Indium-111-Labeled Human Polymorphonuclear Leukocytes: Viability, Random Migration, Chemotaxis, Bactericidal Capacity, and Ultrastructure

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Human polymorphonuclear leukocytes (PMNs) were labeled with indium-111 oxine in ethanol, and the effects of the labeling procedure, radioactivity, and concentrations of oxine and ethanol on PMN function and structure were studied in vitro. The standard labeling procedure did not alter the viability, random migration, chemotaxis, bactericidal capacity, or the ultrastructure of PMNs. Exposure to higher doses of radioactivity, or to higher concentrations of ethanol, had no appreciable effects on random migration and chemotaxis of PMNs. A dose-dependent reduction in their random migration and chemotaxis was observed when higher concentrations of oxine were used. These results indicate that In-111-labeled PMNs are structurally intact and have normal in vitro locomotion and bactericidal activity. Indium-111-labeled PMNs should be suitable for studying the kinetics and distribution of these cells in health and disease.

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Labeling of isolated polymorphonuclear leukocytes (PMNs) with indium–111 (In-111) 8-hydroxyquinoline (oxine) is a useful technique with many potential applications in clinical and biologic investigations (*1*). The oxine-In-111 complex, when dissolved in ethanol, diffuses passively through the PMN plasma membrane and In-111 binds to intracellular components, providing a stable label (*2*). Indium-111 emits two gamma photons of 173 keV (84%) and 247 keV (94%), which are suitable for external detection (*3*). It has a half-life of 67 hr, which is long enough to allow repeated investigations up to a week after injection, while not posing an excessive radiation hazard (*4*).

Indium-111-labeled PMNs have been used successfully to locate abscesses in dogs and humans (*1,3*), and myocardial infarctions in experimental animals (*5,6*). This apparently normal in vivo function of the labeled PMNs suggests that they are not adversely affected by the labeling procedure. To confirm the functional and structural integrity of In-111-labeled PMNs, we have investigated the effect of In-111 labeling on viability, random migration (nondirectional locomotion of cells), chemotaxis (directional locomotion of cells in a chemotactic gradient), bactercidal capacity, and ultrastructure of human PMNs in vitro. An attempt was also made to establish the optimum nontoxic concentrations of oxine, ethanol, and radioactivity to which PMNs are exposed during the labeling procedure.

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MATERIALS AND METHODS

Preparation and labeling of PMNs. Heparinized (40 IU/ml) whole blood from normal human donors was separated into a PMN-rich fraction by Ficoll-Hypaque gradient centrifugation (*7*), followed by dextran sedimentation and hypotonic lysis of the red cells as previously described (*8*). This resulted in 93.87 ± 0.5% (mean ± s.e.m. of 31 preparations) PMNs with the remainder mononuclear cells and eosinophils. The cells were suspended in Hanks' balanced salt solution (HBSS, pH 7.4) and adjusted with Gey's medium (Gey's balanced salt solution, pH 7.4, with 2% bovine serum albumin and 2% penicillin-streptomycin) to a concentration of 10 million PMNs/ml before labeling.

Three populations of PMNs were studied.

1. Some PMNs were kept at 4°C in Gey's medium until used for study; these are referred to as "control" cells.
2. Other PMNs were processed for labeling as described below, but without exposure to In-111-oxine-ethanol; these are referred to as "reference" cells.
3. PMNs labeled with In-111 in oxine and ethanol are referred to as "labeled" cells.

Labeling of PMNs was performed as previously described (*1*). In brief, they were pelleted (450 *g* for 5 min) and washed in normal saline at pH 6.5, repelleted, and resuspended in normal saline to a concentration of 10 million PMNs/ml. A required volume of In-111 oxine solution in 100% ethanol* was diluted fourfold in saline and added dropwise to the PMNs to be labeled at room temperature. After 15 min the PMNs were pelleted and resuspended in Gey's medium to 10 million PMNs/ml. The labeling efficiency—i.e., the cell-associated radioactivity—has been shown to be 80-90% (*1*).

A fresh sample of commercial In-111 oxine in ethanol contains 1 mCi of In-111 and 50 μg of oxine in each 50 μl of ethanol. In clinical studies approximately 100 million PMNs are labeled with a total of 200-500 μCi of In-111, which provides sufficient radioactivity for external detection. In our experiments we tried to keep the radiation dose equivalent to that of the clinical preparations: 2.0-5.0 μCi/million PMNs. Since there is continual decay of In-111, more of the In-111 oxine in ethanol was used for labeling of PMNs as the stock solution decayed, in order to keep the radioactivity within the above range. After the standard labeling procedure, therefore, the final concentrations of oxine and ethanol that the cells were exposed to varied between 0.1 and 1.0 μg oxine/million PMNs (1-10 μg/ml) and 1.5-5.0 μl (≃1.2-4.0 mg) of ethanol per milliliter of cell suspension. Cells were pelleted and resuspended in Gey's medium after labeling; thus, exposure of PMNs to ethanol and oxine was limited to 15 min. Other experiments were designed to determine the effect of higher concentrations of individual labeling components on PMN migration. This was done by addition of extra oxine or ethanol directly to the In-111 oxine stock solution until the desired concentration was achieved.

Control, reference, and labeled PMN populations were studied simultaneously in each experiment, and all experiments were run in duplicate.

Motility assay. Random migration of PMNs was evaluated by the coverslip-motility assay technique. Briefly, a monolayer of adherent PMNs was produced by placing two to three drops of a 10^6 PMN/ml suspension in Gey's medium on glass coverslips. The coverslips were placed on moist gauze in 25- by 10-mm Petri dishes. After 30 min of incubation at 37°C, the coverslips were washed with saline and to the monolayer of adherent PMNs were added either 100 μl of Gey's medium (condition for spontaneous random migration) or 100 μl of a chemotactic agent (condition for activated random migration). In most of the experiments, a 10-n*M* solution of the synthetic chemotactic agent, N-formyl-methionyl-leucyl-phenylalanin† was used. In other experiments, a concentration of 5% *E. coli* endotoxin‡-activated serum was used. This activated serum, the chemotactic activity of which is predominantly the result of production of C5a, was prepared using fresh serum as previously described (*9*). After 25 min of incubation at 37°C, the cells were fixed by adding 1% glutaraldehyde in saline to the coverslips. Motility was evaluated by examining the coverslips directly by phase microscopy, and is expressed as the percentage of cells displaying a "locomotive" structure. As shown in Fig. 1, the locomotive cells are spread out and have a broad, smooth veil (lamellipodium) at the front and a knob-like tail at the rear (*10*). In contrast, the nonlocomotive cells are round and lack both the lamellipodium and the tail.

Chemotactic assay. Chemotaxis of PMNs was assayed by the modified micropore-Boyden-chamber technique of Zigmond and Hirsch (*10*). In brief, the Boyden chambers were prepared by placing PMN suspension (3 million/ml in Gey's medium) in the upper compartments, separated from the lower compartments by a cellulose nitrate filter with 1.2-μm pores. In the lower compartments either Gey's medium was added (condition for random migration) or 5% *E. coli* endotoxin-activated serum (condition for directional migration or chemotaxis). The chambers were then incubated at 37°C for 60-70 min. The filters were removed, fixed in methanol, stained with hematoxylin, dehydrated in a graded ethanol series, and cleared in xylene. Stained filters

were then mounted on glass slides and examined at 500 magnification. Cell migration into the filters was measured by the leading-front technique (*10*), using the optical micrometer on the fine-focus knob of the microscope. The distance in microns from the top of the filter to the farthest plane of focus that contained at least two cells was determined in five fields across the filter. Duplicate filters were always run and the data obtained for each experiment were expressed as the mean of ten measurements in the two filters.

Bactericidal assay. The ability of PMNs to kill ingested *Staphylococcus aureus* was determined by a method described previously (*11*), using a pour-plate technique to determine the number of viable bacteria at 20, 30, and 60 min.

Ultrastructure and electron microscopy. Control, reference, and labeled PMNs were fixed by adding an equal volume of 2% glutaraldehyde in 0.1 *M* sodium cacodylate, pH 7.3, to the cells suspended in Gey's medium at room temperature. After 5 min the cells were gently pelleted, the supernatant removed, and 2% glutaraldehyde in 0.1 *M* Na cacodylate added to the surface of the pellet for an additional 20 min of fixation at room temperature. After a rinse in Na cacodylate buffer for 30 min, the pellet was placed in 1% osmium tetroxide in Na cacodylate for 30 min at room temperature. The pellet was then stained in 0.5% uranyl magnesium acetate in 0.1 *M* saline for 1 hr at room temperature, dehydrated in a graded ethanol series, and given a final wash in propylene oxide before being embedded in Epon.

In other studies, the control or labeled PMNs were allowed to orient in response to a chemotactic stimulus (5% *E. coli* endotoxin-activated serum) at 37°C for 45 min in Boyden chambers in which the cells were separated from the stimulus by a micropore filter with 0.45-μm pores. As previously described (*12*), cells will orient at the surface and protrude pseudopodia into the filter, but are unable to migrate into the filter matrix because of the small pore size. The cells were fixed on the filters in the chambers by removing the medium and overlaying with 2% glutaraldehyde in 0.1 *M* Na cacodylate at 37°C for 15 min. The filters were then removed and handled as described for the pellets above, except that they were embedded in flat embedding molds to allow sectioning perpendicular to the filter surface. The pellets or filters were sectioned with a diamond knife on an ultramicrotome, then picked up on formvar-coated copper grids, stained with uranyl acetate and lead citrate, and examined with an electron microscope at a 60-kV accelerating voltage.

RESULTS

Viability. After labeling, viability as assessed by the trypan blue exclusion test was (94.5 ± 0.4)%—mean ± s.e.m.—for the reference PMNs, and (94.2 ± 0.4)% for the labeled cells.

Random migration. Under standard labeling conditions, the random migration of PMNs was evaluated by the coverslip-motility assay in eight different experiments. The results are expressed as the mean percentage ± s.e.m. of cells displaying "locomotive" configuration (Fig. 1). Two hundred control, reference, and labeled cells were scored in each experiment. For the control, reference, and labeled PMNs respectively, the percentages of cells exhibiting spontaneous random migration were 22.1 ± 2.4, 24.0 ± 2.8, and 21.9 ± 2.2. For activated random migration, the percentages were similarly 45.3 ± 4.0, 49.0 ± 4.7, and 43.6 ± 3.7. These values are not significantly different from each other (Student's t-test).

Chemotaxis. The micropore-Boyden-chamber technique was used to measure both the spontaneous random migration and the chemotaxis of PMNs in seven experiments, using the standard labeling conditions. The leading front of cells undergoing spontaneous random migration penetrated the filters 55.3 ± 2.4 μm, 60.3 ± 3.6 μm, and 52.5 ± 3.5 μm for the control, reference, and labeled PMNs, respectively. Cells responding to chemotactic stimuli similarly traveled 104.8 ± 3.7 μm, 109.6 ± 5.0 μm, and 106.1 ± 6.3 μm. The results of both spontaneous random migration and chemotaxis are not significantly different among the three PMN populations.

Effects of higher concentrations of oxine, ethanol, and radioactivity on random migration and chemotaxis. These effects were evaluated by the micropore-Boyden-chamber technique. Both the random migration and chemotaxis were progressively decreased with increasing concentration of oxine (Fig.

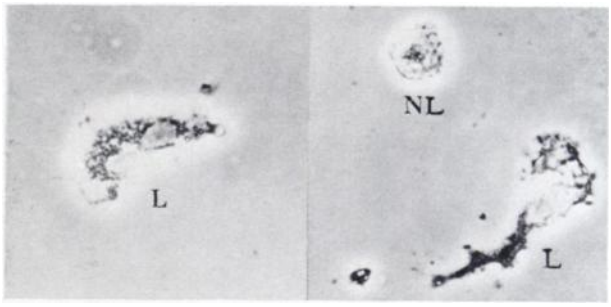

FIG. 1. Typical appearance of locomotive (L) and nonlocomotive (NL) PMNs in coverslip-motility assay. Original magnification x 2600.

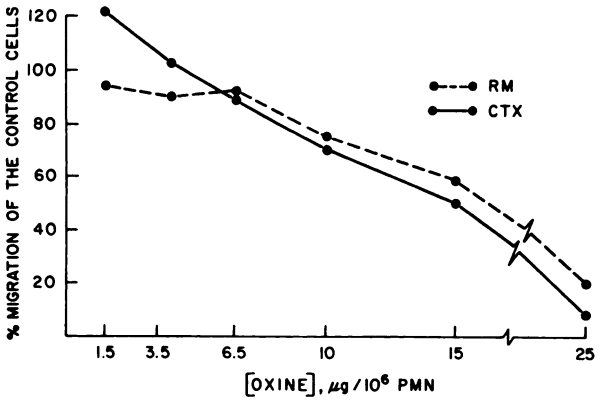

FIG. 2. Effect of oxine concentration on random migration (RM) and chemotaxis (CTX) of In-111-labeled PMNs. Ethanol (1.6–4.0 mg/ml) and radioactivity (< 1 μCi per million PMNs).

2). At a concentration of 25 μg per million PMN, random migration was reduced to 20% and chemotaxis to 8% of the control values. Both random migration and chemotaxis of PMNs exposed to 10 μg or more of oxine per million PMNs are significantly reduced when compared with the cells exposed to lower concentrations of oxine ($P < 0.05$ by Student's t-test for unpaired samples). The effects of increasing ethanol concentration and radioactivity on the random migration and chemotaxis of labeled PMNs were also measured. Ethanol concentrations of up to 80 mg/ml (Fig. 3), and radioactivity of up to 29 μCi per million PMNs (Fig. 4), had no appreciable effects on these PMN parameters.

Bactericidal capacity. The bactericidal capacities of the labeled and reference PMNs were equivalent to those of control PMNs at 20, 30, and 60 min (Fig. 5, data for reference PMNs not shown). In three experiments (97.7 ± 0.4)% (mean ± s.e.m.), (98.0 ± 0.6)%, and (98.3 ± 0.1)% of the staphylococci ingested were killed at the end of 60 min by the control, reference, and labeled PMNs, respectively.

Ultrastructure and orientation. Control, reference, and labeled PMNs fixed in suspension were rounded and showed no appreciable differences in overall shape or fine structure (Fig. 6). The number of cytoplasmic granules, although not quantitated, appeared to be similar in the three populations. Compared with the controls, the reference and labeled cell pellets contained slightly more cellular debris, including membrane fragments, free granules, and an occasional whole cell with a torn plasma membrane.

Experiments in which the control and labeled PMNs were fixed on filters with 0.45-μm pores after orientation in a chemotactic gradient showed no differences between the control and the labeled cells (Fig. 6c). In these studies only viable cells adhere to the filter and therefore as expected the small quantities of damaged cells or cellular debris seen in the pellets of labeled cells were not apparent in the orientation experiments. The labeled PMNs were oriented as previously described for unlabeled cells (*12*), with the nucleus toward the upper end of the cell (i.e., away from the filter surface) and centriole and microtubule array below the nucleus. Although not quantitated, the amounts of subplasmalemmal microfilaments, microtubules, and cytoplasmic granules in the labeled cells appeared to be normal when compared with other preparations.

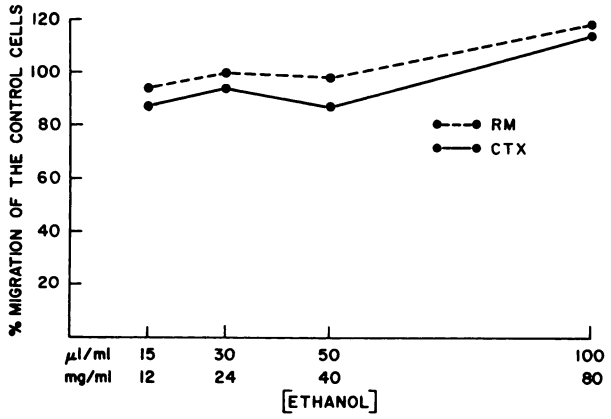

FIG. 3. Effect of ethanol concentration on random migration (RM) and chemotaxis (CTX) of In-111-labeled PMNs. Oxine (1–2 per million PMNs) and radioactivity (< 1 per million PMNs).

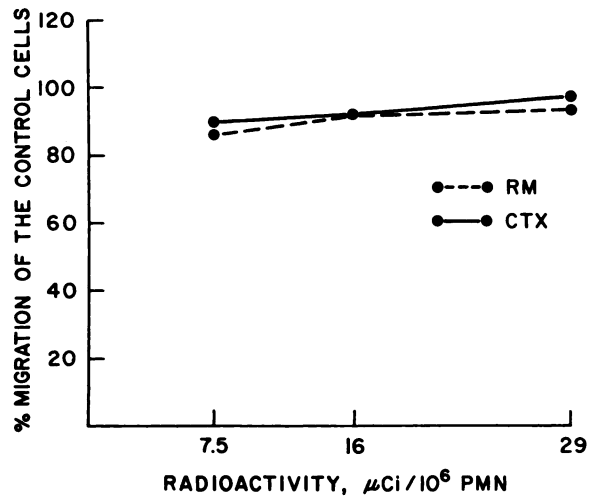

FIG. 4. Effect of radioactivity on random migration (RM) and chemotaxis (CTX) of In-111-labeled PMNs. Oxine (1.0 μg/10^6 PMNs) and ethanol (0.8–4.0 mg per ml).

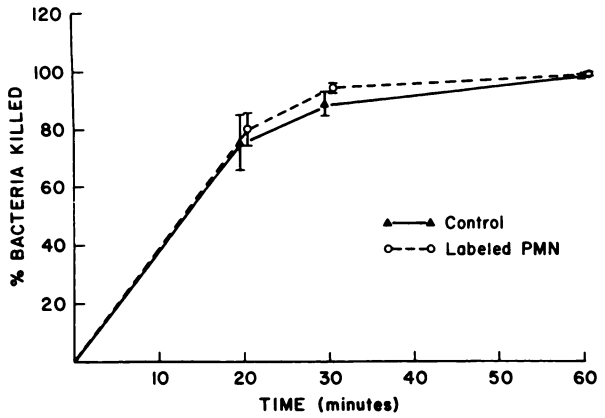

FIG. 5. Bactericidal capacity of control and In-111-labeled PMNs.

DISCUSSION

Indium-111 labeling of PMNs is a highly promising technique for the investigations of the kinetics and distribution of these cells in health and disease. In kinetic and distribution studies, however, the validity of results depends on the demonstration that cellular structure and function are not adversely affected during the labeling procedure. Theoretically, In-111 labeling could alter PMN function by one or more of the following ways: a) direct chemical damage by oxine or ethanol, b) damage due to intracellular radiation, or c) physical damage due to excessive handling of the cells during isolation and labeling procedures.

Our findings indicate that neither the viability, structure, nor function of PMNs is significantly altered when the standard labeling procedure is used—namely, 2-5 μCi In-111 per million PMNs; 0.1-1.0 μg oxine per million cells (=1-10 μg/ml of cell suspension); and 1.2-4 mg ethanol per milliliter of suspension. Specifically, random migration, directional locomotion (chemotaxis), and the ability of the cells to kill staphylococci were unaffected. Whether labeled PMNs are resting or chemotactically stimulated, electron microscopy reveals no alteration in their ultrastructure. The slight increase in cellular debris observed in the reference and labeled PMN pellets can be attributed to the increased handling and pelleting of these PMN populations as compared with the controls.

Our results suggest that oxine is potentially the most injurious of all the labeling components, but the mechanism of injury remains to be determined. Oxine, a lipophilic antibacterial agent, is a nonspecific metal chelator, and could possibly damage the cells by chelating contaminating metal ions such as cadmium and iron (which might be found in the In-111 solution) and transporting them into the cell (*13*). Oxine may also damage the cells by binding the essential intracellular metals and thus rendering them unavailable for metabolic reactions (*14*). We found that a 15-min exposure to oxine in concentrations of 10 μg or more per million PMNs (i.e., 10 times the upper limit of the standard labeling concentration) significantly impaired PMN random migration and chemotaxis, without altering their apparent viability. Smaller concentrations, equivalent to those of our standard labeling technique, did not adversely affect PMN locomotion. Other investigators have found that as little as 0.75 μg oxine per million PMNs reduces random migration and chemotaxis by nearly 50% (*15*). However, the labeling technique, and particularly the source of In-111 used, were different from those in our study, and the differences in results might have been caused by impurities of the In-111 solution.

Previous studies have shown that ethanol at concentrations of 1-10 mg/ml inhibits PMN adherence

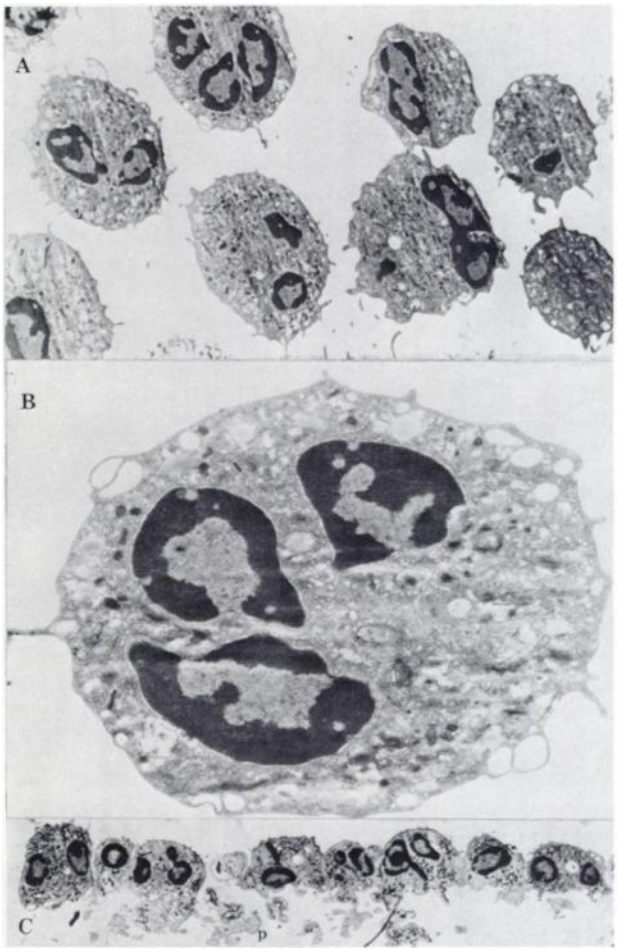

FIG. 6. Electron micrographs of In-111-labeled PMNs. A. Cells in suspension showing normal structure x 6840. B. Higher magnification (original) x 18040. C. Cells oriented in a chemotactic gradient, protruding pseudopods (p) into a 0.45-μm micropore filter x 2280.

to nylon wool in a dose-dependent manner (*16*). Other studies showed no inhibition of phagocytosis and killing after incubation of PMNs in ethanol up to a concentration of 4 mg/ml (*17*). Chemotaxis of PMNs was also not affected by ethanol in concentrations up to 16 mg/ml in one study (*18*) and 8 mg/ml in another (*19*). Note, however, that the duration of exposure to ethanol in these studies was considerably longer than the relatively brief period of 15 min used in our standard In-111-labeling procedure. We have shown that, under our conditions, ethanol concentrations of up to 80 mg/ml in the cell suspension (i.e., 20 times the upper limit of the standard labeling concentration) had no effect on PMN random migration and chemotaxis.

Gamma rays, Auger electrons, and x-rays emitted during the decay of In-111 within the cell could potentially alter PMN function and viability (*20*). Indium-111 appears to bind to DNA to some extent after entering the cell (*2*) and the radiation damage may be greater when the source is in intimate association with the DNA itself. The radiation dose to a PMN resulting from the decay of a single In-111 atom is calculated to be 0.135 rad (*21*). In a typical experiment in which 100 million PMNs are uniformly labeled with 200–500 μCi of In-111, the integrated radiation dose per cell is 3500–8500 rads. Previous studies have shown that PMNs are remarkably resistant to the damaging effects of ionizing radiation (*22–24*). In one study, in vitro external irradiation of up to 50,000 rads had little or no effect on the PMN phagocytic and metabolic functions (*22*). Others have shown that external irradiation up to 10,000 rads has little effect on chemotaxis, phagocytosis, and bactericidal activity of PMNs and doses of less than 75,000 rads did not cause any ultrastructural damage (*24*). In our studies of the effect of intracellular radiation of In-111 on PMN random migration and chemotaxis, the duration of exposure was 2–4 hr. This period began when In-111 was added to the PMN suspension during labeling, and included the 60–70 min during which the cells were incubated in Boyden chambers. We have shown that radioactivity of up to 29 μCi per million PMNs (= 6 times the upper limit of the standard labeling dose) had no detectable adverse effects, up to 4 hr after labeling, on the PMN random migration and chemotaxis. Our findings indicate that circulating PMNs are highly resistant to the short-term effects of intracellular radiation as well, perhaps because these are nondividing "end" cells not engaged in active synthesis of DNA.

One further point of interest with respect to the use of In-111-labeled PMNs is its potential application to in vitro studies of chemotaxis. The conventional radioassay of leukocyte chemotaxis uses chromium-51-labeled cells (PMNs or monocytes) with a double micropore-filter system (*25*). Recently In-111 has been successfully substituted for Cr-51 in this assay (*26*). The results have been shown to compare favorably with the visual assays, and thus the Cr-51 problems with low labeling efficiency (6%) and elution of radioactivity could be avoided by substitution of In-111. Our studies confirm the relative nontoxicity of the standard In-111-labeling procedure to PMN migration, which is essential if this method of assaying leukocyte chemotaxis is to be valid.

In summary, PMNs labeled with In-111 appear to be structurally and functionally unimpaired. None of the PMN functional parameters tested in our study appeared to be modified by our standard labeling method. These studies should help validate the use of In-111 labeled PMNs to locate abscesses and other inflammatory sites and to study PMN kinetics and their survival in blood and tissues in vivo.

FOOTNOTES

* Diagnostic Isotopes, Bloomfield, NJ.

† F-Met-Leu-Phe, Bachem Inc., Torrance, CA.

‡ *E. coli* 0127: B8 lipopolysaccharide B, Difco Laboratories, Detroit, MI.

ACKNOWLEDGMENTS

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