PHYSICS AND RADIATION BIOLOGY

Labeling with Indium-111 has Detrimental Effects on Human Lymphocytes: Concise Communication

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When lymphocytes from human peripheral blood were labeled with In-111 oxinate, several of their properties appeared to be affected. The spontaneous release of the radionuclide was found to be relatively high. Labeled lymphocytes showed a decreased proliferative capacity, dependent on the dose of the label. Cytogenetic studies revealed that In-111 oxinate induces severe chromosomal aberrations. These results emphasize the need for great caution in the use of the In-111 label for studies on lymphocyte traffic in humans.

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The recirculation of lymphocytes in humans can be studied by labeling these cells with radionuclides. However, the label must meet several requirements. First, the spontaneous release of the label should be low. Second, the label should not affect functional activities of the cells. Third, it should not induce cytogenetic aberrations, because most lymphocytes are long-living cells that, even after years, may undergo malignant transformation.

Recently, several investigators have advocated the use of the radionuclide indium-111 (used as indium-111 oxinate) for the labeling of lymphocytes, to study their homing and recirculation (*1–4*). In fact, this method has already been applied in man (*5–9*). Some reports in the literature show that radiation-induced damage may occur after indium-111 labeling of Hela-S3 cells or murine bone-marrow cells, as judged by their colony-forming ability (*10–12*). Also lymphocytes from rat (*11*) and guinea-pig (*13*), labeled with In-111, are affected in their capacity to recirculate or to migrate into an inflammatory site. However, only few data are available on the functional properties of In-111-labeled human lymphocytes (*14*).

In the present study, human lymphocytes were labeled with In-111 oxinate and the spontaneous release of this label was tested. Moreover, the effects of labeling both on the proliferative capacity of lymphocytes in vitro and on the structure of their chromosomes were studied.

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MATERIALS AND METHODS

Isolation and characterization of human lymphocytes. Freshly drawn blood from healthy volunteers was defibrinated and diluted 1:1 with Earle's balanced salt solution. Lymphocytes were isolated by Ficoll-Isopaque density-gradient centrifugation (*15*). The cell suspensions contained about 80% of lymphocytes, about 15% of monocytes, and 2% of granulocytes. Contamination with platelets was always minimal. Viability, as measured by trypan-blue exclusion, was always greater than 95%. The percentage of T lymphocytes was determined by rosette formation with sheep erythrocytes (*16*). Lymphocyte suspensions, enriched or depleted for T cells, were prepared by E-rosette sedimentation (*17*).

Labeling of lymphocytes. The labeling procedure by Frost et al. (*4*) was followed, with minor modifications. Briefly, all cell suspensions were washed three times,

TABLE 1. SPONTANEOUS RELEASE, MEASURED UNDER DIFFERENT CONDITIONS

Spontaneous release (%)	t = 1	t = 24	t = 48 hr	Effect of extra incubation period after labeling† t = 1	t = 24	t = 48 hr
Measured in*						
RPMI 20% HS	3.1 ± 2.6‡	24.7 ± 5.9	45.1 ± 3.4	2.9 ± 0.8	22.9 ± 1.9	45.2 ± 8.2
RPMI 10% HS	2.7 ± 1.0	23.4 ± 1.9	43.3 ± 4.0	3.1 ± 1.3	22.9 ± 1.2	45.8 ± 3.4
RPMI 5% HS	3.4 ± 0.7	26.0 ± 3.0	46.4 ± 4.1	3.2 ± 1.9	27.5 ± 1.7	52.8 ± 8.9
Viability	99 ± 1.9	92 ± 8.2	92 ± 3.8			

* After labeling, cells were washed 3 times and resuspended in RPMI supplemented with different concentrations of human serum.

† After labeling and 3 washings, cells were incubated for 30 min at 37°C, washed twice, then incubated in microtiter plates to measure spontaneous release.

‡ Values represent the mean percentage of release from 8 donors ± s.d.; 10^7 lymphocytes were labeled with 3 μCi In-111 oxinate.

pelleted and resuspended in 300 μl serum-free medium RPMI-1640. In our hands, the labeling was more efficient under serum-free conditions. Labeling was performed with the In-111-oxinate complex. At activity reference time, the specific activity was 1 mCi per 25 μg 8-hydroxyquinoline (oxine). The cells were incubated with varying doses of In-111 oxinate, ranging from 2 to 20 μCi/10^7 lymphocytes, for 30 min at room temperature. In control experiments, decayed label was used to determine the effect of oxinate itself. After three washings, the cells were resuspended in RPMI containing 10% human serum, then counted and resuspended at the required concentrations.

Properties of lymphocytes labeled with In-111 oxinate. After the labeling, several parameters were measured.

1. Incorporation of In-111 per 40,000 lymphocytes in a volume of 100 μl was measured, with a gamma counter, and expressed as counts per minute (cpm).

2. At several time points after the labeling procedure, viability of the cells was determined by trypan-blue exclusion.

3. Spontaneous release of In-111 label was determined by incubating 40,000 lymphocytes, in a volume of 100 μl, in round-bottom microtiter plates with 100 μl of RPMI-1640 containing 10% human serum. The plates were incubated for varying times at 37°C under humidified air containing 5% CO_2. After the incubation, the plates were centrifuged for 10 min at 400 *g*. From the final volume of 200 μl in each well, 100 μl was removed to measure the radioactivity. Five replicate measurements were always performed. The percentage of release was calculated as follows:

$$\frac{\text{cpm in supernatant} \times 2}{\text{total cpm incorporated}} \times 100.$$

The total cpm were always measured in parallel to correct for decay of the radionuclide.

4. Studies on incorporation of tritiated thymidine by lymphocyte cultures of unlabeled cells, by cells labeled with varying doses of In-111 oxinate, and by cells labeled with decayed In-111 oxinate were performed as previously described (*18*). The incorporation of H-3 thymidine was measured after a 24-hr pulse (specific activity 200 mCi/mmole). The total H-3 thymidine counts (at 10 days) were corrected by subtraction of In-111 crossover, which averaged about 5% of the counts present.

5. Cytogenetic studies. Unlabeled lymphocytes, lymphocytes labeled with varying doses of In-111 oxinate, and lymphocytes labeled with decayed In-111 oxinate were grown at 37°C in Hams F-10 medium supplemented with 15% fetal bovine serum. One ml of medium contained 33 IU heparin, 1.7 millimole glutamin, 80 IU penicillin, 80 μg streptomycin, and 32 μl phytohemagglutinin-M reconstituted in 5 ml medium. The lymphocytes were harvested for cytological studies at 48 and 72 hr following initiation. The cells were blocked at mitosis by colcemid for 2 hr and further subjected to hypotonic shock (0.56% NaCl) before fixation in acetic-acid methanol. Standard air-dried preparations were made. The slides were stained with aqueous Giemsa solution and, wherever possible, 100 well-spread metaphases were scored for the presence of chromosomal aberrations.

RESULTS

When 10^7 lymphocytes were incubated with In-111 oxinate at doses ranging from 2 to 20 μCi, the incorporated radioactivity was linearly dependent on the added dose. Up to 48 hr after labeling, cell viability remained higher than 90%. Initial studies revealed a spontaneous release of In-111 oxinate from human lymphocytes, which was of the same magnitude as that of chromate (Cr-51), i.e., about 25% at 24 hr after labeling. Because

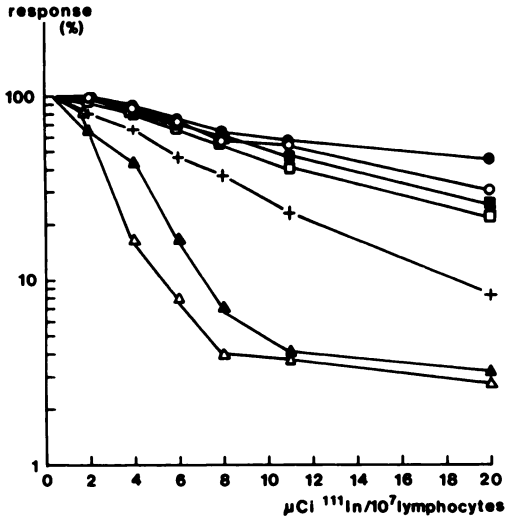

Fig. 1. Relative responses of lymphocytes to several stimuli after labeling with varying concentrations of In-111 oxinate, plotted semilog. Incorporation of H-3 thymidine by unlabeled cells is expressed as 100%. Absolute values of unlabeled cells (cpm × 10^{-3}): antilymphocyte serum (●), 20.7 ± 3.2; phytohemagglutinin (■), 12.7 ± 2.5; concanavalin A (○), 10.1 ± 3.0; pokeweed mitogen (□), 9.1 ± 3.7; antigen cocktail (a mixture of PPD, varidase, mumps, trichophyton, and candida) (△), 4.1 ± 2.8; tetanus toxoid (▲), 7.1 ± 4.9; mixed lymphocyte culture responder capacity +, 7.9 ± 1.6. All values represent the mean ± s.d. of eight experiments.

a low spontaneous release is allegedly one of the main advantages of In-111 in comparison with Cr-51, several experiments were performed to investigate whether a lower spontaneous release might be achieved. Table 1 shows that several modifications in experimental conditions—namely, an additional incubation period followed by further washings to remove any adherent label, and the use of different concentrations of human serum in the culture medium—did not result in a lower spontaneous release. Variation of the number of cells from 5 million to 40 million also did not affect the spontaneous release. The values measured at 24 hr after labeling were consistently about 25%. Finally, a series of experiments in which cells from 50 individuals were labeled and tested, gave largely identical values for the spontaneous release.

To assess the effect of labeling with In-111 oxinate on the proliferative capacity of lymphocytes, dose-response curves were constructed. It is evident from Fig. 1 that the proliferative capacity of lymphocytes in response to several stimuli is affected, although to different extents, depending on the stimulus used. Control experiments revealed that decayed In-111 oxinate did not affect the proliferative capacity of lymphocytes when tested in amounts of about 0.10 to 2 μg per 10^7 lymphocytes, which is in the same order of magnitude as the quantity of oxinate present during labeling. Even when the cells were labeled with In-111 oxinate up to 4 μCi/10^7 cells, the proliferative capacity was diminished, most clearly after stimulation with microbial antigens. Because in our lymphocyte suspensions a low percentage of monocytes is always present, and because the presence of monocytes in the cultures is required for an optimal response of lymphocytes,it could be argued that radiation-induced damage to the monocytes might be responsible for the diminished proliferative capacity of the lymphocytes.

To investigate possible damage to the monocytes, the lymphocyte suspensions of four individuals were separated by E-rosette sedimentation into T and non-T fractions, the latter being enriched for monocytes. The influence of the In-111 label on each of the cell fractions was studied in co-culture experiments. To prevent proliferation in the non-T fraction, these cells were irradiated (2000 rad) before initiation of the cultures. Table 2 shows the results of one such experiment, and it clearly demonstrates that separately labeled T cells themselves showed a depressed proliferative capacity after stimulation with a variety of mitogens and antigens. When unlabeled T cells were co-cultured with In-111-labeled non-T cells, their proliferative responses to mitogens and alloantigens were moderately affected, and their proliferative response to soluble antigens was severely depressed. The results in three other experiments were virtually identical.

Chromosome preparations of the In-111-labeled cells revealed several aberrations: gaps, breaks, and exchanges. In contrast, no effect was detected when lymphocytes were labeled with decayed In-111 oxinate. Most of the aberrations were of chromosome type, indicating that they were induced in the G_0 or G_1 stage of the cell cycle. The frequency of chromosomal aberrations was dependent on the labeling dose. High doses (>8 μCi/10^7 lymphocytes) resulted in a lower proportion of cells in division (see Table 3). Note that one of the donors tested displayed an increased frequency of spontaneously occurring chromosomal aberrations in the lymphocytes. In fact, there was virtually no difference between the two donors regarding the extent of chromosomal aberration induced by labeling with In-111 oxinate.

DISCUSSION

Although a low spontaneous release is generally claimed to be one of the advantages of cell labeling with the nuclide In-111, relative to labeling with Cr-51 (*2*), we found that the spontaneous release is similar for the two nuclides. We were unable to achieve a lower spontaneous release by varying the experimental conditions. However, studying other cells, such as murine P-815 mastocytoma cells and human K-562 cells, the spontaneous release of In-111 label was indeed much lower than that of Cr-51 (data not shown). The reason for this difference, which has also been observed by others (*4*,*19*), is not clear.

According to Frost et al. (*4*), the released In-111

TABLE 2. LYMPHOCYTE CULTURES OF MIXTURES OF T AND NON-T CELLS

	40,000 T cells + 20,000 (non-T cells)$_R$[a]	40,000 T cells*[b] + 20,000 (non-T cells)$_R$	40,000 T cells + 20,000 (non-T cells*)$_R$
PHA	14,3[c]	2,6	7,3
ALS	22,2	4,1	11,1
PWM	11,3	0,3	10,2
ConA	11,2	0,3	5,7
MLC responder capacity	12,6	0,5	10,1
Antigen cocktail	3,3	0,2	0,5
Tetanus toxoid	11,0	0,2	0,2

[a] Non-T cells were irradiated with 2000 rad.

[b] * Indicates labeling with 20 μCi In-111/10^7 cells.

[c] Values represent H-3 thymidine incorporation (cpm $\times 10^{-3}$) measured during last 24 hr of culture either at day 3 (PHA, ALS, ConA) or at day 6 (PWM, MLC, antigens) after initiation of the cultures.

Statistical analysis: all values in Column 2 were significantly different ($p < 0.001$) from those in Column 1; values in Column 3 were significantly different from those in Column 1, except for PWM and MLC (PHA, ALS, ConA $p < 0.05$; antigen cocktail and tetanus toxoid $p < 0.001$; Wilcoxon's signed rank test).

cannot be re-utilized by lymphocytes. It is assumed that free In-111 is preferentially bound to transferrin present in the plasma (*1*); moreover, recent studies in rats revealed that the label is probably transferred to nonrecirculating cells, presumably macrophages (*20*). Thus, in interpreting data from in vivo studies on lymphocyte migration, one should be aware of this rather high spontaneous release of the In-111 label. Indeed, after infusion of In-111-labeled lymphocytes, detected radioactivity is not necessarily associated with lymphocytes.

Labeling with In-111 oxinate leads to a dose-dependent decrease of the proliferative capacity of human lymphocytes, apparently by a direct radiation-induced effect on the T cells themselves. Up to a labeling dose of 2 μCi/10^7 lymphocytes, the proliferative responses are only minimally depressed. However, in the range of 2 to 4 μCi/10^7 lymphocytes, doses that are used for studies in man, the effect is more pronounced. The proliferative responses to the various stimuli are affected to a different extent. This may well be a reflection of the different susceptibility of several subpopulations of T lymphocytes to the effect of irradiation (*21*). In addition, the particularly strong decrease of the response to microbial antigens may be due to an additional radiation-induced damage of the monocytes, for it is well known that the presence of monocytes is required for optimal proliferation of lymphocytes in vitro—at least in response to

TABLE 3. FREQUENCIES OF CHROMOSOMAL ABERRATIONS IN HUMAN LYMPHOCYTES LABELED WITH In-111 OXINATE (RESULTS FROM TWO INDIVIDUALS ARE SHOWN)

	Number of cells analyzed	Percentage of abnormal cells	Aberrations/100 cells				
			Chromatid aberrations			Chromosome aberrations	
			gaps	breaks	exchanges	breaks	dicentrics + rings
Donor A							
0 μCi	100	3	0	2	1	0	0
3 μCi	100	54	4	49	1	33	11
9 μCi	100	90	16	53	9	158	46
15 μCi	73	93	25	86	14	275	90
30 μCi	8	100	63	125	0	413	88
Donor B							
0 μCi	100	14	5	6	0	4	0
2 μCi	100	15	5	6	0	8	3
8 μCi	71	92	17	35	0	155	59
15 μCi	46	94	13	24	0	210	52
30 μCi	61	100	13	128	5	410	157

soluble microbial antigens. The latter possibility is supported by data obtained from the cultures in which unlabeled T cells were mixed with autologous In-111-labeled non-T cells. However, we cannot exclude radiation damage of the unlabeled T cells caused by neighboring labeled non-T cells.

Finally, cytogenetic analysis revealed that In-111 oxinate is an effective inducer of chromosomal aberrations. After labeling with 9 μCi/10^7 cells, lymphocytes with damaged chromosomes were observed at a frequency similar to that observed after 200 to 250 rad of x rays (*22*). Although most of the aberrations were of chromosome type, some 20% appeared as chromatid aberrations, indicating that the radioactivity persists and is capable of inducing lesions even in S and G_2 stages of the cell cycle.

In the extensive data in the literature, there is no indication of a threshold level existing for radiation-induced chromosomal aberrations. Thus, the induction of already high frequencies of chromosomal aberrations by a labeling dose of 2 to 3 μCi In-111 oxinate points out that the doses used for routine labeling of lymphocytes do indeed have a significant radiobiological effect. Because a high labeling dose (15 μCi/10^7 lymphocytes) induces chromosomal aberrations in 93% of the cells, and at the same time their proliferative capacity is decreased by 50%, it appears that lymphocytes with damaged chromosomes are still able to proliferate.

These findings pose the question whether labeling with In-111 oxinate can be safely used in vivo to monitor homing and recirculation of lymphocytes. It is well known that ionizing radiation has mutagenic and carcinogenic properties (*23,24*). Thus, one could envisage a possibility that, by infusing In-111-labeled lymphocytes, a transformed cell is introduced that, after proliferation, may cause a malignant process. Moreover, similar hazards may occur when, for the detection of abcesses in patients, In-111-labeled granulocyte suspensions are infused, because such suspensions may still contain up to 20% of lymphocytes. Further studies are required to establish the viability and oncogenic potential of the lymphocytes contaminating such cell suspensions.

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