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The Radiolabeling of Lymphocytes and Tumor Cells with ^{111}Indium[1] (39991)

PHILIP FROST, JULIAN SMITH, AND HEINER FROST

Departments of Medicine and Immunology, Wayne State University and Harper Hospital, Detroit, Michigan 48201

Introduction. The recirculation of lymphocytes from blood to lymph has been observed in all mammalian species investigated (1, 2). While the major pathways of lymphocyte circulatory patterns have been defined in mice, rats, and sheep, only preliminary data are available regarding lymphocyte recirculation in man (3–5). One reason for this difficulty has been the lack of a radioisotopic label useful in humans. Animal lymphocyte recirculation studies have relied extensively on radiolabeling of lymphocytes with [^{51}Cr]sodium chromate (^{51}Cr) (1, 6, 7). Unfortunately, this label has inherent deficiencies which make it less useful in man. ^{51}Cr is a low-energy γ photon emitter, only 9% of its energy being emitted as γ. This makes ^{51}Cr less suitable for external scanning by a γ camera or rectilinear scanner. Because of its relatively weak γ emissions, high doses of ^{51}Cr would have to be used in labeling lymphocytes for use in external scanning. The use of high-dose ^{51}Cr in association with its half-life of 27.8 days would expose subjects to unnecessarily high levels of irradiation (300 μCi of ^{51}Cr results in a spleen dose of 10 rads and a total body dose of 0.12 rads). In our hands, doses of ^{51}Cr in the range of 100 μCi/10^8 lymphocytes result in a loss of 40–50% of the cells. In addition, ^{51}Cr does not label lymphocytes uniformly and any migration studies may not truly reflect the circulatory properties of lymphocyte populations (6, 8). For these reasons, we investigated other radioisotopes as labels for lymphoid cells. Our choice of ^{111}indium is based on the work of McAfee and Thakur (9, 10), Thakur *et al.* (11), and Segal *et al.* (12), who labeled polymorphonuclear leukocytes with ^{111}indium for use in localizing abscesses.

Methods. Animals. Balb/c mice, 6–10 weeks of age, were purchased from a commercial supplier (Cumberland View Farms, Clinton, Tenn.). Male Lewis rats (150–250 g) were obtained from Microbiological Associates (Walkersville, Md.) and splenectomized at the time of thoracic duct cannulation.

Lymphocytes. Balb/c thymus was used as a source of T cells. B cells were obtained from the lymph node and spleen of Balb/c nude mice from our own colony. Rat thoracic duct lymphocytes were obtained by cannulation of the thoracic duct using the method of Bollman *et al.* (13). Macrophages were obtained from the peritoneum or spleen by the adherence of these cell suspensions to glass. One hundred million spleen or peritoneal exudate cells were placed in glass petri dishes in minimal essential medium (MEM) with 10% fetal calf serum (FCS) and allowed to incubate at 37° for 30 min. The supernatant was discarded and adherent cells were removed with a silicone rubber policeman. This procedure was repeated and macrophage purity was found to be >95% by morphologic criteria.

Radioisotopes. ^{111}Indium chloride (^{111}InCl) was obtained from Medi-Physics, Inc. (Emeryville, Calif.). It has a half-life of 67 hr and emits γ photons at 173 keV (89%) and 247 keV (93%). ^{51}Cr (as sodium chromate) was obtained from New England Nuclear (Boston, Mass.). It has a half-life of 27.8 days and emits a single γ photon at 320 keV (9%).

The preparation of ^{111}indium–oxine complex. The method of Thakur *et al.* was used (11). Briefly, 3 mCi of ^{111}InCl at pH 1–3 was added to an equal volume of sterile distilled water followed by 200 μl of 0.3 *M* acetate buffer, pH 5. One hundred and fifty micrograms of 8-hydroxyquinoline (oxine; Sigma Chemical Co., St. Louis, Mo.) was dissolved in absolute ethanol (Commercial Solvents Corporation, Terra Haute, Ind.) and added to the mixture. The tube

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was mixed well and allowed to stand at room temperature for 15 min. An equal volume of chloroform (Baker Chemical Company, Phillipsburg, N.J.) was then added. The solution was mixed and allowed to stand at room temperature for 10 min. The water layer was discarded and the chloroform evaporated in a boiling water bath. The remaining ^{111}indium-oxine chelate was dissolved in 100 μl of absolute ethanol and diluted to 500 μl with normal saline prior to use. In all preparations of the ^{111}indium-oxine complex, excess oxine was added to saturate any cadmium impurities. Under these conditions, approximately 75-85% of the original γ activity of the ^{111}InCl is extracted as the chelated ^{111}indium-oxine complex (^{111}InOx). This was determined by comparing counts per minute (cpm) of an aliquot of the original ^{111}InCl (from a known volume). By this means, we can determine the number of microcuries of ^{111}InOx added to any individual cell suspension.

Results. The effect of ^{111}indium-oxine dose. Figure 1 demonstrates that with the addition of increased amounts of ^{111}InOx to a constant number of cells in a standard volume, the number of counts per cell increases from 2.5×10^5 cpm/10^7 cells at 2.5 μCi to 2.5×10^6 cpm/10^7 cells at 10μCi. While the counts per minute per 10^7 cells increases steadily, it is not exponential and the difference in labeling efficiency at 50 and 100 μCi is increased by only 24% for the 100% increase in available label. The viability of the cells (as measured by trypan blue) remains relatively constant at >90%, indicating that the ^{111}InOx complex is relatively nontoxic. Labeling with ^{111}InOx that had passed through four half-lives yielded identical viabilities, reflecting the nontoxic nature of the decay product. Cell viability has also been confirmed by *in vivo* lymphocyte migration studies (see below).

Effect of cell concentration and ^{111}InOx dose on labeling efficiency. Efficiency of labeling with ^{111}InOx is related to two variables, the cell concentration and ^{111}InOx dose. Figure 1 describes the effect of changing the ^{111}InOx dose added to a standard cell concentration. If the ^{111}InOx dose is kept constant and the cell concentration is increased or decreased above or below 10^7 cells/ml, labeling efficiency per cell decreases. Though optimal labeling is achieved at 10^7/ml, our labeling procedure is standardized at a cell concentration of 5×10^7/ml for practical reasons. The difference in labeling per cell at this higher cell concentration is insignificant.

Effect of incubation time and temperature. Figure 2 demonstrates that the advantage gained in labeling efficiency per cell by longer incubation times is lost in viable cell recovery. Because of these observations, we have opted for a 15-min incubation time. Labeling at 22 or 37° produced almost identical labeling efficiencies and viable cell recoveries.

Effect of medium and fetal calf serum. Cells can be labeled in PBS, MEM, and tissue culture media 199 or RPMI-1640, providing 10% FCS is added as a media supplement. Media not containing FCS result in 30% less efficient labeling. RPMI-1640 with 10% FCS gave the most consistent and highest labeling efficiency. In 12 successive experiments, the addition of 10 μCi of ^{111}InOx to 5×10^7 lymph node lymphocytes in 0.5 ml of RPMI-1640 with 10% FCS and 20 m*M* Hepes resulted in $2.71 \times 10^6 \pm 0.5 \times 10^6$ cpm/10^7 cells. Similar results were obtained when labeling tumor cells or spleen cells in RPMI-1640.

Cell type Labeled. We have observed efficient labeling of adherent and nonadherent (>95% macrophages) spleen cells, thymic and lymph node cells from normal mice, and lymph node cells from nude Balb/c mice, as well as lymphocytes from the thoracic duct of rats. DBA/2 mastocytoma cells and human cell lines [EB-33, H494, 14975 (prostate); malme and miwa (melanoma)] are also efficiently labeled and release ^{111}InOx in cytotoxicity assays (14) (Wiltrout and Frost, unpublished).

Spontaneous release. Table I summarizes the spontaneous release of ^{111}InOx and ^{51}Cr from lymphocytes and DBA/2 mastocytoma cells incubated in culture for 24 hr. While the spontaneous release of ^{111}InOx from lymphocytes is more gradual than that observed with ^{51}Cr, the total release is comparable at 24 hr. However, this is not true with mastocytoma cells where the spontaneous release of ^{111}InOx is at least fourfold

less than that of ^{51}Cr at 24 hr. The reason why mastocytoma cells should release ^{111}InOx at a much slower rate than lymphocytes is currently unclear. Similar low spontaneous release has been observed with human tumor lines (14).

Reutilization. The lysis of ^{111}InOx-labeled DBA/2 mastocytoma cells by cell rupture (15) results in the release of isotope in an unknown form. The addition of released isotope to unlabeled DBA/2 mastocytoma cells or lymphocytes results in less than 1% being taken up by these cells. This implies that once the ^{111}InOx complex is taken up by cells, it is altered sufficiently so as to render it nonreutilizable.

TABLE I. PERCENTAGE SPONTANEOUS RELEASE OF ^{51}Cr AND ^{111}In FOR LYMPHOCYTES AND TUMOR CELLS.[a]

Time (hr)	Lymph node lymphocytes		DBA/2 mastocytoma cells	
	^{51}Cr	^{111}InOx	^{51}Cr	^{111}InOx
2	13.5	7.5	5.3	1.3
4	21.6	13.6	9.5	2.2
6	32.7	17.2	12.6	2.6
24	67.2	59.8	24.8	6.3

[a] Five × 10^7 lymphocytes or DBA/2 mastocytoma cells were labeled with either ^{51}Cr or ^{111}InOx and placed in culture. Culture samples were assessed for spontaneous release of label at varying times thereafter.

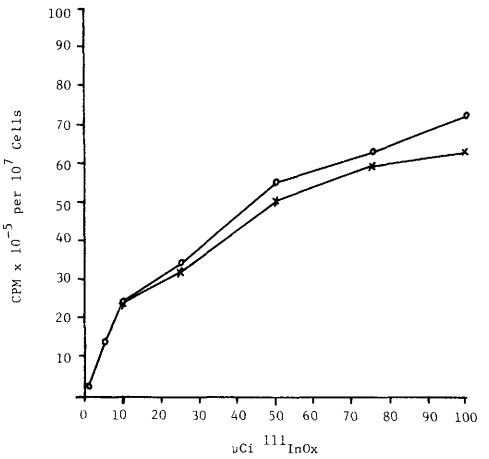

FIG. 1. The effect of ^{111}InOx dose on lymphocyte labeling. Increasing amounts of ^{111}InOx were added to 5 × 10^7 lymphocytes in 0.5 ml of RPMI with 10% FCS and 20 m*M* Hepes buffer. The cells were incubated for 15 min at 37°. O——O, Expt. 1; ×——×, Expt. 2.

Membrane vs cytoplasmic labeling. To demonstrate whether ^{111}InOx labeled membrane or cytoplasm, labeled cells were dispersed in a cell rupturing pump (15). The cellular constituents were separated by differential centrifugation (15, 16) and less than 10% of the label was found to be associated with membrane. In addition, attempts at labeling purified membrane fragments resulted in poor labeling efficiency.

Comparison of ^{111}indium-oxine and ^{51}Cr as lymphocyte labels. Table II summarizes the labeling efficiency of ^{51}Cr and ^{111}InOx on lymph node, spleen, and thymic cells, utilizing threefold more ^{51}Cr than ^{111}InOx (30 μCi of ^{51}Cr as sodium chromate and 10 μCi of ^{111}InOx). The increase in labeling efficiency with ^{111}InOx is 47-fold, 65-fold,

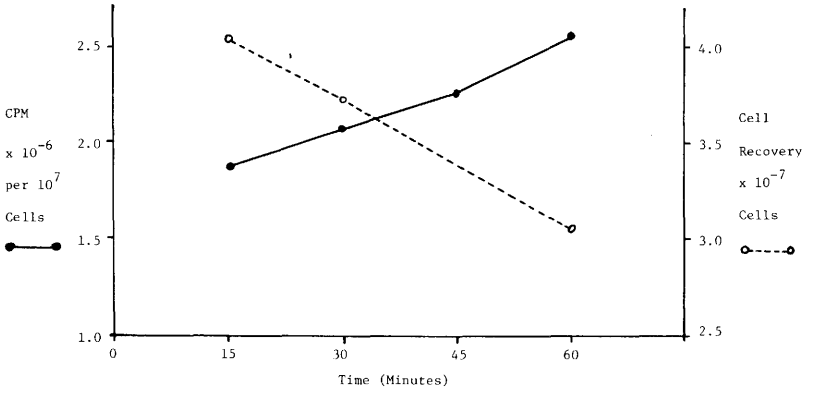

FIG. 2. The effect of incubation time on labeling efficiency. Ten microcuries of ^{111}InOx was added to 5 × 10^7 lymphocytes in 0.5 ml of RPMI with 10% FCS. The mixture was allowed to incubate for 15, 30, 45, or 60 min.

and 13.3-fold with lymph node cells, spleen cells, and thymic cells, respectively.

The recirculation of ^{111}indium-oxine lymphocytes in the rat. ^{111}InOx-labeled rat thoracic duct lymphocytes (TDL) recirculate from blood to lymph and distribute to organs in a manner almost identical to that seen with TDL labeled with other isotopes (Table III). In these experiments, cells were labeled with 20 μCi of ^{111}InOx, a dose sufficient for detection by nuclear scanning (17). Doses of ^{111}InOx in excess of 100 μCi/10^8 result in a normal initial organ distribution (useful in scanning) but diminished recirculation of the cells.

Discussion. Optimal conditions for labeling lymphocytes, macrophages, and tumor cells with ^{111}InOx have been outlined. The ^{111}InOx primarily labels cell cytoplasmic components, probably by passive diffusion of the highly lipophilic metal oxine complex (11). The spontaneous release of ^{111}InOx for cells is low and the label released is nonreutilizable.

^{111}InOx offers several advantages over other labels in studies of lymphocyte recirculation and tumor cell cytotoxicity. It has a short half-life (67 hr), far shorter than that of ^{51}Cr (27.8 days) and longer that that of ^{99}technetium (6 hr). Because it emits two γ photons, it can be detected by a γ camera and rectilinear scanner (17). A variety of cells are labeled to high specific activities with minimal loss in viability, and finally, labeled cells show significantly lower spontaneous release of label when compared with ^{51}Cr.

These observations, taken together with evidence that ^{111}InOx-labeled lymphocytes recirculate in rats and distribute to lymphoid organs normally, make it of potential value in the study of lymphocyte recirculation in man. The low spontaneous release from human and animal tumor cells may provide the long-term isotope release assay needed in the study of cytotoxicity to human tumors.

TABLE II. COMPARISON OF ^{51}Cr AND ^{111}InOx AS LYMPHOCYTE LABELS.[a]

	Counts per minute per 10^7 cells		Ratio of ^{111}InOx to ^{51}Cr
	^{51}Cr	^{111}InOx	
Lymph node cells	5.8×10^4	275×10^4	47
Spleen cells	6.9×10^4	451×10^4	65
Thymocytes	13×10^4	173×10^4	13.3

[a] Five $\times$ 10^7 lymph node, spleen, or thymic lymphocytes were labeled with 30 μCi of ^{51}Cr or 10 μCi of ^{111}InOx and the cpm/10^7 cells were assessed after incubation at 37° for 15 min in RPMI and 10% FCS.

Summary. ^{111}Indium when complexed

TABLE III. RECIRCULATION AND ORGAN DISTRIBUTION OF ^{111}InOx-LABELED RAT THORACIC DUCT LYMPHOCYTES.[a]

(a) Recirculation				(b) Organ distribution (30 hr)	
Time (hr)	Volume of lymph (ml)	CFL[b]	TDL[c]		
0–6	16 ± 5	0.58 ± 0.14	1.99 ± 1.3	Lung	0.58 ± 0.08
6–12	25 ± 7	1.14 ± 0.46	6.03 ± 2.6	Liver	17.56 ± 3.66
12–18	16 ± 3	0.35 ± 0.40	6.00 ± 1.63	Thymus	0.84 ± 0.38
18–24	24 ± 5	0.31 ± 0.13	5.11 ± 0.56	Small bowel	7.84 ± 2.50
24–30	23 ± 4	0.43 ± 0.10	3.38 ± 0.37	Femur	0.90 ± 0.28
				Kidney	0.64 ± 0.09
				Testes	0.04 ± 0.01
				Peripheral lymph nodes	4.89 ± 0.82
				Mesenteric lymph nodes	6.08 ± 1.82
				Whole blood, 1 ml	0.77 ± 0.68
				Total recovery (in thoracic duct lymph and sampled organs)	65.39 ± 6.56

[a] Mean ± SD (expressed as percentage of counts injected) of recovered ^{111}InOx activity from four adult splenectomized rats. (a) Time course of appearance of recirculating cells in the thoracic duct. (b) Organ distribution of labeled cells at 30 hr after intravenous injection. Cells were labeled with 20 μCi of ^{111}InOx/10^8 cells.

[b] Thoracic duct lymphocytes.

[c] Cell-free lymph.

with oxine is an efficient label of lymphocytes and tumor cells. ^{111}Indium–oxine complex (^{111}InOx) is not reutilizable, making it useful for short-term lymphocyte recirculation studies. The spontaneous release of ^{111}InOx from tumor cells is very low, making it a useful isotope in cytotoxicity studies. ^{111}InOx emits two γ photons detectable by nuclear scanning, making this label of potential use in the study of lymphocyte migration in man.

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