High-Level Iodination of Monoclonal Antibody Fragments for Radiotherapy

John M. Ferens, Kenneth A. Krohn, Paul L. Beaumier, Joseph P. Brown, Ingegerd Hellström, Karl Erik Hellström, Jorge A. Carrasquillo,* and Steven M. Larson*

University of Washington School of Medicine, Fred Hutchinson Cancer Research Center, and Seattle Veterans Administration Medical Center, Seattle, Washington

Two different murine monoclonal antibody Fab fragments specific for p97, a melanoma-associated antigen, were labeled with I-131 at high activity levels without excessive chemical damage. Up to 20 mg of Fab were labeled with up to 300 mCi of I-131 using the chloramine-T method and large working volumes at room temperature. As much as 90% of the initial activity was recovered as labeled product. The labeled Fabs varied in their sensitivity to radioiodination damage, as measured by an in vitro cell-binding assay. Radioiodination was performed safely using a remote iodination apparatus. The final product was of radiopharmaceutical quality suitable for clinical diagnosis and experimental radiotherapy in humans.

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We have been investigating the potential use of radioiodinated monoclonal antibody, both whole and fragment (Fab), for diagnosis and experimental radiotherapy of metastatic melanoma in humans (*1–3*). The development of labeled Fabs for clinical application had several facets. First, high-affinity monoclonal antibodies were produced to react with the p97 antigen of human melanoma (*4,5*). Second, a sensitive assay was developed to measure the retained immunoreactivity of the radioiodinated Fabs (*6*). Third, toxicity testing was performed in animals (*1*), and approval obtained for use of radioiodinated Fab as an Investigational New Drug. Finally, radioiodinated anti-p97 Fabs were tested for localization in vivo in human tumor (*7*).

Several radiochemistry, radiopharmacy, and radiation safety problems arose in accomplishing radioiodinations with hundreds of mCi of I-131. First, to retain their antigen recognition capacity, the antibodies had to be iodinated at high specific activity with minimal chemical or radiation damage. Second, safety hazards to those performing the labeling had to be minimized, including both direct radiation and potential thyroid accumulation of radioiodine. Third, an efficient iodination technique had to be used, allowing both large reaction volumes and simple manipulations for labeling and purification. This paper describes the methods and semiautomated apparatus used to label monoclonal antibody fragments within these specifications.

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For reprints contact: Kenneth A. Krohn, PhD, Div. of Nuc. Med., RC-70, University of Washington, Seattle, WA 98195.

* Present address: Nuclear Medicine Department, NIH Clinical Center, Bldg 10, ACRF, 9000 Rockville Place, Bethesda, MD 20205.

METHODS AND MATERIALS

Reagents. The required reagents were obtained as reagent-grade chemicals and used as supplied commercially. All buffers were prepared sterile and pyrogen-free with USP or reagent-grade chemicals.

Radioiodine. Iodine-131 was obtained at high specific activity as reductant-free labeling-grade material in 25 to 300 μl of 0.1*N* NaOH, in a 5-ml, flat-bottomed vial.

Antibody fragments. The murine (mouse/mouse) monoclonal whole antibodies 96.5 (IgG2a) and 8.2 (IgG1) and their Fab fragments (50,000 daltons) were obtained as described previously (*1,6*). Both Fabs have exhibited high affinity ($10^{10} M^{-1}$) for the same human melanoma surface antigen (p97), but against different regions (epitopes) of this antigen (*1,4,6*). The fragments were stored at −70°C before labeling.

Radioiodination. Fabs were radioiodinated using a modification of the standard chloramine-T (C-T) method (*8,9*). Labeling was done in 1 to 4 ml at room temperature for 5 min with 20 μg C-T per mg of protein. One to 20 mg of protein was reacted with 1–300 mCi of I-131 in 0.1 *M* phosphate-buffered saline (PBS), pH 7.2. The reaction was quenched with sodium thiosulfate (3:1 molar ratio to C-T) and sodium iodide (1 mg per mg protein). The reaction mixture was gel-filtered on Sephadex G-10 with 0.05 *M* PBS, pH 7.2. The labeled product was immediately stored at 2 to 8°C.

Remote labeling apparatus. Figure 1 illustrates schematically the semiautomated remote radioiodination apparatus. The components included the shipping vial (which became the reaction vial), a needle block with two spinal needles, an eluant reservoir of 0.05 *M* PBS, a 2-channel peristaltic pump, a G-M radiation

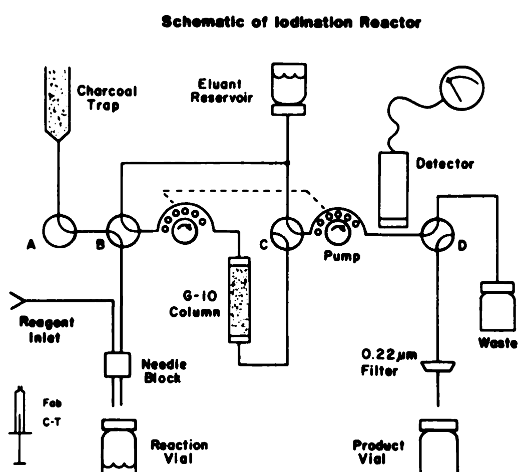

FIG 1. Labeling apparatus for protein iodination using chloramine-T. Four valves (A through D) are controlled individually from outside hood, and are shown as positioned at beginning of reaction sequence. Reagent inlet is 3-way stopcock. Peristaltic pump is run at 1 ml per min during transfer and elution. G-10 column (1.5 X 13 cm) provides good separation of labeled protein from free iodine up to 25 mg of protein in 5 ml of volume.

detector, and four Teflon valves. The system was assembled in a fume hood with all fluid pathways sterile and pyrogen-free. Three inches (76 mm) of leaded glass and 4 in. (102 mm) of lead provided shielding.

The labeling procedure was begun by remotely pushing the needles about 1/4" through the septum of the unopened shipping vial. Buffer, antibody, and freshly prepared C-T were added to the vial through the reagent inlet line as the vial was mechanically agitated. The charcoal trapped any iodine vapors released from the vial during reaction and equilibrated the reaction's pressure with atmospheric.

When complete, the reaction was quenched through the inlet line, and the needles pushed to the bottom of the vial. The peristaltic pump was used to transfer the reaction mixture onto the chromatographic column. Once the transfer was complete, the column was eluted with buffer from the reservoir. The first peak, as detected by the radiation monitor, was directed through the sterilizing filter into the product-collection vial. All other eluate was directed to the waste vial for disposal.

After use, the apparatus was stored containing 0.1% sodium azide in normal saline. The reaction vial, needles, and reagent inlet line were removed, and the entire system, except for the charcoal trap, was filled with the azide solution. Before the next use, the system was completely flushed with three system volumes of 0.05 *M* PBS.

Quality-control tests. The p97-antigen-binding capacity of freshly radioiodinated Fab was tested with an in vitro cell-binding assay (CBA), and the results expressed as percentage of initial activity present in the cell pellet (*6*). The nonspecific binding of this assay system was less than 3% with radioiodinated Fab 1.4, a nonspecific control. To verify correct peak collection from the column, and ensure that no contamination by the waste peak had occurred, the percent radioiodine bound to protein was measured by electrophoresis on cellulose polyacetate strips. Product sterility and apyrogenicity were tested, respectively, by the USP sterility test and the Limulus amebocyte lysate (LAL) method.

RESULTS

Labeling. Table 1 presents a comparison between the reaction parameters and results of several C-T iodination procedures. Shown are: (a) the iodination of human growth hormone (HGH) by Hunter (*10*); (b) a typical early benchtop iodination for diagnostic imaging using Fab 8.2, patterned after McConahey and

TABLE 1. CHLORAMINE-T REACTION COMPARISON

		Classical* example	Initial† diagnostic	Present‡ therapeutic
Reagents:	Protein type	HGH	Fab 8.2	Fab 96.5
	Protein (mg)	0.005	2	10
	C-T (µg)	100	25	230
	I-131 (mCi)	2	9	250
Molar ratios:	C-T/protein (rxn)§	1900	3	5
	C-T/iodine (rxn)	490	8	5
	Iodine/protein (prod)	2.5	0.25	0.75
Reaction:	Volume (ml)	0.1	0.5	2.5
	Time (min)	0.5	10	5
	Temp. (°C)	20	0	20
	pH	7.5	7.1	7.3
	Quench	Yes	No	Yes
Product:	Chemical yield (%)	55–75	60–90	85–95
	Immunoreactivity (%)	100	60–70	40–55

* From Hunter (*10*).
† Early benchtop work patterned after McConahey and Dixon (*9*).
‡ Using remote iodination apparatus.
§ Reactants.

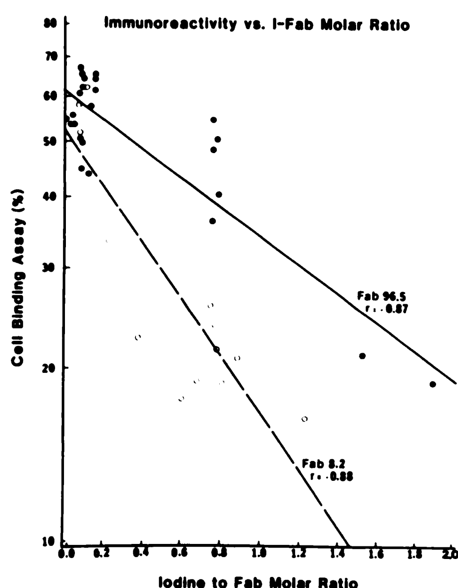

FIG. 2. Cell-binding assay (CBA) percentages for iodinated Fab 8.2 and 96.5 are plotted against iodine-to-Fab molar ratio in labeled product. Labelings were performed at identical ratios of C-T to Fab, and at constant time, temperature, and pH.

Dixon (*9*); and (c) a typical iodination of Fab 96.5 for experimental radiotherapy using the present remote iodination apparatus. All recent diagnostic labelings and all therapeutic labelings were performed in this apparatus with 10 to 20 mg of Fab.

Labelings have been achieved with approximately 2,000 to 4,000 times as much protein and 125 times as much radioactive iodine as in the classical method (*10*). Molar ratios of chloramine-T to both protein and iodine in the reaction mixture have been about 0.3% and 1%, respectively, of classical levels. As shown in Table 1, radiochemical yields for some runs have reached as high as 95%, as measured by electrophoresis of the reaction mixture. As much as 90% of the initial activity was recovered as labeled product suitable for injection. The unrecovered activity was distributed as: unlabeled iodine (5% or more); labeled product retained on the Millipore filter (3–4%); and product lost in the reaction vial, lines, and fittings of the apparatus. The molar ratio of iodine to protein in the labeled product was typically between 0.05 and 0.2 for diagnostic preparations and 0.75 for therapeutic preparations. To date, 16 labelings for human use have been performed with this protocol in the potentially therapeutic range of >150 mCi I-131.

Quality control. As shown in Fig. 2, the immunoreactivity of iodinated Fab 96.5 has ranged from 45–68% in the diagnostic labelings, and from 37–55% in the therapeutic. When labeled under identical conditions, including molar ratio of iodine to Fab, the CBA revealed that Fabs 96.5 and 8.2 did not exhibit the same immunoreactivity. In addition, CBA results for both Fabs varied with the ratio of iodine to Fab in the labeled product.

The percentage bound measured by electrophoresis was typically greater than 99%. USP sterility test results were not available until 14 days after batch preparation, and the labeled Fab was used before test results. Terminal sterilization of the product by Millipore filtration proved very reliable, and sterility test results were routinely negative. The LAL test was consistently negative for pyrogenicity at the acceptance level of 1.25 endotoxin unit per ml.

Radiation exposure. The radiation exposure experience for the 4.2 Ci of I-131 handled by our labeling chemist over the course of 1 yr was monitored by film badges and hand thermoluminescent dosimeters. Thyroid radioactivity was monitored after each labeling by the radiation safety office using a calibrated scintillation detector, and never exceeded 1 nCi. Table 2 summarizes these data.

DISCUSSION

There was a differential sensitivity of the two Fab fragments to iodination, as evaluated by the cell-binding assay (CBA). When the labeling was done with I-125 using the C-T method at low levels (iodine/Fab molar ratio about 1:80, unpublished data), CBA percentages of ~70% were routinely obtained for both Fabs. As the iodine-to-Fab ratio increased, the measured immunoreactivity of both Fabs decreased exponentially, as shown in Fig. 2. However, Fab 8.2 was found more sensitive to iodine incorporation than Fab 96.5. This may be a consequence of a tyrosine residue situated at or near the binding pocket of Fab 8.2, which, when iodinated, dramatically affects its immunoreactivity. The CBA was vital in assessing and optimizing the quality of the labeled product.

The radiation doses received by our labeling chemist were low during high-level iodinations with the labeling apparatus. These doses compared very favorably with our experience for an average laboratory technician doing open-vial C-T labeling in a fume hood with a few mCi of I-125. Measured activity in the charcoal trap after an iodination was up to 0.1% of the total activity handled. Much of the dose to whole body and hands (Table 2) was received during routine handling of the radioactive shipment, not during the iodination process.

The labeling apparatus described has several distinct advantages over more traditional radioiodination setups for a process working at high activity levels (*11,12*). Since the shipping vial provided by the manufacturer was suitable for closed-system iodination, volatile iodine was never exposed to the atmosphere. No significant positive pressures were developed within the system, eliminating the hazard of leaking radioactive material. The ability to maintain system's sterility, including the Sephadex column, was a great advantage, eliminating the need to disassemble a contaminated setup. The apparatus maintained its sterility and apyrogenicity with weekly use for more than 6 mos, and did not require a large fume hood. As currently constructed, we anticipate that the apparatus will be useful with up to 1000 mCi of I-131 activity.

A number of other chemical techniques do exist for the radioiodination of antibodies (*13*). With minor internal modifications the apparatus has been useful for iodinations with several of these, including lactoperoxidase (LPO), 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril (iodogen), and iodine monochloride (ICl). We have also successfully iodinated Fabs with I-125 and I-123 in the apparatus (*2,3*).

Further modifications of the iodination reactor can be made easily. Reactions could be performed at reduced temperature, or the apparatus placed under the control of a microprocessor for

TABLE 2. RADIATION DOSE TO LABELING CHEMIST

Organ	Total* (mrem)	Annual limit (mrem)	Percentage of annual limit
Hand	880	75,000	1.2
Body	210	5,000	4.2
Thyroid	220	30,000	0.7

* From first year's experience, totaling 4,200 mCi of I-131.

routine operation. We see no reason why this apparatus could not be easily adapted for use in the conjugation of bifunctional metal ligands and other radiochemical reactions at high activity levels.

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