125}I, it is more modest/mCi as shown by HPLC and immunoreactivity data. The use of freezing at −70°C also slows ^{125}I autoradiolysis.

Decreasing the storage temperature to −70°C drastically reduced the radiolysis-induced drop in immunoreactivity and considerably slowed the appearance of free iodine versus storage at 4°C. The beneficial effect of low temperature on immunoreactivity is presumedly due to the immobilization of ^{131}I decay-induced free radicals in the ice crystal lattice preventing the hydrogen peroxide and perhydroxyl radical from damaging antibodies (*5*). The radioprotective effect of the −70°C appears analogous to that reported for human serum albumin used when antibodies are externally irradiated, but is achieved without adding human blood products to the antibody injection mixture (*4*). The combination of the two approaches, cryopreservation and albumin or a free radical scavenger might be even more effective than −70°C alone and may warrant further study (*16*). It should also be noted that freezing of hormones decreases radiolysis, and that the possibility of freezing antibodies for storage has been previously suggested (*6*, *17*). Simple absorbed dose calculations indicate that storage at larger, rather than smaller volumes, may also be beneficial and suggest that a combination of these approaches may ultimately be most useful.

The more modest effects of ^{125}I on autoradiolysis despite the presence of low-energy, low range, high L.E.T., ^{125}I Auger electrons suggests that the free-radical effect may be predominant and the extent of autoradioloysis parallels the low estimated radiation dose by MIRD for ^{125}I. While we have observed the benefits of cryopreservation with each antibody, the radiolytic effect of varying radiolabel and storage conditions likely should be determined for each antibody and label, as it has been reported that some yttrium-90-labeled antibodies experience relatively little autoradiolysis when stored over days at a 2 mCi/ml concentration (*18*).

In summary, for the three monoclonal antibodies studied, freezing at −70°C *radioiodinated* monoclonal antibodies preserves their immunoreactivity and molecular integrity much better than storage at 4°. These data suggest that for high-dose radioimmunotherapy with monoclonal antibodies, freezing at −70°C might rationally be performed immediately after radiolabeling, while quality control procedures are performed on a small aliquot of the antibody. If the quality control results are satisfactory, the frozen product, which could have been transported to another site, might then be thawed and infused to the patient. Obviously, such an approach will need to be tailored to the individual antibody and label being studied, but it appears that for therapeutic doses of radioantibody, cryopreservation immediately after labeling may be a generally useful approach to maintain the immunological viability of a variety of antibodies and radiolabels.

ACKNOWLEDGMENTS

This work was supported by CA 41531, CA 33802, CA 40497, and POI CA 42768-04 awarded by NCI/PHS. The technical assistance of Anaira Clavo, Gayle Jackson, Pam Sullivan, Patricia Scott, Steve Kronberg, and Andrew Kucharski is greatly appreciated. The secretarial expertise of Michele Curro, Mary Miller, and Lisa Donahue is recognized.

REFERENCES

1. Goldenberg DM. Targeting of cancer with radiolabeled antibodies. Prospects for imaging and therapy. *Arch Pathol Lab Med* 1988;112:580–587.
2. Sitzmann JV, Order SE, Klein JL, Leichner PK, Fishman EK, Smith GW. Conversion by new treatment modalities of nonresectable to resectable hepatocellular cancer. *J Clin Oncol* 1987;5:1566–1573.
3. DeNardo SJ, DeNardo GL, O'Grady LF, et al. Treatment of a patient with B cel lymphoma by I-131 LYM-1 monoclonal antibodies. *Int J Biol Markers* 1987;2:49–53.
4. Kishore R, Early JF, Krohn KA, et al. Autoradiolysis of iodinated monoclonal antibody preparations. *Int J of Radiat Appl Instrum Part B, Nucl Med and Biol* 1986;4:457–459.
5. Pizarello DJ. Direct and indirect action. In: Pizzarello DJ, Witcofski RL. *Basic radiation biology.* Second edition. Philadelphia: Lea & Febiger; 1975:20–29.
6. Bartolini P, De Assiss LM, Fonseca MLQ. Radioiodination of human growth hormone with characterization and minimization of the commonly defined "damaged products." *Clin Chim Acta* 1981;110:177–185.
7. Wahl RL, Liebert M, Roberts J, Jackson G, Kronberg S, Laino L. Production and characterization of a murine monoclonal antibody reactive with ovarian and other epithelial carcinomas. *Proceedings of AACR* 1986;27:355.
8. Wilson BS, Giacomini P, Imai K, et al. Human melanoma-associated antigens identified with monoclonal antibodies. *La Ricerca in Clinica e in Laboratorio* 1982;12:517–538.
9. Wilson BS, Imai K, Natali PG, Ferrone S. Distribution and molecular characterization of a cell surface and cytoplasmic antigen detectable in human melanoma cells with monoclonal antibodies. *Int J Cancer* 1981;28:293.
10. Ey P, Prowse S, Jenkins C. Isolation of pure IgG_1, IgG_{2a}, and IgG_{2b} from mouse serum using protein A-sepharose. *Immunochemistry* 1987;15:429–436.
11. Wahl RL, Parker CW, Philpott GW. Improved radioimaging and tumor localization with monoclonal F(ab') 2. *J Nucl Med* 1983;24:316–325.

12. Laemmli VK. Cleavage of structural proteins during assembly of the head bacteriophage T4. *Nature* 1970;222:680.
13. Fraker PJ, Speck JC. Protein and cell membrane iodinations with a sparingly soluble chloramide, 1,3,4,6-tetrachloro-3a,6a diphenylglycoluril. *Biochem Biophys Res Commun* 1987; 80:849–857.
14. MIRD Pamphlet 10, Society of Nuclear Medicine, 1975.
15. MIRD Pamphlet 8. *J Nucl Med* 1971;25:(suppl 5).
16. Yalow RS, Berson SA. Labeling of proteins-problems and practices. *Trans New York Acad Sci* 1966;28:1033–1044.
17. Saha GB. Radioiodination of Antibodies for Tumor Imaging. In: Burchiel SW, Rhodes BA, eds. *Radioimmunimaging and Radioimmunotherapy* New York: Elsevier, 1983:171–184.
18. Frincke JM, Halpern SE, Hagan PL, et al. The effect of radioautolysis on 90Y antibody (Ab) preparations. *J Nucl Med* 1987;28:711.