RADIOCHEMISTRY AND RADIOPHARMACEUTICALS

Indium-111 Labeling of Leukocytes: A Detrimental Effect on Neutrophil and Lymphocyte Function and an Improved Method of Cell Labeling

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A technique for the labeling of cells with the gamma emitter indium-111 has recently been developed. In this study the effects of the labeling procedure on some in vitro functions of human neutrophils and lymphocytes were investigated. With the standard labeling procedure, neutrophil chemotaxis was reduced to approximately 50% of normal and lymphocytes lost surface receptors and failed to respond to stimulation with phytohemagglutinin. The 8-hydroxyquinoline that is used to chelate the indium is toxic to lymphocytes; accordingly the relationship between the quantity of oxine, the chelation of indium, and cell labeling were investigated. Optimal conditions for In-111 cell labeling were established: 100 million cells in 10 ml Hanks' balanced salt solution are mixed with 5 μg of oxine in a mixture of 50 μl of ethanol and 200 μl of saline; they are incubated at 37°C for 10 min and then washed. Initially, neutrophils and lymphocytes appear functionally normal, but after 24–48 hr lymphocyte function is impaired as a result of radiation damage. This toxicity may limit studies by external scanning on the distribution and kinetics of lymphocytes labeled with In-111.

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The labeling of isolated cells with indium-111 is a new technique with important applications in clinical medicine and biologic investigation (*1*). When In-111 is administered to human patients, its emitted photons can be detected externally with a gamma camera or scanning equipment (*2*). It has a half-life of 68 hr, which is long enough to allow repeated investigations for up to a week while not posing an excessive radiation hazard (*3*). The mechanism of labeling (*4*) results in a firm association of the label with the cell. Indium-111 can be transported through the plasma membrane and into the cell as a lipid-soluble chelate with 8-hydroxyquinoline (oxine). Once within the cell the In-111 dissociates from the oxine and binds to compounds in the cytosol, and possibly to DNA.

Labeled granulocytes have been successfully employed in man in the detection of abscesses (*2*), and

lymphocytes migrate to the lymph nodes of patients with Hodgkins disease (*5*). The apparently normal function of these cells suggested that they are not adversely affected by the labeling procedure. Indium-111 labeling appears to be the only practical method for the external detection of cell kinetics and distribution. The present study was conducted to investigate the effects of the standard method of labeling human leukocytes with In-111 oxine on various parameters of their function because it is essential that the cells remain undamaged by the labeling procedure if meaningful conclusions are to be drawn from such investigations.

MATERIALS, METHODS, AND RESULTS

Cell separation. Leukocytes were purified from venous blood (50–200 ml) taken from healthy volunteers into heparin (10 IU/ml)—and also from single and pooled buffy-coat preparations in citrate phosphate dextrose—by dextran sedimentation of erythrocytes and centrifugation on ficoll/sodium diatrizoate* of density 1.077 (6). Erythrocytes were removed from the neutrophil pellet by hypotonic lysis with distilled water for 30 sec, after which the neutrophils were resuspended in Hanks' balanced salt solution containing heparin 5 IU/ml. The lymphocytes, which remain above the ficoll/sodium diatrizoate, were washed twice in RPMI medium containing (100 units/ml) penicillin, (100 μg/ml) streptomycin, (0.25 millimols/l) glutamine, and 10% fetal calf serum. They were then allowed to stand in glass Ehlenmeyer flasks at room temperature for 2 hr, thereby reducing the contamination by monocytes, most of which adhere to the glass.

Effect of standard labeling procedure on lymphocyte and neutrophil function. Lymphocytes (100 million) suspended in 10 ml saline (0.15 mol/l) were mixed with 40 μCi of In-111 oxine weighing 75 μg before extraction, chelated in the standard way (1), left for 10 min at room temperature, and washed twice in the medium, after which various parameters of integrity and function were studied. Surface receptors were examined by rosetting techniques (7), spontaneous and antibody-dependent (ADCC) cytotoxicity by the release of chromium (8) from target cells [a myeloid (K562) cell line (9)], and transformation in response to the mixed lymphocyte reaction and stimulation with mitogens (10).

Lymphocytes were incubated with (0.1 μg/ml) phytohemagglutinin or pokeweed mitogen in the RPMI medium for 2–3 days, and ([³H]-methyl) thymidine (1 μCi/ml) was included for the last 3–6 hr. They were harvested on GF/C glass microfiber paper†, washed with 0.15 mol/l saline buffered with 8 millimol/l phosphate (pH 7.4), then with 5% trichloracetic acid, and finally with methanol. Radioactivity was counted using a toluene-based scintillation fluid (10).

Neutrophils were labeled in a similar manner and their function assessed by chemotaxis under agar (11) using as attractant the supernatant of a 24-hr culture of E. coli in broth.

The labeling efficiencies for the lymphocytes and neutrophils were 88% and 50%, respectively, giving final specific activities of 35 and 20 μCi per 10^8 cells. The functions of both cell types were adversely affected. The incorporation of [³H] thymidine into lymphocytes stimulated with PHA was initially normal, but fell to 30% of normal at 48 hr. Spontaneous migration of neutrophils was depressed to 55.4% (s.e. = 8.4%, n = 5) of the control, and chemotactic migration, expressed as the percentage increase over spontaneous migration, was 45.2% ($\pm$4.5%, n = 5) as compared with 86% ($\pm$12%, n = 5) in control cells.

Experiments were accordingly performed to identify and eliminate the factors responsible for the cell damage.

Effect of ethanol and oxine on lymphocyte function. The effect of ethanol was first investigated by the addition of absolute ethanol to a suspension of lymphocytes in culture medium. One million lymphocytes were stimulated with 1 μg PHA/ml, and [³H] thymidine incorporation in to DNA was measured 65 hr later. Ethanol was without effect at a concentration of 0.2%, whereas 1% reduced thymidine incorporation to 26% of control.

Using a nontoxic concentration of ethanol (0.1%) the effect of various amounts of oxine on lymphocyte transformation was then studied. Oxine was added to the cell suspensions, which were washed in saline after 15 min at room temperature and then resuspended in medium and stimulated with PHA (1 μg/ml), pokeweed mitogen or by the mixed lymphocyte reaction alone.

TABLE 1. EFFECT OF OXINE ON LYMPHOCYTE TRANSFORMATION (EXPRESSED AS RADIOACTIVITY COUNTS/MIN)*

Mitogenic stimulus	42 hr			65 hr		
	Oxine (μg/ml)					
	0	0.2	1.0	0	0.2	1.0
Mixed lymphocyte reaction	13,900	13,830	360	34,100	35,640	310
Phytohemagglutinin (1 μg/ml)	100,370	94,390	460	110,280	108,630	3,760
Pokeweed	64,440	58,040	280	95,430	73,430	895

* Oxine was administered in two concentrations as a 15-min pulse. Tritiated thymidine incorporation into the cells (4-hr pulse, 1 μCi/ml) was measured as detailed in the methods.

Oxine was found to be toxic at a concentration of 1.0 μg per million cells which completely inhibited thymidine incorporation (Table 1), whereas a concentration of 0.2 μg was almost without effect. The toxicity of oxine was shown to be directly related to the number of cells in the suspension (data not shown) rather than to the volume of the suspending medium.

Relationship between oxine concentration and the chelation of In-111. To 0.5 ml aliquots of [111]In Cl_3 were added 0.5 ml of 0.3 mol/l sodium acetate buffer, pH 5.0; 0.05 ml of ethanol containing 0.5–100 μg of oxine was added and the mixture was well shaken. The chelated In-111 was extracted into 5 ml $CHCl_3$. There was a linear relationship between the extraction of radioactivity and the logarithm of the amount of oxine (Fig. 1). Approximately 50% of the radioactivity was chelated by 10 μg of oxine.

Effect of incubation conditions on cell labeling. One million Meth A cells (12) (a methylcholanthrene-induced mouse cell line) were suspended in 1.0 ml of various media that included: 0.15 mol/l NaCl, with or without phosphate buffer (8 millimols/l phosphate, pH 7.4); Hanks' balanced salt solution; RPMI containing 10% fetal calf serum; L15; and minimum essential medium containing a low concentration of Ca^{2+} and Mg^{2+}‡. One μCi In-111 chelated to 3.33 μg oxine in 10 μl ethanol was mixed with 40 μl of 0.15 mol/l NaCl and added
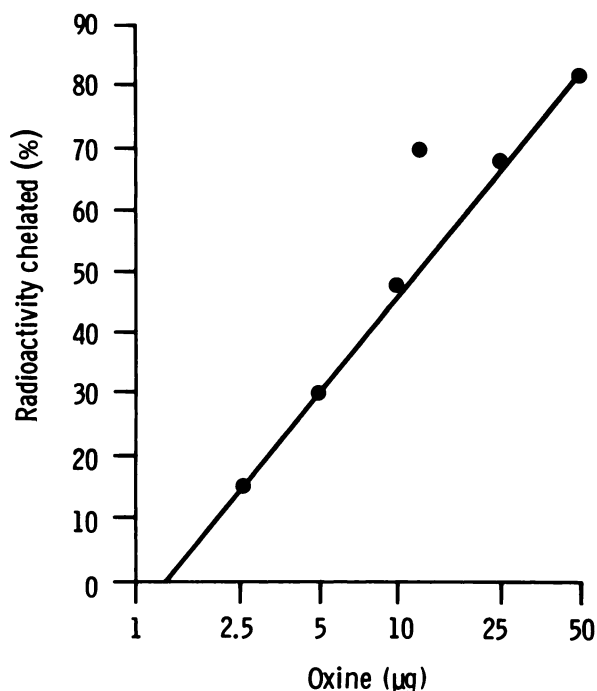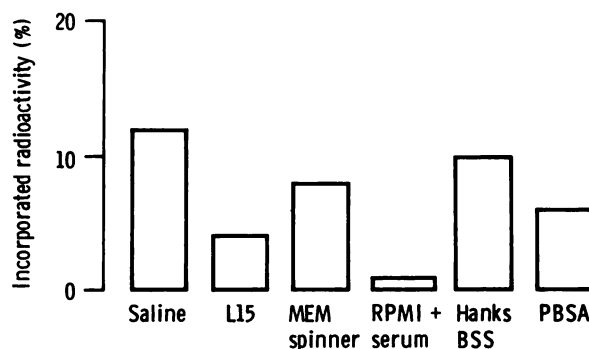
FIG. 2. Effect of varying incubation medium upon labeling of Meth A cells[12] with In-111 oxine. Cells were suspended either in saline alone or with one of the following added: phosphate buffer (PBSA), minimum essential medium for suspension culture (MEM spinner), Leibovitz medium (L15), Hanks' balanced salt solution, or Roswell Park Memorial Institute medium 1640 (RPMI) containing 10% fetal calf serum.

to each cell suspension. After 10 min at room temperature, the radioactivity in a sample of the cell suspension was taken and compared with the residual radioactivity in the supernatant after centrifugation at 8,000 g.
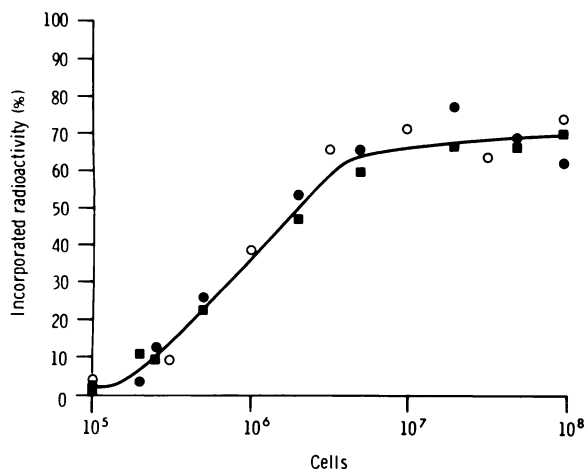
Cell labeling was most efficient in saline (Fig. 2). It was least reduced in Hanks' BSS where it was 81.4% of the saline level, and it was almost abolished by serum because the indium binds to transferrin.

Five million mouse mastocytoma cells (13) in 0.5 ml Hanks' BSS were mixed with 5 μl of In-111 oxine chelate (0.42 μg) and incubated at 21°C and 37°C for 5, 10, 20, and 30 min. The cells were then centrifuged as described above and the radioactivities in the cell suspension and the supernatant were compared.

Incubation time and temperature hardly affected the degree of cell labeling, which ranged from 48.0% after 5 min at 21°C to 53.8% after 30 min at 37°C.

Relationship between cell numbers and uptake of radioactivity. Aliquots (0.5 ml) of suspensions of lymphocytes, neutrophils, and mouse mastocytoma cells, each containing 200,000–200 million cells/ml, in Hanks' BSS were mixed with (0.1 μCi) In-111 oxine (1.7 μg) in ethanol (5 μl) and saline (20 μl) and incubated at 37° for 10 min. The cells were centrifuged at 8,000 g for 2 min and the radioactivity in the supernatant was compared with that in the cell suspension.

For all three cell types, a linear relationship existed between the uptake of radioactivity by the cells and the logarithm of the number of cells between 5×10^5 and 5×10^6 (Fig. 3). With larger numbers of cells, the percentage of incorporated activity was not increased.

FIG. 1. Relationship between quantity of oxine (log scale) and percentage of In-111 chelated. See text for experimental details.

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FIG. 3. Relationship between numbers of granulocytes (■), lymphocytes (●), and mouse mastocytoma cells (○) and percentage of total radioactivity that becomes incorporated into the cells. See text for experimental details.

Effect of different concentrations of oxine on efficiency of cell labeling. To 10 million neutrophils in 500 μl Hanks' BSS were added 25 μl of a mixture of ethanol (5 μl) and saline (20 μl) containing between 0.2 and 5.0 μg oxine chelated to tracer amounts of In-111. The mixture was incubated for 10 min at 37°C and the cells were then washed in Hanks' BSS.

A linear relationship was found to exist between the incorporation of radioactivity into the cells and the logarithm of the concentration of oxine (Fig. 4).

Effect of optimal labeling conditions on the function of lymphocytes and neutrophils. The optimal labeling conditions require that an excess of cells, suspended in a physiologic medium, be exposed to as low a concentration of ethanol and oxine as will achieve efficient cell labeling. The foregoing experiments indicated that the most practical formula for In-111 cell labeling was to mix 100 million cells suspended in 10 ml of Hanks' BSS with 250 μl of a mixture of In-111 oxine (5 μg) in 50 μl of ethanol and 200 μl of 0.15 mol/l NaCl. The mixture was incubated at 37°C for 10 min, then centrifuged at 400 g for 5 min and the cells resuspended in medium.

Generally about 30% of the radioactivity in 1 ml of ^{111}InCl$_3$ preparation can be chelated with 5 μg oxine, and 70.7% (s.e. = 2.0, n = 5) of this was incorporated into neutrophils and 57.7% (s.e. = 3.4, n =5) into lymphocytes.

The rate of oxygen consumption by 100 million neutrophils—measured in the chamber of a Clark oxygen electrode at 37°C after stimulation with IgG-coated latex particles§—was 0.211 (±0.018, n = 3) fmols/min per cell for control cells. This rate

was similar to that of cells that had just been labeled with In-111 oxine whose radioactivity had decayed almost completely (0.226 ± 0.019, n = 3), or with 48 μCi of In-111 oxine (0.227 ± 0.015, n = 3). Spontaneous and chemotactic migration under agarose was also similar in the three groups.

The effect of the labeling procedure and of In-111 radioactivity on lymphocyte functions was determined by comparing the function of unlabeled lymphocytes (100 million cells) with those labeled with 40 μCi of In-111 or with the same amount of indium (Cd) from which the radioactivity had decayed (<0.1 μCi).

The parameters of function of all three groups were similar 27 hr after labeling, but then there was a progressive deterioration in the function of cells labeled with the radioactive In-111 (Table 2). Although the rosetting characteristics of these cells were only marginally affected by 60 hr, [³H] thymidine incorporation was almost completely inhibited at 70 hr. The behavior of the cells labeled with the cold In-111 (Cd) was very similar to that of the unlabeled control cells.

The toxic radiation effect was further investigated by examining the function at 18 hr of 100 million lymphocytes labeled by the standard technique with either 150 μCi or with 400 μCi of radioactivity. Those cells labeled with 150 μCi demonstrated nor-
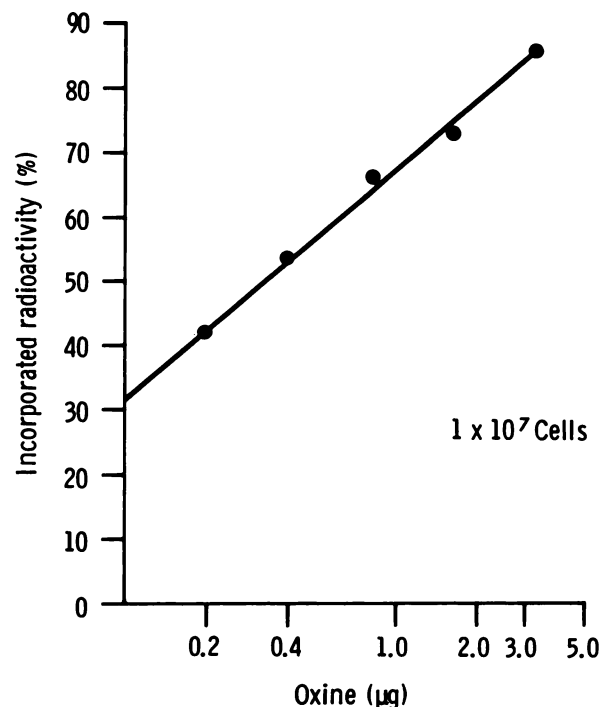
FIG. 4. Relationship between concentration of oxine (log scale) and percentage of radioactivity incorporated into a fixed number of neutrophils.

TABLE 2. EFFECT OF LABELING PROCEDURE AND RADIATION DOSE ON VARIOUS PARAMETERS OF LYMPHOCYTE FUNCTION*

Test	Cell preparation	Time (hr)				
		27	40	48	60	70
E rosettes	Control	76		78	63	
(%)	Cold In-111	86		53	68	
	Hot In-111	74		46	51	
Trypan blue	Control	2		9	20	
(% staining)	Cold In-111	4		10	22	
	Hot In-111	6		13	44	
[³H] thymidine incorporation	Control		70			110
(TCA insoluble	Cold In111		40			95
CPM × 10⁻³)	Hot In-111		40			15
Blast trans-formation	Control					64
(% of viable	Cold In-111					65
cells)	Hot In-111					42

* See text for experimental details.

mal E, EA, and EAC rosetting characteristics, and ADCC and spontaneous cytotoxicity of the target cells were normal. By contrast, the cells labeled with 400 μCi of radioactivity were damaged. E, EA, and EAC rosettes were 35, 24, and 43% of the control values, respectively, and ADCC and spontaneous cytotoxicity were completely abolished.

DISCUSSION

Indium-111 labeling of cells is a major advance, as the properties of this nuclide make it suitable for external scanning in man, while the fairly rapid rate of radiodecay ($T_{1/2} = 68$ hr) reduces the whole-body radiation hazard. Labeled neutrophils are valuable for the location of abscesses (2), and lymphocytes have been shown to accumulate in lymph nodes (5). This technique has also proved useful to label target cells in cytotoxicity assays because it firmly labels the cell and does not show the delayed spontaneous leakage from the cell that is observed with chromium, the tracer classically used for this purpose (14).

The labeling technique appeared to offer for the first time a method of directly measuring the kinetics and distribution of blood cells in man with an external detector. In order for these investigations of the movements of cells under physiologic or pathologic conditions to have any significance, it is essential to ensure that the labeling process does not itself grossly change the properties of the cells.

The initial investigations revealed that the cells were in fact damaged by the labeling process as a whole, and steps were then taken to identify the

toxic factors. The possible causes of the toxicity included direct chemical damage by the ethanol or oxine, the transport of metals—including indium and contaminating trace metals—into the cell by oxine, which is a nonspecific metal chelator (15), and the radioactivity. The first observation was that the amount of oxine used in the standard labeling procedure (100 μg) is toxic to neutrophils and lymphocytes. The toxicity of oxine was only systematically studied using the purest commercially available material obtained from a single source. Oxine obtained from another source was also shown to reduce neutrophil migration when used at an initial concentration of 100 μg per 100 million cells, which indicates that the toxicity of oxine is a general property of this compound rather than a batch-related phenomenon. Having investigated the factors governing the chelation of In-111 and cell labeling, we modified the labeling procedure, reducing the concentrations of ethanol and oxine by suspending the cells in a balanced salt solution rather than in saline. Neutrophils labeled with this procedure appeared to behave normally with regard to mobility and oxygen consumption. Lymphocytes, however, still showed evidence of damage and the cause of this was identified as the radiation emitted by the In-111. The reason for the different effect in neutrophils and lymphocytes may be that the lymphocytes can be maintained for longer times in vitro, and experiments can thus be more prolonged, but it is probably due to the differential sensitivity of the two cell types toward irradiation (16). Indium-111 appears to bind to DNA to some extent after entering the cell (4). It releases soft Auger electrons in addition to gamma photons (17), and the Auger electrons have considerable destructive potential, particularly when in intimate association with the DNA itself. An approximate average dose/cell in a typical labeling procedure for injection in a patient (1 mCi of In-111 per 100 million cells) can be calculated using the data in Table 3. A cell of radius 6 μm (mass $= 9 \times 10^{-10}$ g) will receive $0.0107/(9 \times 10^{-10}) = 1.19 \times 10^{7}$ rad/μCi-hr. Since the average radioactivity per cell is 10^{-5} μCi (10^8 μCi/10^8 cells), the dose rate per cell will be $1.19 \times 10^{7} \times 10^{-5} = 93$ rad/hr or about 1700 rads/day when the decay of In-111 is taken into account. This calculation is likely to be an overestimate since it assumes that all the In-111 is at or near the center of the cell. If, as is very likely, the In-111 is not centrally located, a more realistic figure would be 1000 rads/day, which is sufficient to cause extensive damage to lymphocytes (18,19). Although patient data on which to base whole-body and organ dose calculations is meagre, it has been estimated (5) that the doses from an injection of 1 mCi In-111-

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oxine-leukocytes are approximately 0.5 rads to the whole body, 3 rads to the liver and 5 rads to the spleen.

The use of neutrophils labeled with In-111 to locate abscesses is an established and clinically useful technique. These cells are resistant to radiation damage (20) and do not appear to be modified by the labeling technique, which should be useful for the investigation of their kinetics and distribution in health and disease. Lymphocytes, however, are damaged by the incorporated radioactivity. As it stands, this technique does not appear suitable for long-term studies on lymphocytes using external detection of radioactivity, where the administered radiation must of necessity be large enough to allow gamma imaging. Short-term studies may be feasible, particularly if large numbers of cells are employed so as to reduce the specific radioactivity of each cell. Unfortunately the mechanism of radiation damage to lymphocytes is unknown. Most of the radiation comes from Auger electrons with a very short range in the tissues (Table 3) and thus a change in the labeling procedure resulting in a different subcellular distribution of the In-111 could possibly reduce cell damage. The possibility of the induction of mutagenic change by irradiation of these long-lived cells, which have a proliferative potential, must also be considered. It is quite clear that irradiation can induce malignant mutation in lymphocytes (19). We cannot predict the likelihood of mutagenic changes in lymphocytes labeled with In-111. The data in this study suggest that if the lymphocytes are labeled with sufficient radioactivity to permit external detection, most of them will be killed and the danger of mutation thereby eliminated. However, it seems logical that if the labeling procedure is modified to reduce radiation damage to lymphocytes, their survival must be associated with a greater risk of mutagenesis.

The optimal conditions for the labeling of cells with In-111 oxine have been determined. The label-ing procedure itself does not appear to modify the parameters of neutrophil and lymphocyte integrity and function that were examined. Radiation from the In-111 damages lymphocytes, and measures to reduce this toxicity will have to be taken if observations made on cells labeled in this way are to be related to their normal physiology. The principle of the labeling technique—in which oxine chelates the indium and transports it through the plasma membrane of the cell, where it attaches to various binding substances—may possibly be applied to the labeling of cells with radioactive metals that have less damaging radiation and that are also chelated by oxine.

The radiotoxicity of radioindium may find clinical application as a means of differentially targeting radiation to organs such as the spleen and lymph nodes for the irradiation of diseased tissue. The high intracellular radiation from In-111 is a result of the emission of very soft electrons with a range of one cell diameter or less. Thus the damage to neighboring cells should not be severe. The indium isotope In-113m, however, emits energetic conversion electrons, which could be much more damaging to surrounding tissues. Indium-113m should be incorporated into cells in exactly the same way as In-111. If this method of tissue irradiation were to be attempted, cells could be labeled with a mixture of the two isotopes, the In-111 to enable the radiation source to be located and quantified, and the In-113m to administer damaging radiation. Starting with 35 mCi of In-113m from a 50-mCi generator, we incorporated 10 mCi in 10^9 lymphocytes. Assuming that if these cells were injected into a patient, 50% would go to the liver, 25% to the spleen, and 25% to the lymph nodes, the total doses to these organs would be 2, 11, and 3 rads, respectively (based on organ data and geometric factors given in references 21 and 22). Lymphoid cells are relatively radiosensitive (18,19) and it may be possible to damage these and other radiosensitive cells in these organs if some

TABLE 3. DATA USED IN THE DOSIMETRY CALCULATIONS

Auger electron	Mean energy (keV)[17]	Range in tissue (μm)*	Pi†	Di[17] g-rad/μCi-h	Pi Di
KLL	19.2	7.8	0.45	0.0045	0.00202
KLX	22.3	10.1	0.21	0.0021	0.00044
KXY	25.4	12.8	0.10	0.0003	0.00003
LMM	2.4	0.4	1.0	0.0052	0.00520
MXY	0.6	0.1	1.0	0.0030	0.00300
					0.01069

* Extrapolated from tables in reference 23.

† Ratio of cell volume (radius = 6 μm) to volume of sphere of radius equal to electron range.

50 mCi of In-113m were injected. Selective damage to cells in these situations could have important implications for immunosuppression and for therapy in malignant disease.

FOOTNOTES

* Ficollpaque, Pharmacia.
† Whatman.
‡ MEM Spinner Flow Labs.
§ 50 per cell, Orga, Behring.

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