Technetium-99m-Labeled Lymphocytes: A Radiotoxicity Study

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Radiolabeled white blood cells offer a unique clinical diagnostic technique. However, radiotoxicity to these radiosensitive cells must be carefully considered. Indium-111 has been shown to produce significant chromosomal alteration even at subscintigraphic doses. It has been suggested that ^{99m}Tc is less radiotoxic and may be desirable even though labeling is less efficient. The radiotoxicity of bound ^{99m}Tc was investigated in human lymphocytes by determination of division delay and chromosomal aberration as a function of increasing ^{99m}Tc activity concentration. This data revealed that human lymphocytes demonstrate significant division delay above 0.580 mCi/10^7 cells with chromosomal damage equivalent to 1 Gy of 250 keV x-ray at this level.

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Various techniques for the labeling of leukocytes with technetium-99m (^{99m}Tc) have been reported (*1–3*) and in at least one publication the migratory pattern of ^{99m}Tc-labeled cells has been described in humans (*4*). Although most of these techniques have suffered from extremely poor labeling efficiency, it has been reported that nucleated cells, including lymphocytes, labeled with ^{99m}Tc do not demonstrate significant alteration in DNA or protein synthesis (*1*). A recent report has claimed improved ^{99m}Tc labeling efficiency using a modified stannous chloride labeling technique (*5*). As lymphocytes are a relatively radiation sensitive population, these results encourage an investigation of the utilization of ^{99m}Tc as a label for these cells. Although previous reports have suggested that the ^{99m}Tc label is less radiotoxic to nucleated cells than indium-111 (^{111}In), quantitation of the degree of damage associated with the ^{99m}Tc label has not been established. In this study we attempted to delineate the alteration in genetic material and related cellular function associated with increasing bound activity concentrations. These alterations were measured by cell division delay and chromosomal damage in lymphocytes labeled with ^{99m}Tc.

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MATERIALS AND METHODS

Lymphocyte Isolation

Human lymphocytes were aseptically isolated from 60 ml of heparinized blood by the Ficoll-Hypaque gradient technique. A modified procedure of Farid et al. (*5*) was employed in a laminar flow hood using sterile, pyrogen-free glassware and solutions. Heparinized blood was divided into four aliquots and mixed with an equal volume of 0.1*M* phosphate buffer (pH 7.4) containing 0.85% sodium chloride, in a 50-ml conical centrifuge tube. Fifteen milliliters of Ficoll-Hypaque solution were delivered to the bottom of each tube by syringe fitted with a 0.22-μ membrane filter and 16G cannula. Tubes were centrifuged at room temperature for 25 min at 400 *g*. The lymphocytes, present at the interface of the first and second layers, were aspirated using a syringe fitted with a 16G cannula. Lymphocyte suspensions were combined in a 50-ml conical tube and the cell population was measured on a Coulter counter. Lymphocyte yield was $6.13 \pm 1.69 \times 10^7$ (n = 14).

The cell suspension was centrifuged at room temperature for 15 min at 400 g and the supernate removed. The cell pellet was washed with 5 ml of normal saline, centrifuged, and the supernate removed.

Lymphocyte Labeling

The cell pellet was resuspended and incubated for 15

min at 37°C with 3.0 ml saline containing 6.0 mg sodium pyrophosphate and 1.7 mg stannous chloride. Following centrifugation, the supernate was removed, the cells washed in 3.0 ml saline, recentrifuged, and the supernate discarded.

The pretinned cell pellet was resuspended and incubated for 10 min at room temperature with the required ^{99m}Tc activity in 1.0 ml saline. The suspension was centrifuged and the supernate (A) retained. Following a 3.0-ml saline rinse, the cells were centrifuged and supernate (B) retained.

Labeling efficiency was calculated by the following formula:

$$\frac{\text{cell activity}}{\text{cell activity} + (A) + (B)} \times 100.$$

Tissue Culture

All cultures were initiated with 10^7 lymphocytes in a 25-ml Falcon culture flask. Following stimulation with PHA, the cells were grown by incubation at 37.5°C and with 5% CO_2 in McCoy's 5A media supplemented with 15% fetal calf serum.

Division Delay

Radiation-induced division delay in the lymphocyte population was determined in parallel studies.

1. *Mitotic index.* Mitotic figures/1,000 cells were counted at various harvest times following the initiation of the culture.

2. *Harlequin staining.* Sister chromatid differential staining, which allows determination of division status of the mitotic figure, i.e., first, second, or third division, following culture initiation was performed. This technique, which was first described by Latt (*6*) and later modified by Perry and Wolf (*7*) consists of initiating the culture in flasks containing 5-bromodeoxy-uridine (BrdU) at a final concentration of 2.5 μg/ml and allowing incubation in the dark or exposed only to 60W yellow "bug lights." Following harvest and slide preparation, slides were stained (*8*) with fresh Hoechst 33,258 at 10^{-4} *M* in distilled water for 15 min and then rinsed. Cover slips were mounted on McIlvains' buffer at pH 8.0 and sealed. Slide preparations were then exposed to black light at a distance of 5 cm for 15 min at 55°C. The slides were rinsed and stained with 2% giemsa stain in 0.01 phosphate buffer at pH 6.8 for 15 to 20 min. In this technique, chromatids with BrdU substituted in one DNA strand stain dark, while substituted in both strands stain light, making it possible to determine the number of divisions BrdU have been incorporated into the DNA (Fig. 1).

Chromosomal Damage

The presence and degree of chromosomal damage were determined by counting micronuclei (MN). When pieces of chromosomal material are broken or deleted from the main body of the chromosome by radiation events, they are no longer associated with the kinetochore and, therefore, are no longer associated with the division apparatus. When a cell subsequently divides, these fragments are usually included in the cell, but not in the main nucleus. As a result, they form small regular nuclei within the cell (Fig. 2) which may be easily counted and usually expressed as MN/1,000 dividing cells. These data provide good estimates of the quantity of damage that has occurred prior to the previous division.

RESULTS

Utilizing the described stannous chloride labeling technique, ten experiments demonstrated a labeling efficiency of 57.78 ± 4.9%. A preliminary series of experiments was carried out to determine the highest feasible concentration of bound ^{99m}Tc on PHA-stimulated lymphocytes. Cells were harvested at 72 hr, following 6 hr incubation in the presence of colcemid to collect metaphases. The results, detailed in Table 1, demonstrate that activities above 0.580 $\mu Ci/10^7$ cells produced marked impairment of division rate and, therefore, yielded too few divisions to quantitate damage by MN assay or to accurately measure radiation-induced division delay by mitotic index and Harlequin staining. For this reason, 0.580 $\mu Ci/10^7$ cells was the highest activity level utilized in subsequent experiments.

The effect of increasing ^{99m}Tc concentrations on the mitotic index is presented in Table 1 and Figs. 3 and 4. Figure 3 shows the effect of increasing ^{99m}Tc doses on cells grown for 72 hr. Figure 4 shows the effect of each of three doses at three different time points. Three

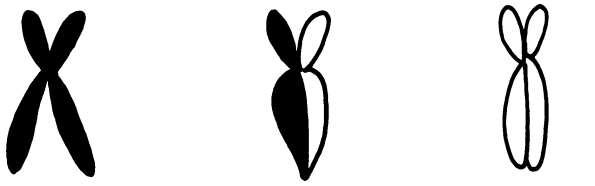

FIGURE 1
Diagrammatic representation of Harlequin staining produced by lack of stain in chromatids that have incorporated BrdU in both DNA strands. When DNA is allowed to synthesize in presence of BrdU, first division chromatids incorporate BrdU in only one of two strands and both strain normally. In second division, one chromatid will incorporate BrdU in both strands, other chromatid will incorporate BrdU in only one strand. This results in Harlequin effect. In third division, both chromatids incorporate BrdU in both strands. There is lack of stain in both chromatids

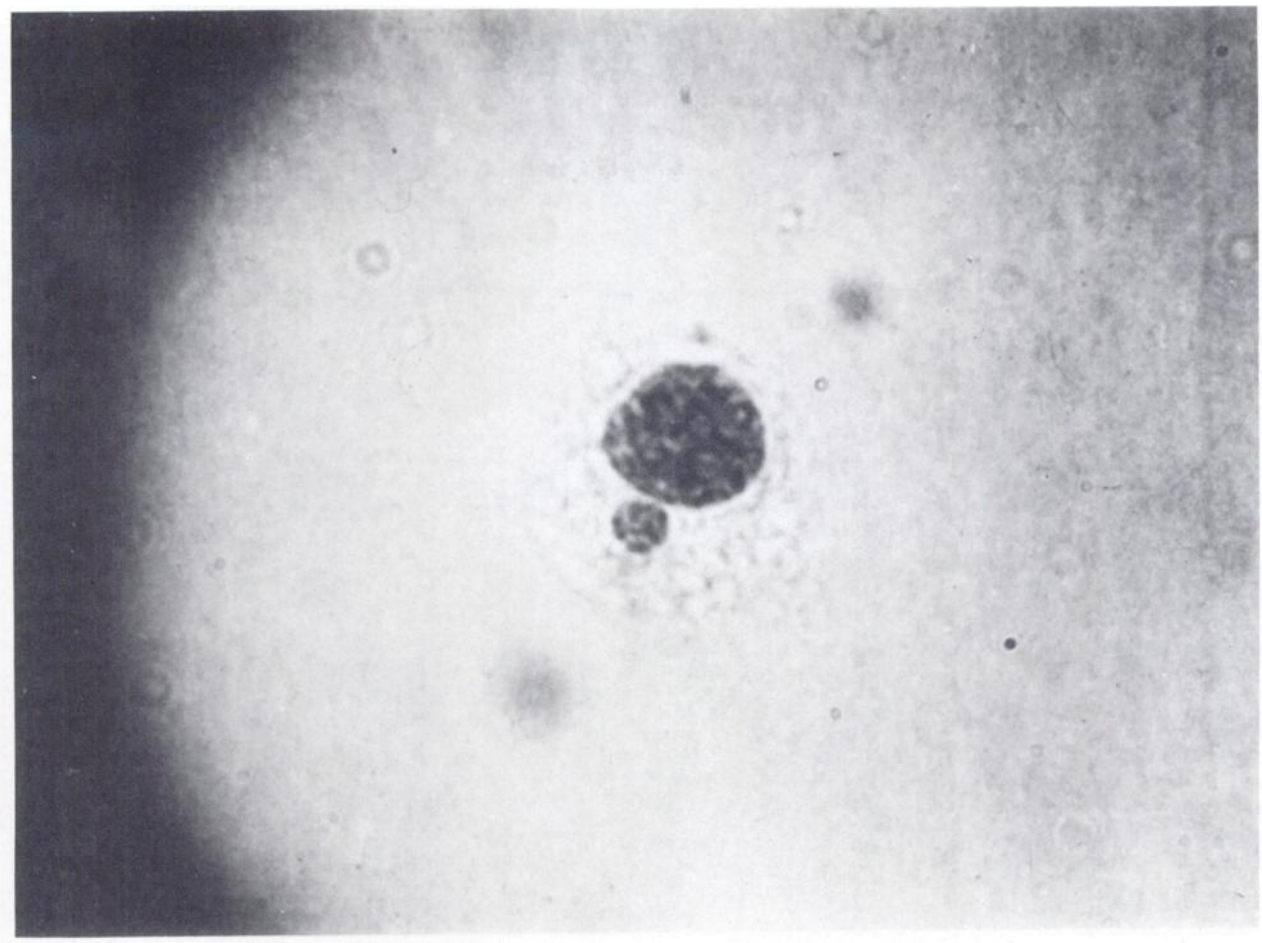

FIGURE 2
Photograph of MN, micronuclei are result of radiation-induced chromosomal fragments not incorporated in main nucleus. They are easy to recognize and score. They are proportional in frequency to amount of chromosomal damage induced and are therefore useful quantitative assay of damage

thousand cells were scored at each point. Although the mitotic index was reduced at all ^{99m}Tc concentrations, there were sufficient mitotic figures in each group, including 0.580 mCi, to provide valid data. As would be expected, low doses of ^{99m}Tc were observed not only to decrease the total number of mitotic figures but also to delay their peak appearance.

The Harlequin labeling experiments provided further evidence of mitotic delay. Fifty divisions were scored for the controls; however, the number of divisions available in the two samples treated with ^{99m}Tc was only 20 at both 63 and 66 hr. There were no second divisions at 72 hr in the exposed cultures as against 22% in the control group. These data are presented in Table 2.

Assessment of genetic damage by scoring micronuclei (MN) at harvest times of 72 and 76 hr is presented in Table 3, and the data from 76 hr are also presented in Fig. 5. Increasing concentrations of ^{99m}Tc resulted in proportional increases in MN. The later harvest times had greater numbers of MN associated with them as a result of division delay. The exception to this observation was the 0.580 mCi exposure. The increased division delay associated with this tracer concentration is responsible for the flat response at 72 hr and 76 hr. A later harvest time would have produced an increased number of MN.

TABLE 1
Effect of Increasing Concentrations of ^{99m}Tc on Division Rate (72 hr harvest)

No. of cultures	No. of cells/culture	mCi/10^7 cells	% of control mean ± s.e.e.
8	1×10^7	0.00	100 (control)
14	1×10^7	0.155	90 ± 11
8	1×10^7	0.242	80 ± 12
7	1×10^7	0.580	60 ± 11
7	1×10^7	0.723	40 ± 14
8	1×10^7	0.910	10 ± 3
9	1×10^7	1.06	0 (dead)

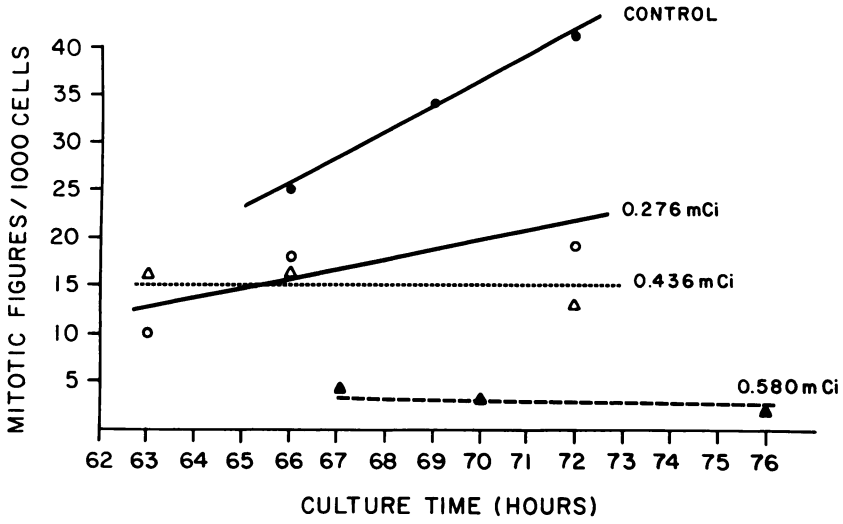

FIGURE 3
Mitotic indices (mitotic figure/1,000 cells) after culture with three concentrations of ^{99m}Tc/1 × 10^7 cells and control. Variable culture time periods were chosen in anticipation of increasing mitotic delay induced by increasing concentrations of ^{99m}Tc

DISCUSSION

While various blood cellular components have been successfully labeled with ^{111}In and have purportedly provided important information on distribution, migration, and survival in various animal studies (*9–14*), other applications, including clinical utilization (*15–19*) have remained controversial. This controversy has centered on the implication from studies performed with regard to the relative radiotoxicity of [^{111}In]oxine on cellular components, especially lymphocytes (*20,21*).

In the case of ^{111}In-labeled lymphocytes, a number of reports have detailed significant functional impairment due to radiation. Mild to severe aberrations in lymphocyte migration patterns were demonstrated by Chisholm et al. (*22*) using cells labeled with doses of ^{111}In as low as 10 μCi/10^8 cells. In another report, guinea pig T-lymphocytes labeled with extremely low doses of [^{111}In]oxine (1 to 10 Ci/10^8 cells) demonstrated marked reduction in migration into inflammatory sites while not impairing lymph node localization (*23*). This report also revealed that a significant portion of the isotope found at the inflammatory site was noncell-bound label. Others have demonstrated that even when no effect on viability or cell surface phenotype is evi-

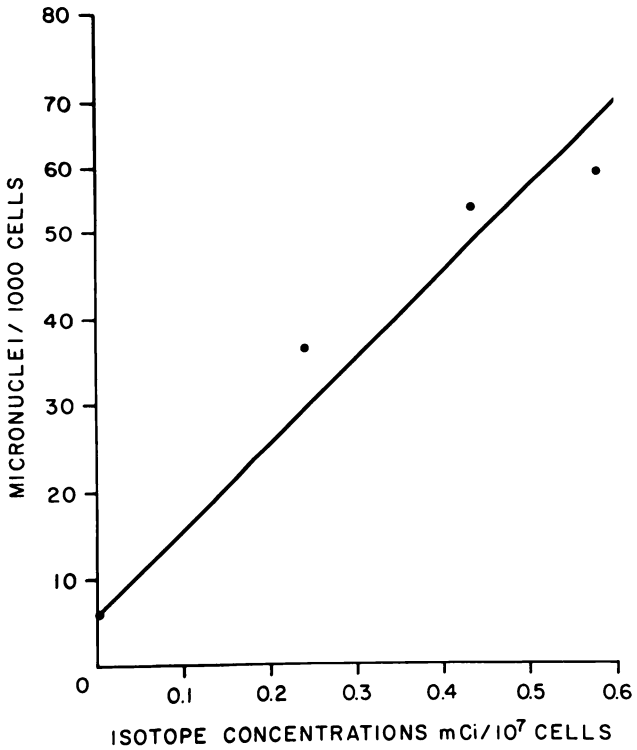

FIGURE 4
Micronuclei per 1,000 cells 76 hr following culture initiation with various concentrations of ^{99m}Tc 10^7 cells. 2,000 cells were counted for each point.

TABLE 2
Percentage of Cells Reaching Their Second Division at Various Times Following Initiation and Stimulation of Cultures

Isotope concentration/ 10^7 cells	Culture time (hr)	% Cells in first division	% Cells in second division
0.00 (control)	63	100	0
	66	100	0
	72	78	22
0.276 mCi	63	100	0
	66	100	0
	72	100	0
0.436 mCi	63	100	0
	66	100	0
	72	100	0

TABLE 3
Micronuclei Production After 72 and 76 hr of Culture at Various Isotope Concentrations (mCi/10^7 cells)

Isotope concentration	Hours of culture	Micronuclei/ 1,000 cells mean ± s.e.e.
None	72	6.80 ± 3.6
	76	6.00 ± 4.1
0.236 mCi	72	27.13 ± 9.6
	76	36.50 ± 11.3
0.435 mCi	72	35.00 ± 13.1
	76	53.00 ± 13.8
0.580 mCi	72	55.00 ± 14.2
	76	56.80 ± 16.7

dent, there may be a significant reduction in antibody dependent cellular cytotoxicity at 20 μCi/10^8 human lymphocytes (*24*). There is also evidence that even when labeled lymphocytes appear normal initially, they may exhibit significant functional abnormality by 24 to 48 hr postlabeling, (*25*) the most acceptable times for scintigraphic imaging.

In addition to the functional aberrations described, studies have demonstrated associated alteration of proliferative capacity and chromosomal abnormality. Chisholm et al. reported a significant reduction in colony forming ability in Hela S3 tumor cells labeled with doses of ^{111}In far less than that used for imaging studies (*22*). Krall and Geldof demonstrated impaired colony formation capacity of bone marrow cells when labeled with [^{111}In]oxine in doses greater than 1.0 μCi/10^8 cells (*26*). Most significantly, a recent publication by ten Berg et al. (*21*) reported lymphocyte chromosomal abnormality similar in frequency to that observed after 2 to 2.5 Gy after labeling with 9 μCi/10^7 cells. They further reported that at usual imaging labeling doses (150 μCi/10^8 lymphocytes) they observed chromosomal aberration in 95% of the cells. These reports clearly demonstrate that lymphocytes labeled with ^{111}In in the minimum quantities necessary for scintigraphic imaging will not maintain normal functional capacities.

Previous reports have also documented alterations in normal lymphocyte recirculation following ^{99m}Tc labeling (*27,28*). These alterations occurred despite low cell labeling efficiencies and are attributable to their subjection to high radiation burden during incubation. In this study we have quantitated the damage to lymphocyte genetic material and proliferative capacity at increasing ^{99m}Tc-labeling activities. The data demonstrate that a human lymphocyte isolate labeled with ^{99m}Tc in a concentration of 242 μCi/10^7 cells has a 20% loss of proliferative capacity and damage to genetic material approximating an exposure of 60 cGray of 250 keV x-ray. While this compares favorably with ^{111}In, which produces damage in lymphocyte genetic material equivalent to 2 to 2.5 Gy at only 9 μCi/10^7 cells, the radiation damage from ^{99m}Tc labeling remains significant. Although we have not investigated the in vivo distribution of lymphocytes labeled with ^{99m}Tc at the activity concentrations studied in vitro, these data demonstrate that lymphocytes labeled with ^{99m}Tc in tracer concentrations sufficient for scintigraphy develop significantly less radiation equivalent chromosomal damage than with subscintigraphic concentrations of ^{111}In. The feasibility of utilizing ^{99m}Tc-labeled lymphocytes in certain specific applications is currently being investigated.

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