Leukocyte Labeling with Technetium-99m Tin Colloids

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Triple density gradients of metrizamide in plasma (MP) were used to characterize label distribution in human leukocyte preparations incubated with ^{99m}Tc tin colloids. Less than 50% of the cell-associated radioactivity was specifically bound to leukocytes when heparinized blood was rotated with stannous fluoride colloid ([Tc]SFC). Labeling efficiency in leukocyte rich plasma (LRP) averaged 44%, of which >90% was specifically bound to leukocytes. MP-gradient analysis also revealed that leukocyte labeling did not occur with stannous chloride colloid, nor when citrate was present during rotation with [Tc]SFC. When citrate was added after labeling to "solubilize" unbound [Tc]SFC, radiocolloid was removed from the leukocytes, indicating that the mechanism of [Tc]SFC labeling is adherence rather than phagocytosis. Technetium-labeled neutrophils exhibited normal in vitro chemotaxis and no lung uptake in vivo. Technetium-labeled mononuclear leukocytes, on the other hand, exhibited prolonged lung transit in vivo. Neither [Tc]SFC cell preparation showed signs of in vivo reoxidation to pertechnetate.

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Over 10 years ago, methods were developed to label leukocytes with particulate radiopharmaceuticals. English and Andersen (*1*) utilized technetium-99m (^{99m}Tc) sulfur colloid ([Tc]SC), while Fisher et al. (*2*) and McAfee and Thakur (*3*) used ^{99m}Tc millimicrospheres. By incubating isolated leukocytes, buffy coat cells, or whole blood with the radiocolloid, radioactivity quickly and specifically became fixed to the phagocytic leukocytes, namely neutrophils and monocytes. In vitro viability studies showed that there was no detriment of function with radiocolloid labeling (*1*), while in vivo studies demonstrated that such labeled cells were capable of visualizing foci of acute inflammation (*4–6*) and experimentally induced abscesses (*2*).

Leukocyte labeling research soon shifted to the use of lipid soluble chelates of indium-111 (^{111}In) with the introduction of the oxine ligand by McAfee and Thakur in 1976 (*7*). The labeling yields were much higher and purification much easier with [^{111}In]oxine than had been reported with Tc colloids. But, since the mechanism of [^{111}In]oxine labeling is nonspecific (*8*), it is necessary to purify leukocytes prior to labeling. To obtain the desired high labeling efficiencies with the oxine chelate requires that leukocytes be washed free of plasma proteins before incubation. Such treatment has been implicated in altering cell function (*9*).

In 1981, Schroth et al. (*10*) revived interest in the labeling of phagocytic white blood cells with a new Tc-colloid. They reported successful labeling of leukocytes when heparinized whole blood was incubated with [^{99m}Tc]stannous fluoride colloid prepared from a commercial radiopharmaceutical kit. After a 1-hr incubation, trisodium citrate was added to the mixture to "solubilize" unbound colloid and facilitate its removal from the labeled blood cells. In 1984, Hanna et al. (*11*) reported on a modification of the Schroth procedure. While reporting that stannous fluoride solution must be freshly prepared for each dose of [^{99m}Tc]colloid, this group presented very promising clinical studies utilizing leukocytes labeled with this new radiocolloid (*12*).

Because of the many advantages that ^{99m}Tc-labeled leukocytes would have over ^{111}In-labeled leukocytes, we decided to re-evaluate the feasibility of labeling leukocytes with ^{99m}Tc tin colloids. Our initial goals were (a) to reproduce the labeling method of Hanna et al. (*11*) using whole blood and fresh stannous fluoride solution, (b) to develop a stable kit form of stannous fluoride, (c) to determine if stannous chloride could also be used to prepare Tc-colloid suitable for leukocyte labeling, and

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(d) to investigate labeling in leukocyte rich plasma (LRP) instead of whole blood.

In order to aid the analysis of specific leukocyte uptake of radiocolloid, our overriding concern was to develop and utilize a more appropriate quality control method to assure that the ^{99m}Tc radioactivity that is centrifuged with the leukocytes is truly cell-associated and not merely an artifact of the sedimentation properties of the colloid.

MATERIALS AND METHODS

Density Gradient Analysis of Tc-Colloid-Labeled Leukocytes

Aliquots of various Tc-labeled leukocyte suspensions were centrifuged through triple discontinuous density gradients of metrizamide* in plasma (MP) in order to separate mononuclear leukocytes from neutrophils, erythrocytes, and free colloid.

Three MP-gradient solutions were prepared as follows: 40%, 50%, and 60% v/v (i.e., 40% MP = 4 volumes of a stock 35% w/v metrizamide solution + 6 volumes of plasma). The discontinuous gradients were prepared in 7 ml tubes by *under*laying 1–2 ml of plasma (containing an aliquot of the test suspension) with 1.5 ml of 40% MP by using a 22 g × 3.5 in. disposable spinal needle. Then 1.5 ml of 50% MP and 1.5 ml of 60% MP were added, respectively. Loaded gradient tubes were centrifuged in a swinging bucket rotor for 20 min at 2,000 *g* to separate the leukocytes.

After centrifugation, two distinct leukocyte bands were observed; one at the interface between the plasma and the 40% MP, and the second between the 40% and 50% MP-gradient solutions. Microscopic analysis revealed that the upper band consisted of mononuclear leukocytes and platelets, with <5% neutrophil contamination. The second band contained >95% of the applied neutrophils (PMNs). Erythrocytes sedimented to the interface between the 50% and 60% MP solutions. As demonstrated below, free radiocolloid pelleted at the bottom of the triple density gradient tubes.

The distribution of radioactivity in the tubes was determined by imaging with a gamma camera. Applying region of interest analysis to the counts at each interface and the entire tube, we were able to determine the portion of radioactivity associated with each specific cell population.

Preparation of ^{99m}Tc Tin Colloids

A stock solution of stannous fluoride (SF) was prepared according to the method of Hanna et al. (*11*), and consisted of an aqueous solution of stannous fluoride† (0.125 mg/ml) and sodium fluoride† (1.00 mg/ml). The solution was sterilized by 0.22-μm membrane filtration and stored frozen in 1-ml portions to enhance tin(II) stability. A 1-ml portion is chemically equivalent to a single vial of the kit used by Schroth et al. (*10*).

Technetium-99m stannous fluoride colloid ([Tc]SFC) was prepared by mixing 1 ml [^{99m}Tc]pertechnetate (100–500 μCi) with 1 ml of SF and rotating the mixture for 1 hr. Technetium-99m stannous chloride colloid ([Tc]SCC) was prepared in like manner using equimolar concentrations of stannous chloride‡ and pertechnetate. Fresh dilutions of stannous chloride were prepared from a concentrated stock solution as previously described (*13*).

The chemical concentration of technetium was held constant at 0.10 μM by including 200 pmol of carrier [^{99}Tc] pertechnetate.§ This amount of chemical pertechnetate is equivalent to ~30 mCi of ^{99m}Tc radioactivity at elution of a ^{99}Mo/^{99m}Tc generator with 24 hr of buildup time (*14*). Radiochemical purity of ^{99m}Tc tin colloids was determined using ITLC-SG¶ thin layer chromatography with acetone as the solvent.

Cell Labeling

Leukocyte rich plasma (LRP) was generated by adding 2 ml of 6% hydroxyethylstarch** in saline (HES) to 20 ml heparinized whole blood (5 IU/ml) and allowing erythrocyte sedimentation to proceed for 1 hr at room temperature, during which time the radiocolloids were also prepared.

To compare the relative cell labeling properties of [Tc]SFC and [Tc]SCC, equimolar quantities of the respective colloids were rotated for 1 hr at room temperature in the ratio of 1 ml colloid to 5 ml LRP. The labeled LRP suspensions were then transfered to clean centrifuge tubes, softly spun (10 min at 100 *g*), and washed with plasma to determine the "apparent" labeling efficiency. Aliquots were then taken from each mixture and analyzed with the MP-gradient system to determine the true leukocyte labeling yield.

To compare the relative leukocyte labeling yields in whole blood and LRP, equal 1-ml amounts of [Tc]SFC were rotated for 1 hr with duplicate 10-ml samples of heparinized blood and with duplicate 5-ml samples of LRP. Aliquots were taken from each incubation mixture for MP-gradient analysis. All mixtures were softly spun and the supernatant plasma discarded. The packed whole blood cells were washed twice with saline, while the LRP cell pellets were washed once with plasma. Aliquots of the washed cells were then applied to the MP-gradient tubes for analysis of labeled cell purity.

Viability of Labeled Leukocytes

We examined the chemotactic viability of [Tc]SFC-labeled leukocytes in dual membrane chemotaxis chambers as previously described (*15*). Following the incubation, both membrane filters were carefully removed from the chambers and washed. The radioactivity in the lower membrane of each chamber (the filter *not* contacting the labeled cell suspension) was quantified in a sodium iodide detector to determine the target to nontarget ratios.

Biodistribution of Tc-SFC-Labeled Leukocytes

On three separate occasions, three distinct [^{99m}Tc]leukocyte formulations were prepared from blood of a single volunteer: (a) plasma-washed mixed leukocytes (from LRP), (b) MP gradient-purified granulocytes, and (c) purified mononuclear leukocytes. For each study, 2 mCi of the respective ^{99m}Tc-labeled leukocytes were injected, and images were obtained hourly (from 0.5 to 5.5 hr) of the anterior thorax, liver and spleen, and pelvis.

RESULTS

Radiocolloid Sedimentation Properties

One of the problems associated with using a radiocolloid to label leukocytes is the probability that free

colloid will spin down with the cells, making it difficult to determine the extent to which the colloid is truly affixed to the leukocytes. In our initial experiments, substantial portions of both Tc tin colloids pelleted to the bottom of the tubes when diluted in plasma or saline and softly centrifuged (Fig. 1). Even though some radioactivity remained in the supernatant, it was apparent that soft centrifugation would not completely remove unbound radiocolloid from labeled cells.

The sedimentation properties of both colloids in the MP-gradient system are illustrated in Figures 2A (SCC) and 2C (SFC). Both Tc-colloids penetrated all interfaces and pelleted at the bottom after centrifuging for 20 min at 2,000 *g*. Figure 2B is included to illustrate the effect of adding ~0.2 ml of blood to a [Tc]SFC-plasma mixture immediately prior to centrifugation. The mere addition of whole blood caused ~10% of the radioactivity to collect at the 50–60% MP interface, which is the sedimentation position of erythrocytes in the MP gradient system. Thus, a substantial portion of the radiocolloid quickly became nonspecifically associated with the red blood cells.

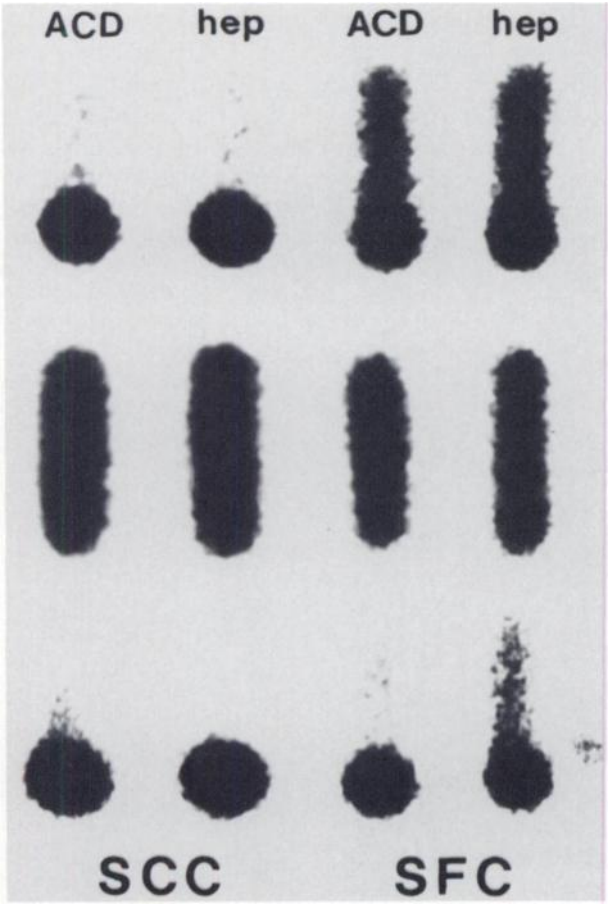

FIGURE 1
Sedimentation of ^{99m}Tc tin colloids after soft centrifugation (10 min at 100 *g*). Top: >95% of SCC radioactivity pelleted in both ACD- and heparinized-plasma, whereas ~70% of SFC activity pelleted in both plasmas. Middle: Saline resuspension of the plasma colloid pellets above, indicating that colloid does not stick to the tube or fail to redisperse. Bottom: Soft-spin pelleting of ^{99m}Tc tin colloids through the above saline wash solution.

Leukocyte Labeling with [Tc]SCC and [Tc]SFC

We observed a marked disparity in the leukocyte labeling properties of the two colloids in LRP. The labeling efficiency of [Tc]SCC initially appeared to be more than twice that of [Tc]SFC (as determined by soft centrifugation). However, using the MP-gradient system (Fig. 3), we found that nearly all of the SCC radioactivity was still free colloid and not cell bound. From this observation, we concluded that the *chloride* form of Tc tin colloid ([Tc]SCC) was not suitable for leukocyte labeling. Accordingly, no further labeling studies were carried out with [Tc]SCC. The *fluoride* form ([Tc]SFC), on the other hand, did label leukocytes selectively during LRP rotation. Less than 10% of the radioactivity in the plasma-washed cell pellet was found to be free colloid. Activity was seldom found in the erythrocyte layer.

Effect of Citrate on [Tc]SFC and Cell Labeling

In control experiments, 10% v/v of 3.8% trisodium citrate was rotated for 1 hr with [Tc]SFC in plasma. The citrate appeared to dissolve much of the colloid, since minimal sedimentation was seen when tubes were softly spun (Fig. 4). However, the presence of colloid was still apparent, even after 1 hr, when the centrifugation was increased to 1,000 *g*. Obviously, citrate *does* alter the sedimentation of [Tc]SFC in plasma, but it does *not* solubilize [Tc]SFC when in plasma.

We also added citrate both before and after the labeling rotation to determine if this altered colloid sedimentation had any beneficial effect on the final labeled cell purity. Using soft centrifugation, we obtained a higher "apparent" labeling efficiency with ACD-LRP than with heparinized LRP (61% versus 45%). However, MP-gradient analysis (Fig. 5) revealed instead that virtually no leukocyte labeling occured in the presence of ACD, whereas selective labeling was seen in the heparinized sample. When citrate was added to each LRP mixture *after* the labeling rotation, the [Tc]SFC was effectively removed from the heparinized leukocytes as free colloid.

This experiment clearly illustrates that serious errors in interpretation can occur when relying only upon simple centrifugation to analyze preparations of labeled cells, and further attests to the value of the MP density gradient approach for this purpose.

[Tc]SFC Labeling of Whole Blood Versus LRP

When heparinized whole blood was incubated with [Tc]SFC (n = 4), 85–95% of the radioactivity appeared to be cell-bound after simple centrifugation. When LRP was incubated with [Tc]SFC (n = 18), only 44 ± 11%

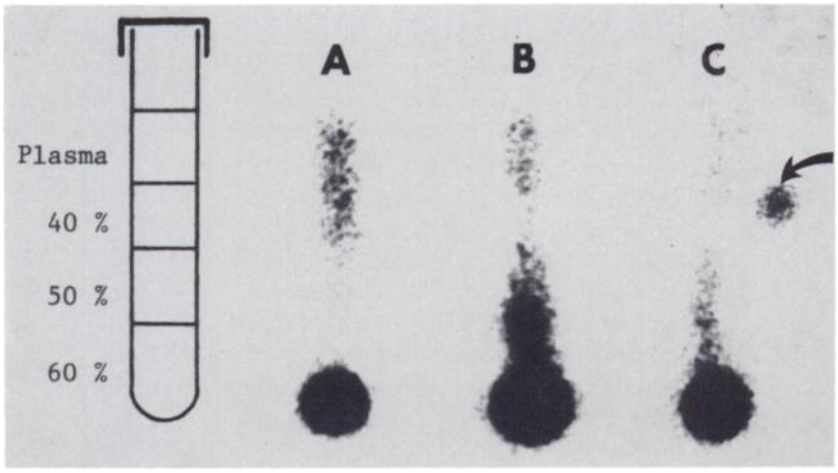

FIGURE 2
Sedimentation of [Tc]SCC (A) and [Tc]SFC (B, C) through the triple MP-gradient after 10 min of centrifugation at 2,000 *g*. Tube B illustrates effect of adding a small volume of whole blood to SFC-plasma suspension immediately prior to centrifugation. Activity seen at 50%:60% gradient interface represents nonspecific RBC binding of SFC. Marker (arrow) at level of first gradient interface.

of the radioactivity appeared to be cell-associated after simple centrifugation. MP-gradient analysis, however, revealed very different purities for the two labeling media. Whole blood (Fig. 6A) showed incomplete leukocyte labeling with low specificity. Washing the blood cells twice with saline (Fig. 6B) appeared to remove most of the free colloid, but it did not remove the colloid bound to the red blood cells, a large yet variable fraction of the cell-bound radioactivity. Labeling in LRP resulted in more selective leukocyte uptake of colloid. Some free colloid could be seen in the unwashed LRP mixture (Fig. 6C), but very little activity was associated with the red cell layer. A single plasma wash of the LRP cell pellet surprisingly removed most of the unbound Tc-colloid, yielding a [^{99m}Tc]leukocyte preparation of consistantly high purity (Fig. 6D).

Biostability of [Tc]SFC-Labeled Leukocytes

After overnight rotation of [Tc]SFC labeled cells in autologous plasma, only 15% of the radioactivity was eluted. Most of the eluted activity remained as Tc-colloid, as determined by ITLC-SG chromatography.

Using the chemotaxis radioassay method (*15*), we found that leukocytes labeled with [Tc]SFC responded normally to chemotactic stimulation in vitro. More importantly, the dual membrane chemotaxis chambers

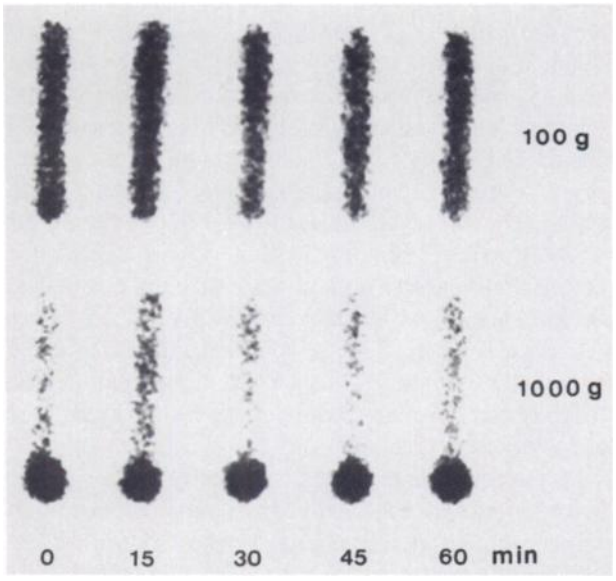

FIGURE 3
Labeled cell purity of plasma washed leukocyte pellets after 1 hr ^{99m}Tc colloid rotation with heparinized leukocyte rich plasma. The majority of SCC radioactivity remained as free nonbound colloid, whereas little free colloid was present when SFC was used to label leukocytes in LRP suspension. Note the lack of RBC activity at 50%:60% gradient interface.

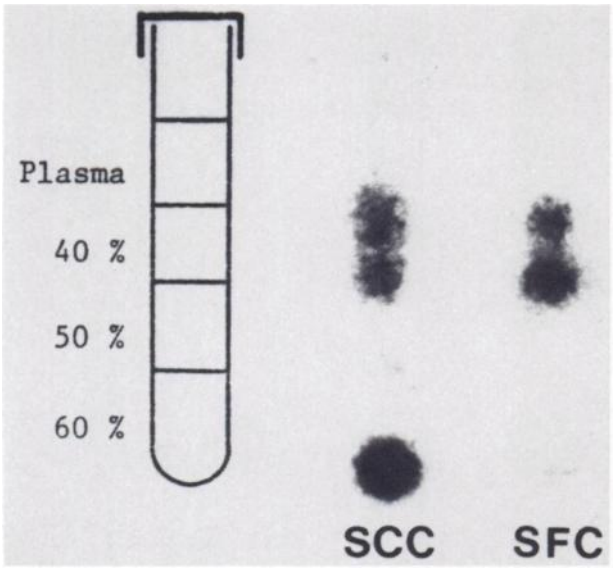

FIGURE 4
Effect of citrate incubation on [^{99m}Tc]SFC sedimentation through heparinized cell free plasma. Top: Minimal colloid pelleting observed after 10 min of soft centrifugation, giving the appearance of colloid solubilization. Bottom: Re-spinning of the tubes above for 10 min at 1,000 *g* revealed the continued presence of radiocolloid. Altered sedimentation property of [Tc]SFC (cf Fig. 1) is consistent with smaller particle size.

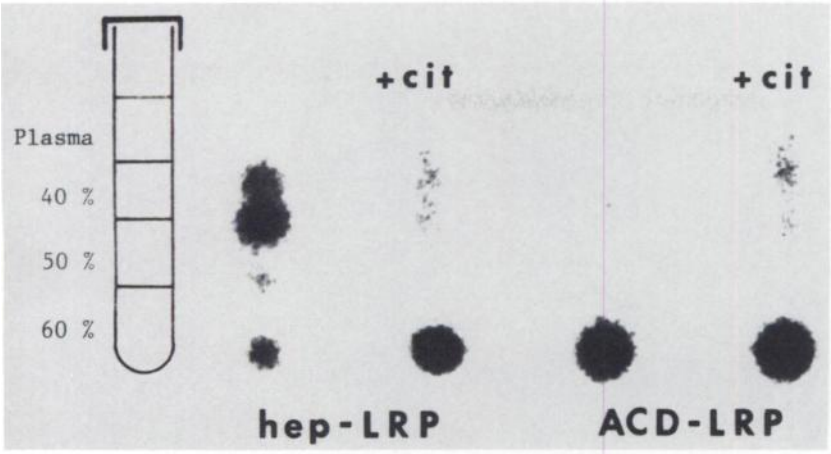

FIGURE 5
The effect of citrate on leukocyte uptake and retention of [^{99m}Tc]SFC. Specific leukocyte uptake occured when labeling was carried out in heparinized LRP (Tube 1), but not when ACD was used as the anticoagulant (Tube 3). After citrate was added and rotation continued for 1 hr (+cit, Tubes 2, 4), radiocolloid was removed from the heparinized leukocytes, suggesting that the mechanism of cell uptake is surface adherence rather than phagocytosis.

demonstrated that the specific cells that migrated in response to the chemoattractant were in fact labeled with ^{99m}Tc. In quantitative experiments (n = 4), we measured target to nontarget ratios of 22.1 ± 6.5 to 1, indicating that 22.1-fold more labeled neutrophils migrated toward the chemoattractant than migrated randomly in the absence of chemoattractant. Thus, the labeled leukocytes were judged to be viable.

Biodistribution of [Tc]-Labeled Leukocytes

Thirty minutes after the injection of ^{99m}Tc-mixed leukocytes (mWBCs, Fig. 7), images revealed lung uptake of the same intensity as the liver/spleen; however, this lung accumulation disappeared by 3–4 hr. When purified [^{99m}Tc]granulocytes were injected (GRAN, Fig. 7), virtually no lung uptake of tracer was visualized, either at 0.5 or 5.5 hr postinjection. To confirm that monocytes were responsible for this initial lung uptake, purified ^{99m}Tc-labeled mononuclear leukocytes were injected in the third in vivo study. Ten minutes after injection, the lung activity was greater than that in the liver or spleen, while at 30 min, the lung activity was equal to that of the liver. By 4 hr postinjection, the lung activity was virtually gone, and there was apparently normal distribution of tracer in the liver, spleen, and bone marrow. We did not see uptake or accumulation of radioactivity in the thyroid, stomach, kidney or bladder during the first 6 hr postinjection, suggesting no in vivo reoxidation of label to free [^{99m}Tc]pertechnetate.

DISCUSSION

While several groups have reported successful selective labeling of blood leukocytes with [^{99m}Tc]radiocolloids (*1–6,10–12*), techniques based on this approach have not gained wide acceptance in spite of the imaging and dosimetry advantages of ^{99m}Tc over ^{111}In. Many of the problems associated with interpretation, reproducibility, and acceptance of these techniques stem from the unusual and often anomalous sedimentation properties of the radiocolloids used. There are a number of other problems inherent in colloid-based leukocyte labeling techniques. In their original work, McAfee and Thakur found that less than half of the radioactivity in preparation of cells previously exposed to [^{99m}Tc]sulfur colloid was cell bound, and that much of this activity resulted from nonspecific adsorption of the colloid to the cells (*3*). In addition, McAfee et al. (*16*) have reported very poor results with the ^{99m}Tc-colloid technique of Schroth et al., and others have had similar

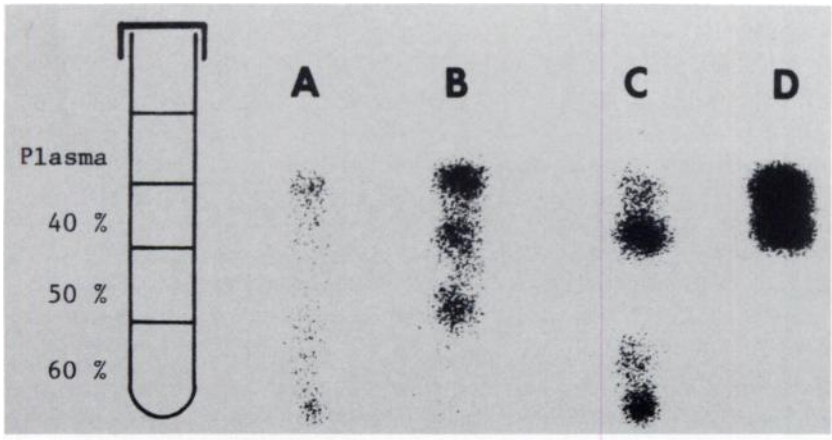

FIGURE 6
Radiocellular purity of [^{99m}Tc]SFC labeling in heparinized whole blood versus leukocyte rich plasma. A = Whole blood labeling without citrate addition; B = Whole blood labeling after two saline washes; C = LRP labeling without citrate or washing; D = LRP pellet after only one plasma wash.

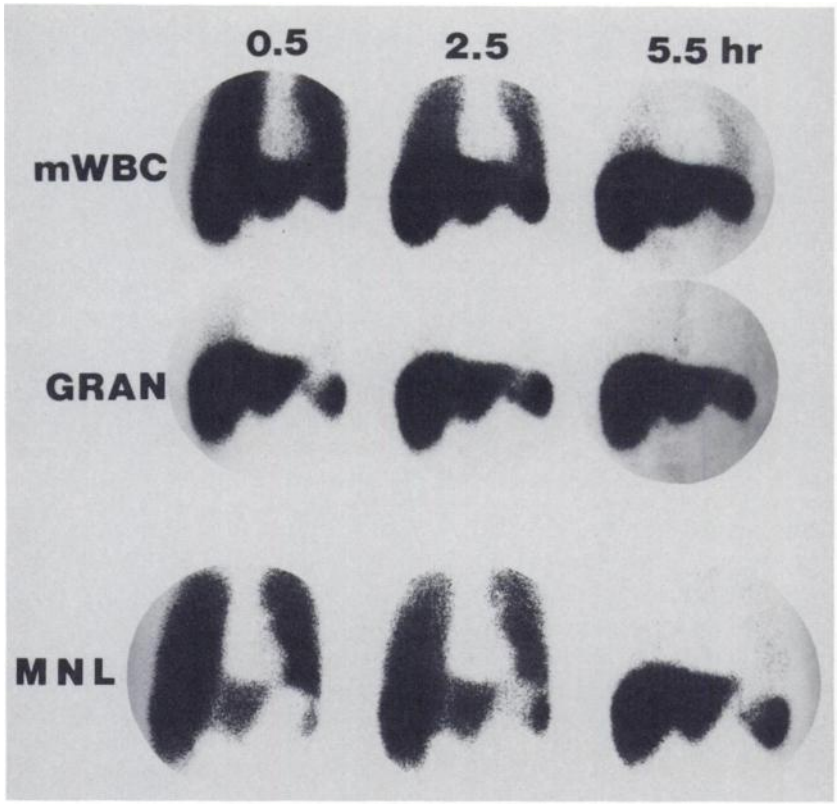

FIGURE 7
In vivo images of [^{99m}Tc]SFC-labeled leukocytes in a normal human subject. Early lung uptake was evident when plasma washed mixed leukocytes (mWBC) were re-injected. However, this lung activity cleared within a few hours. When granulocytes were purified with MP-gradient separation and re-injected (GRAN), no lung accumulation of tracer was seen, suggesting that monocytes were responsible for the initial lung uptake. When purified mononuclear leukocytes (MNL) were separated from granulocytes using MP-gradient centrifugation and re-injected, intense lung uptake of tracer was evident. However, lung activity cleared at the same rate as mWBC, confirming that monocytes were responsible for initial lung uptake.

problems (*17*). Furthermore, if radiocolloids are taken up by phagocytosis, subsequent structural or metabolic alterations may alter the cells' viability and in vivo distribution. Finally, McAfee has stressed that, for analysis and use of radiocolloid-labeled leukocytes, refined techniques will be necessary to separate cells that have phagocytized radiocolloid from the free colloid in the labeling mixture. Radiocolloids do, however, provide a potential mechanism to *selectively* label phagocytic cells within a mixed cellular population, and thereby merit further investigation as cell labeling reagents.

The present study has specifically addressed two of the concerns stated above—namely the reproducibility of previous results and the adequate separation of free radiocolloid from labeled cells. From our previous work with dual Ficoll:Hypaque density gradients (*1,4,6,18–20*), we observed that both mononuclear leukocytes and neutrophils band in high purity at the two gradient interfaces. However, not all of the neutrophils in blood or LRP are recovered in one band. Instead, a significant number of the neutrophils penetrate the second interface with the erythrocytes. In effect, while the Ficoll:Hypaque dual density gradient system will purify granulocytes for subsequent labeling, it is not an optimal technique for the accurate determination of radioactivity distribution in preparations of labeled leukocytes or whole blood.

We obtained better results with the triple density gradient system of metrizamide:plasma described in this report. The MP-gradient system easily and reproducibly separated neutrophils from mononuclear leukocytes, erythrocytes and free Tc-SFC, provided that a small sample of LRP (≤1 ml) or whole blood (≤0.1 ml) was applied to the gradient. This method should be useful to evaluate other labeling procedures, including those that use soluble tracers.

We successfully reproduced the ^{99m}Tc tin colloid labeling method described by Hanna et al. (*11*), using both freshly prepared and frozen stannous fluoride solution to prepare the radiocolloid. However, when the labeled whole blood mixture was analyzed by our MP-gradient method, we found that much of the apparent cell bound activity was instead nonspecifically associated with erythrocytes. Less than half of the cell-bound activity was leukocyte associated. This may help explain the conclusions of others that, when leukocytes are labeled with Tc-colloids and re-injected, the rapid blood clearance, low level of circulating activity, and high liver uptake represent cell activation and/or damage (*16*). If more than half of the injected radioactivity is instead free or loosely bound Tc-colloid, one would expect the blood clearance to be rapid, the circulating activity to be low, and liver uptake to be high.

We found that the addition of trisodium citrate caused the release of label from the leukocytes, precluding its use as a Tc-colloid solubilizing agent. Instead, this observation refutes the hypothesis that leukocytes are labeled with [Tc]SFC by the process of phagocytosis. Specific cell surface adherence is the more likely mech-

anism. Our results are also consistent with the work of McAfee and Thakur (*3*) who used ACD to remove other radiocolloids from blood cell surfaces.

Marked differences were seen in the biodistribution kinetics of ^{99m}Tc-labeled leukocytes, purified granulocytes and purified mononuclear leukocytes. The assumption that lung uptake of labeled leukocytes represents cell activation or cell damage (*9,16*) may not be entirely valid. In the case of [Tc]SFC-labeled white blood cells, monocytes seem to have a prolonged transit time through the lungs, while granulocytes are not retained. As reported by Clay et al. (*21*), when human monocytes were elutriated from 500 ml of whole blood, labeled with [^{111}In]oxine and re-injected, the cells were seen to have virtually complete lung retention with only 2–3% in the circulation. By 5 hr after injection, the lungs had cleared. This supports our observations with ^{99m}Tc-labeled monocytes obtained from only 20 ml of whole blood.

The potential clinical value of ^{99m}Tc-labeled neutrophils is apparent from the widespread clinical acceptance of ^{111}In-labeled leukocytes to localize infection. The value of ^{99m}Tc-labeled monocytes, however, has yet to be established. Recent experiments indicate that Tc-labeled monocytes may be useful for imaging neoplastic tissue (*22*). Labeled monocytes may also be useful for localizing areas of immune reactivity and for studying monocyte kinetics in normal individuals or patients with hematological disorders (*23*). The favorable in vitro and in vivo stability results of this study should encourage the continued clinical investigations of [^{99m}Tc]SFC-labeled leukocytes.

NOTES

* Nyegaard & Company, Oslo, Norway.

† Sigma Chemical Company, St. Louis, MO.

‡ Aldrich Chemical Company, Milwaukee, WI.

§ DuPont Company, No. Billerica, MA.

¶ Gelman Sciences, Inc., Ann Arbor, MI.

** American McGaw, Division of American Hospital Supply Corp, Irving, CA.

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