TECHNICAL NOTES

Evaluation of Neutrophil Labeling Techniques Using the Chemotaxis Radioassay

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Neutrophils isolated from human blood were labeled by various methods and exposed to a chemotactic gradient. The chemotactically functional cells that migrated into the gradient were isolated. The portion of radioactivity of the original cell suspension carried with the chemotactically responsive cells was related to the relative number of migrating cells as determined microscopically. Of the radionuclides used, P-32 diisopropylfluorophosphate (DFP), In-111 oxine, and Tc-99m sulfur colloid provided cell preparations with the highest relative portion of radioactivity confined to functionally intact (chemotactic) neutrophils. Results with $Na_2{}^{51}CrO_4$ and with $SnCl_2$-reduced $^{99m}TcO_4^-$ were less than optimal. Neutrophils exposed to Ga-67 citrate apparently took up the label and retained chemotactic responsiveness. However, little or no radioactivity was detected in the neutrophils that migrated from the suspensions of Ga-67-labeled cells. The results indicate that the chemotaxis radioassay can yield unique information pertaining to the extent to which a radiotracer is specifically associated with viable neutrophils in a suspension of labeled cells.

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Almost without exception, evaluations of new cell-labeling techniques document the labeling yield obtained and offer data comparing some function of labeled cells with that of cells not exposed to the radionuclide. However, the possibility that the majority of the label associates with cells of diminished function cannot be dismissed. For example, the radiopharmaceutical may be toxic, and uneven distribution of the radiotracer may preferentially damage those cells that become more heavily labeled. It is also possible that deleterious radiation effects may preferentially disrupt the function of cells most heavily labeled by the tracer.

Recent interest in the diagnostic utility of labeled autologous neutrophils has led to the development of several methods for labeling these cells (*1–10*). We undertook this investigation to assess comparatively the functional integrity of labeled neutrophils and to determine the amount of radioactivity confined to viable cells. To accomplish this, we exploited the ability of chemotactically viable neutrophils in labeled cell suspensions to migrate toward a chemoattractant. We exposed neutrophils labeled by several methods to chemoattractant, isolated the responsive cells, and compared the extent to which the chemotactically viable cells held radioactivity.

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MATERIALS AND METHODS

Radioagents. The following were procured commercially: $Na_2{}^{51}CrO_4$ (1 mCi/ml); Ga-67 citrate (2 mCi/ml); In-111 8-hydroxyquinoline (In-111 oxine); P-32 diisopropylfluorophosphate [^{32}P]DFP); and Mo-99 → Tc-99m generator. The Ga-67 citrate was diluted with sterile saline, before use, to 20 μCi/ml; the [^{32}P]DFP was suspended in saline at a concentration of 100 μCi/ml. Technetium-99m sulfur colloid (TcSC) was prepared from $Na^{99m}TcO_4$ using a commercial thiosulfate kit.*

Neutrophils. Polymorphonuclear leukocytes were isolated from heparinized blood obtained from healthy adults by centrifugation over cushions of Ficoll-Hypaque,† followed by isotonic lysis of erythrocytes as detailed previously (*11*). Final cell preparations contained no erythrocytes, few detectable platelets, and <3% mononuclear leukocytes. Over 98% of the cells were judged viable as assessed by their ability to exclude trypan blue dye. Cells were washed three times with Hanks' balanced salt solution and resuspended in phosphate-buffered saline at a concentration of 30 million cells/ml before incubation with the radiotracers.

Neutrophil labeling. Neutrophils were incubated with the various radionuclides for 30 min at room temperature, according to previously described protocols. These procedures involved incubation with Ga-67 citrate (*4*), TcSC (*1*), $Na_2{}^{51}CrO_4$ (*3*), In-111 oxine (*10*), and [^{32}P]DFP (*12*), with continuous gentle agitation. Three additional cell-labeling methods involving $^{99m}TcO_4^-$ were used

(*5,6,13*). These required prior reduction of the anion with $SnCl_2$. The $SnCl_2$ required exposure of cells to various concentrations of stannous chloride dissolved in either saline at pH 4.0 (*6*) or acid citrate dextrose at neutral pH (*5,13*). For each labeling method, after incubation, the radioactivity not associated with cells was removed by washing the cells three times with BSS. Cells were resuspended in BSS at 20 million/ml for use in chemotaxis assays.

Chemotaxis. Cells were labeled, washed, and placed in chemotaxis chambers within 2 hr of blood collection. The granulocyte chemotaxis radioassay was performed as described by Gallin et al. (*14*), using 10% endotoxin-activated serum (1 μg *E. coli* 055:B5 endotoxin to 1 ml serum) as the chemoattractant in the lower compartment of the chemotaxis chamber, and approximately 4 million labeled neutrophils (200 μl) in the upper compartment. Two 5-μm cellulose acetate Millipore filters separated the lower compartment from the upper. After 3 hr of incubation at 37°C, the two filters were removed. The upper filter was stained with hematoxylin and the average number of cells responding to the chemoattractant (viewed on the underside of the filter) per high-power field (HPF, 420×) was determined, giving a comparative estimate of the functional competence of the labeled neutrophils. Lower filters were rinsed in saline, placed in polypropylene tubes, and their radioactivity determined (cpm), reflecting the amount of radioactivity carried by migrating neutrophils. In an ancillary study, the number of cells on the upper filter was found to be directly proprotional to the number of cells on the lower filter ($r = 0.96$). In all experiments, appropriate consideration was given to radionuclide decay and background counts. Assays for chemotaxis were also performed by the conventional Boyden chamber method using one filter to separate the cells from the chemoattractant. After 75 min of incubation, the filter was removed, stained, and the number of responding cells (per HPF) determined microscopically. The chemotactic response of the neutrophils exposed to the radionuclide being investigated was compared with that of unlabeled cells from the same suspension in order to determine the effects of the labeling procedure on chemotaxis.

Data expression. Chemotactic response of the cells exposed to radionuclides was determined microscopically by counting cells per high-power field and is expressed as a percentage of the response of the concomitantly run control chambers, which contained unlabeled neutrophils. The relative radioactivity of labeled cells responding to the chemoattractant is represented by a chemotactic index (CI), where:

$$CI = \frac{\text{cpm of lower filter}}{\text{cpm of cells originally placed in chamber}} \times 10^6$$

By using the corrective factor of 10^6, the CI reflects the cpm that would have been recovered on lower filters if 1 million cpm were carried on the cells placed in each chamber's upper compartment (*14*). In all experiments, chambers containing BSS instead of chemoattractant were used for controls. Under the conditions used, neutrophils did not migrate through the filter separating the cells from BSS in control chambers. In order to correct for spontaneous elution of the tracer to the lower filter from the cells in the upper compartment, a chemotactic index of migration (CIm) was determined, where:

CIm = CIpos (of chambers with chemoattractant)—CIneg (of chambers with BSS only)

In order to give an indication of the magnitude of radioactivity carried by the labeled neutrophils to the lower filter relative to the amount of radioactivity that eluted from the upper compartment, a ratio of radiotracer uptake to background uptake was derived from the chemotactic indices (CIpos ÷ CIneg). A low relative target-to-nontarget ratio is indicative of high spontaneous elution or low labeling of chemotactically responsive cells. A high T/NT would therefore indicate that efficient labeling of chemotactically viable cells was obtained and that a low level of spontaneous elution of tracer from labeled cells occurred.

RESULTS

Chemotactic characteristics of [^{32}P]DFP-labeled neutrophils. Thirty to 55% of the P-32 activity in [^{32}P]DFP mixed with neutrophil suspensions remained cell-associated after three washes. These cells had no apparent chemotactic defect in comparison with labeled cells (Table 1). In the radiometric assay of chemotaxis, lower filters from chambers with chemotactic factor were found to contain an average of 37 times as much radioactivity as filters from chambers without chemotactic factor. This was associated with a migratory chemotactic index of 75,900 (i.e., 75,900 cpm would have been recovered on lower filters if 1 million cpm were associated with cells in the upper compartment and no spontaneous elution occurred).

Tc-99m sulfur colloid, Na_2-51CrO_4, and In-111 oxine. Neutrophils labeled with TcSC, Cr-51, and In-111 oxine using the methods evaluated in this report have been successfully utilized

TABLE I. CHEMOTACTIC CHARACTERISTICS OF LABELED NEUTROPHILS*

Radionuclide	μCi	Labeling yield†	Chemotactic‡ response	CIpos§	CIneg	CIm¶	CIpos/CIneg ratio
[^{32}P]DFP	10–20	30–55%	98%	78,000	2,100	75,900	37.1
Tc-99m SC	50–100	15–34%	96%	62,000	1,300	60,700	47.6
In-111 oxine	50–100	31–65%	94%	55,000	3,700	51,300	14.9
Cr-51	10–20	3.2–8.9%	93%	37,050	9,500	27,550	3.9
Ga-67 citrate	2–10	0.3–2.5%	103%	1,290	1,450	0	0.9

* Results are based on at least seven separate experiments for each radionuclide.

† % of tracer added to cell suspension that remained after three washes.

‡ % of chemotactic response of cells not exposed to radiotracer. Control response of cells not exposed to tracer. Control response was 108 cells/field (range 86–132), determined by microscopic examination of lower surface of upper filters.

§ CIpos and CIneg represent radiometric chemotactic indices for chambers with and without chemotactic factor, respectively.

¶ CIpos—mean CIneg.

to delineate areas of infection and inflammation by external imaging (*2–4, 9*). As shown in Table 1, none of these radiotracers caused a measurable decrease in the chemotactic response (cells/field) of human neutrophils. In the radiometric analysis of chemotaxis, the methods using TcSC and In-111 oxine resulted in higher T:NT values than did the technique with Cr-51 (Table 1). None of the methods resulted in higher T:NT values than those obtained with [^{32}P]DFP. The lower T:NT value for Cr-51 was a consequence of the higher CIneg for this tracer, a finding indicating that a substantial amount of the label was eluted from the cells during incubation in the chemotactic chambers. The moderately low CIneg obtained with In-111 oxine indicates that only a modest amount of tracer elution occurred during incubation at 37°C.

Failure of Ga-67 citrate to label chemotactically viable cells. As reported by others (*4*), incubation of neutrophils with Ga-67 citrate left a low but reproducible amount of radioactivity associated with the cells after several washes (Table 1). Cells exposed to Ga-67 citrate were not found to be inhibited in their subsequent chemotactic responsiveness. However, using Ga-67-labeled neutrophils, we found no more radioactivity on the filters from chambers that contained chemoattractants than on the filters from control chambers (Table 1). Thus, CIpos:CIneg ratios using Ga-67 were close to 1. Although the cells in suspensions incubated with Ga-67 displayed chemotactic activity, we found that the chemotactic cells were not associated with a significant or measurable portion of the radioactivity of the original cell preparations. Since Ga-67 citrate did not inhibit neutrophil variability, we conclude that Ga-67 did not associate with chemotactically viable cells or, alternatively, that the label was lost during migration.

Chemotactic characteristics of neutrophils labeled with stannous chloride—sodium pertechnetate. Three previously described methods for labeling neutrophils with $SnCl_2$ and $^{99m}TcO_4^-$ were evaluated. The first method required a low concentration of $SnCl_2$ (1 μg/ml) and low pH (4.0) to dissolve the salt and label the cells (*6*). Neutrophil chemotaxis determined radiometrically and visually was completely inhibited when this technique was used, and the inhibition of function was determined to result from exposure of cells to low pH. When $SnCl_2$ was used at 1 μg/ml at pH 7.0, chemotaxis (determined visually) was not impaired, but the labeling yield was quite low (<5.0%) and the CIm was <1000.

A second technique required a higher concentration of $SnCl_2$ (2 μg/ml) and an acid citrate dextrose (ACD) solution to dissolve the salt (*13*). A labeling yield of 42% was obtained with this method, but chemotaxis was completely inhibited. Chemotactic inhibition resulted both from $SnCl_2$ toxicity and incubation of cells in ACD.

A third method involved the use of 100 μg/ml $SnCl_2$ in ACD (*15*). This method also resulted in nearly complete inhibition of chemotaxis, although the labeling yield was relatively high (47.0%). If ACD was replaced with saline, the labeling yield decreased to 13.5%. Cells labeled in this manner retained 75% of the chemotactic response of unlabeled cells, with a chemotactic index of migration of 2780.

DISCUSSION

This investigation was designed to compare neutrophil labeling techniques. We attempted to determine the viability of labeled cells and the portion of radioactivity in the cell suspensions specifically associated with these viable cells. Although this information is of critical importance in a reliable evaluation of cells labeled by any method, it has seldom been obtained. It is not often technically possible to obtain this information. Fortunately, viable polymorphonuclear leukocytes can migrate chemotactically—a biological function exploited herein and elsewhere (*16*) to separate viable cells specifically from the original cell preparation. Studies associating chemotactic defects and inhibitors with defective neutrophil mobilization, inflammatory responses, and host defense to infection (*17–19*) justify chemotaxis as the basis for cell selection. Chemotactically competent neutrophils, injected i.v., would be more likely than impaired cells to find the site of infection.

The characteristics of neutrophils incubated with Ga-67 demonstrate the usefulness of the technique. The data suggest that few, if any, chemotactically viable cells accumulate the tracer. Since the Ga-67 did associate with cells in suspension, and as the cells in suspension retained chemotactic function after incubation with Ga-67, the conclusion that the tracer was not associated with chemotactically viable cells would have been difficult to obtain in the absence of this type of analysis.

With respect to labeling techniques involving [^{32}P]DFP and In-111 oxine, our results are supported by numerous in vivo observations. Both procedures yielded cells that performed satisfactorily in the chemotaxis radioassay, and both have been extensively utilized for studies of neutrophil distribution and kinetics in experimental animals and in humans. The labeling method involving Tc-99m sulfur colloid was also found acceptable. Although similar methods have been useful for diagnostic purposes (20), the short half-life of Tc-99m has resulted in a growing acceptance of In-111 oxine for studies where neutrophils labeled with a gamma-emitting nuclide are required. Experiments with [^{32}P]DFP gave the highest labeling and viability yields in this study. As it is apparently difficult to use neutrophils labeled with In-111 oxine for assessing neutrophil kinetics (*21*), it may not be advisable to abandon completely the use of the more hazardous [^{32}P]DFP as a labeling method in favor of In-111 oxine. Perhaps new methods using DFP labeled with other nuclides will be useful to avert the radiation burden associated with P-32. Currently, methods involving $^{99m}TcO_4^-$ and $SnCl_2$ seem to be ill-advised. Although chemotactically competent labeled cells can be obtained, the adverse effects of $SnCl_2$ on neutrophil function may be encountered.

There are certain limitations to the chemotaxis radioassay that may not be entirely avoidable. First, to minimize variables, we prepared cells by a standard method that involved erythrocyte lysis and several washings in buffer. When neutrophils are labeled for diagnostic purposes, much gentler methods are used. The development of new radiopharmaceuticals such as In-111 tropolone has allowed cell labeling to be accomplished in plasma (*22*). It may not be possible to assess such modifications adequately by the chemotaxis radioassay, since plasma factors may inhibit or stimulate chemotaxis. Second, cell performance in the chemotaxis assay may be directly affected by the dose of radiation absorbed after labeling. This may vary widely from one radionuclide to another, especially in light of the variations in labeling yield observed. In a recent study, ten Berge et al. reported that the proliferative response of lymphocytes decreased sharply after exposure to 5 μCi In-111 per 10^7 cells (23). At a dose of 9 μCi/10^7 cells, In-111 oxine caused chromosomal damage to a degree similar to that obtained with 200 rad of x-rays. We note, however, that neutrophils are remarkably radioresistant: doses of ionizing radiation of up to 20,000 rad have only a slight inhibitory influence on neutrophil chemotaxis (*24*).

In conclusion, our results suggest that pertinent data relating to the viability of labeled cells may not be readily obtained by independent analysis of viability and the total radioactivity of the cell suspension. With neutrophils, this problem can be circumvented by exploiting chemotactic function in selecting viable cells from the preparation. Improvements in existing methods for neutrophil labeling may be facilitated by assessing any alterations in viable cell labeling caused by the new method. The chemotaxis radioassay could also be applied periodically to ensure that a labeling method used for clinical purposes provides labeled cells that retain chemotactic viability.

FOOTNOTES

* Syncor, Inc., Sylman, CA.
† Histopaque, Sigma, St. Louis, MO.

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