In Vitro and In Vivo Evaluation of Granulocyte Labeling with [^{99m}Tc]d,l-HM-PAO

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The functional integrity of white blood cells labeled with [^{99m}Tc]d,l-HM-PAO containing variable amounts of the ligand or of the ^{99m}Tc activity was evaluated by enzymatic tests and by measuring random migration, chemotaxis, phagocytosis, killing, and adhesion. The ultrastructure of labeled cells was studied by electron microscopy. The tracer dose and the HM-PAO concentration did not significantly affect phagocytosis and killing. The results of the other tests remained normal. A maximum labeling efficiency of 80% was reached by incubating the granulocytes for 20 min with 10–20 mCi of [^{99m}Tc]d,1-HM-PAO containing 50 μg of the ligand in 1 ml of saline. There was only a slow washout of 20% of activity from the labeled cells in 24 hr. The ultrastructure was not influenced by the labeling technique. Proven infection sites of 17 orthopedic patients were clearly visualized. After a short transient lung uptake, there was a clear spleen and moderate liver uptake with early bladder and late prominent colon visualization. Because of the lower cost, favorable radiation dose and more suitable tracer characteristics, this technique is a promising alternative for ^{111}In labeling of white blood cells.

J Nucl Med 30:2022–2028, 1989

Indium-111- (^{111}In) labeled white blood cells have been used for years in the detection of infection. This technique combines both high sensitivity and specificity but the nonideal physical characteristics of the radionuclide are a disadvantage. As technetium-99m (^{99m}Tc) is the preferred radioisotope in view of its availability, ideal physical characteristics and low cost, many attempts have been made for more than 15 years to replace indium by technetium (*1,2*).

The most successful approaches to incorporate ^{99m}Tc in granulocytes have been the reduction of pertechnetate by stannous compounds diffused in the WBC in a similar way as used in red blood cell labeling (*3–5*) and the incorporation of ^{99m}Tc colloids by phagocytosis (*6,7*). In an animal study with ^{99m}Tc-labeled granulocytes obtained by the stannous pyrophosphate reduction method, Harper et al. mentioned the same radiation dose and less of a tendency to accumulate in the pus as compared to ^{111}In-labeled cells (*3*). Using WBC labeled with ^{99m}Tc colloids, Hanna and coworkers found a prolonged lung transit time that can be explained by aggregation or activation of the neutrophils (*6*). The results reported by Gil et al. when using stannous glucoheptonate as reducing agent could not be confirmed in our laboratory (*8*).

Recently, Peters et al. (*9*) described the possibility of labeling granulocytes with [^{99m}Tc]hexamethylpropylene amineoxime ([^{99m}Tc]-d,1-HM-PAO), a new radiopharmaceutical introduced for brain imaging (*10,11*). Because of its lipophilic character this agent is able to diffuse through the cell membrane and apparently binds intracellularly. The underlying mechanism is not fully understood.

The aim of the present study is the evaluation of this new labeling technique by checking the in-vitro and in vivo behavior of the technetium-labeled cells. The integrity of the cells was tested by means of electron

Received Feb. 24, 1988; revision accepted July 28, 1989.

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microscopy and enzymatic and functional tests. Clinical studies have been performed in orthopedic patients with proven infections to demonstrate the clinical usefulness of the labeled granulocytes.

MATERIALS AND METHODS

Separation of White Blood Cells

The separation method is schematically shown in Figure 1.

Sedimentation of red blood cells for 1 hr to obtain leukocyte and platelet-rich plasma (LRP) was facilitated by the addition of 2 ml of 2% (w/v) methylcellulose to a mixture of 10 ml ACD and 50 ml whole blood. After centrifugation (150 *g*, 5 min) of the LRP, a second centrifugation (2500 *g*, 7 min) of the supernatant (platelet-rich plasma or PRP) was performed follows by filtration of the supernatant (platelet-poor plasma or PPP) through a 0.45 μm filter resulting in cell-free plasma (CFP).

The leukocyte-rich pellet was resuspended in 1 ml of Hanks' balanced salt solution (HBSS) containing 0.1% albumin, and the remaining red blood cells (RBCs) were eliminated by hypotonic lysis for 20 sec. The debris of the RBC on the surface of the pellet obtained after a subsequent centrifugation (150 *g*, 5 min) was eliminated by gently pipetting 1 ml of HBSS and aspirating the fluid. The remaining pellet was resuspended in 2 ml of cell-free plasma.

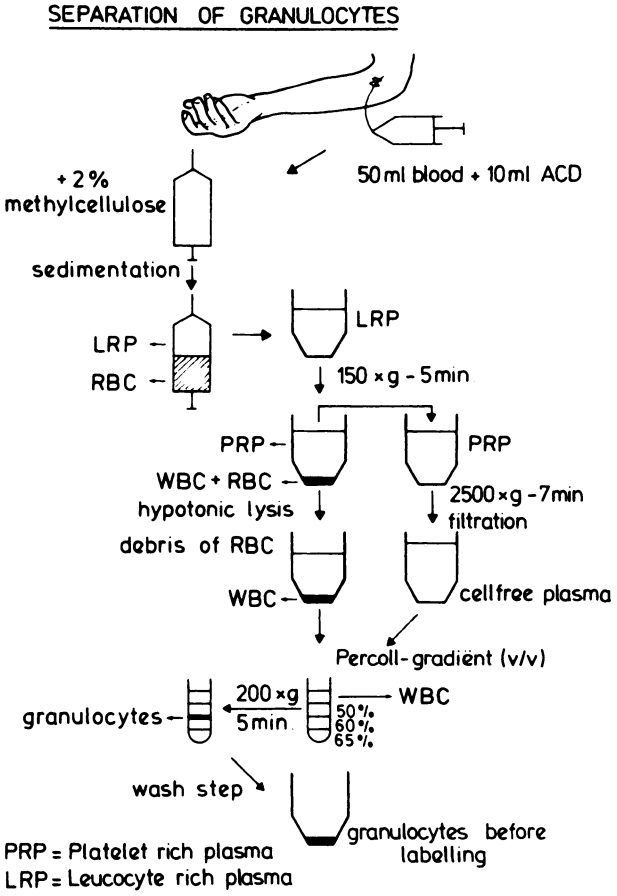

FIGURE 1
Schematic survey of separation procedure.

Further purification was performed by discontinous gradient centrifugation of the mixed WBC pellet applied on the top of three layers of Percoll-plasma mixtures of increasing density (65%, 60%, 50% (v/v) Percoll with a density of 1.129 g/ml). The bottom layer was washed with 4 ml of 0.9% (w/v) NaCl and centrifuged (150 *g*, 5 min) to obtain a WBC pellet.

Labeling Method

d,l-HM-PAO was synthesized following a published procedure (*11*). Five micrograms of $SnCl_2 \cdot 2H_2O$ in 2.5 μl HCl 0.05 N was added to 0.5 mg HM-PAO dissolved in 1 ml of water. Neutralization of the very small amount of HCl during labeling was not necessary as the final pH was still slightly alkaline. 120 mCi of [^{99m}Tc]-$NaTcO_4$ dissolved in 4 ml saline was added. The preparation was diluted with saline to a concentration of 20 mCi/ml. The pellet of granulocytes after Percoll separation was resuspended immediately in 20 mCi of [^{99m}Tc]d,l-HM-PAO in saline (1 ml) or was first resuspended in 1 ml of 0,9% NaCl and then added to 20 mCi of [^{99m}Tc] d,l-HM-PAO in 1 ml. The incubation time was 20 min. After centrifugation, the radioactivity associated with the cells was determined. Before use, each preparation of [^{99m}Tc]-d,l-HMPAO was analyzed by thin-layer chromatography on ITLC SG sheets with acetone and saline as mobile phases to check for "polar" [^{99m}Tc]HM-PAO and pertechnetate, respectively. Paper chromatography with acetonitrile-water (1:1) as the mobile phase was used to determine the content of colloidal ^{99m}Tc. Only preparations with a radiochemical purity over 88% were used for WBC labeling.

Evaluation of the Incorporation and the Washout of the Tracer

Aliquots of the incubation mixture were withdrawn at different time intervals (5, 10, 15, 20, 25, 30, 40, 50, and 60 min), and the radioactivity in the cells and the supernatant was measured after centrifugation.

After labeling, the cells were resuspended in plasma and preserved at 37° C for 24 hr after labeling. Aliquots were taken at 1, 2, 3, 4, 5, and 24 hr, and the radioactivity associated with the cells was measured after centrifugation.

In Vitro Tests of Granulocyte Function

1. In the first experiment (n = 3), the cells (30×10^6) were incubated for 20 min with different concentrations of HM-PAO (0.05, 0.25, and 0.5 mg HM-PAO/ml). The added amount of tracer varied between 1 and 15 mCi.

2. In a second experiment (n = 3), the tracer dose, dissolved in a constant volume, was varied (0, 1, 5, and 15 mCi) while the concentration of HM-PAO (0.25 mg/ml), the incubation volume (1 ml), and the number of cells (40×10^6) were kept constant.

The labeled cells obtained in these experiments were evaluated with the following enzymatic and functional tests.

The myeloperoxidase (MPO) content of granulocytes was assayed spectrophotometrically using a horseradish peroxidase standard. Enzyme activity is expressed in units of MPO/2.10^6 PMN. Normal values: 11.28 ± 2.91 units/2×10^6 granulocytes.

Superoxide generation (SO) was quantified spectrophotometrically at 550 nm by the superoxide dismutase-sensitive reduction of ferricytochrome C. Zymosan activated by autologous serum (SAZ, 0.5 ml/10^7 PMN) was used as standard stimulant in a concentration of 2.5 ng/dl. The results are

expressed as nmol of cytochrome C reduced per 5.10^6 PMN in 15 minutes. Normal values: 26.91 ± 3.31.

Chemotaxis was evaluated by measuring the migration (μm) under agarose (0.9% w/v agarose). ZAS (zymosan activated serum) and FMLP (formyl-methionyl-leucyl-phenylaline) were used as chemoattractants. Normal values: random migration 430 ± 90 μm; chemotaxis ZAS 1040 ± 310 μm; FMLP 1340 ± 270 μm.

Phagocytosis and intracellular killing capacity of granulocytes were assayed using a pour-plating technique. The results are expressed as a percentage of *Staphylococcus aureus* phagocytosed and of *Staphylococcus aureus* killed after 20 min. The killing/phagocytosis index is an accurate measure of granulocyte antibacterial efficiency. Normal values are: phagocytosis: 83.03 ± 5.07%; killing: 72.7 ± 6.26%; killing/phagocytosis index: 0.86 ± 0.04.

Adherence was assayed by the ability of PMN to adhere to gelatin-coated plastic surfaces (overnight coating of culture plates with 1% gelatin solution). Labeled PMN and a free chelator were added to each well and preincubated at 37° C; after 10 min, the stimulant (FMLP $10^{-7}M$) was added and the plates incubated for another 30 min. The supernatant was then carefully removed, and the pellet was washed twice with warm HBSS-ALB, and then lysed twice with NaOH 0.1 N. The radioactivity in the supernatant and pellet was counted and results expressed as percent adherence. Each assay was performed in quadruplicate, and the results were calculated as the mean percentage of adherence or the mean percentage of control.

Electron Microscopy

Cell pellets were resuspended in 1.5% v/v glutaraldehyde in 0.1M cacodylate buffer (pH 7.3; 370 mOsm/l) for 30 min at room temperature. After washing in 0.1M cacodylate buffer containing 4% w/v sucrose (pH 7.3; 320 mOsm/l), the cells were postfixed for 1 hr in 1% w/v OsO_4 in Sörensen phosphate buffer (pH 7.3; 350 mOsm/l). Cells were washed in cacodylate buffer containing sucrose and stored overnight in 70% v/v alcohol in water. After dehydration in a graded series of ethanol and washing in propylene oxide, the cells were resuspended in a mixture of 1 volume of propylene oxide and 4 volumes of epon for 1 hr. After centrifugation, the propylene-oxide-epon mixture was replaced by epon. The cells were embedded in Beem polyethylene capsules and centrifuged at 9,000 g for 30 min before polymerization.

The cell pellets were sectioned with a diamond knife on an LKB III microtome. Ultrathin sections, stained with uranyl acetate and lead citrate, were examined with an electron microscope (Zeiss EM 10).

Patient Studies

Seventeen orthopedic patients with proven infections were injected with leukocytes labeled with 10–17 mCi of [^{99m}Tc]-d,1-HM-PAO: eight patients with an infected fracture, seven with an infected total hip prothesis, one with a malum perforans, and one with an infected joint after an intra-articular injection. All the infections were confirmed by cultures, clinical evaluation, radiology, or surgical findings. Eleven patients were injected under a double-headed camera (SELO GAMMA CAT) interfaced to a computer system (PDP 11/34 (DEC)) and were continuously monitored to evaluate lung, liver, and spleen uptake. Delayed images were made 3–4 hr and 21 hr postinjection. Two experienced readers graded the images as follows: 0: negative; 1: possibly positive; 2: suspect; 3: positive.

RESULTS

The incorporation of [^{99m}Tc]d,1-HM-PAO in the white blood cells and the washout of the radionuclide in function of time is shown in Figures 2 and 3. The results of the functional tests of granulocytes labeled with varying amounts of HM-PAO are presented in Table 1. The results of the same tests obtained with a constant amount of HM-PAO but varying tracer activities are shown in Table 2.

The ultrastructure of a neutrophil granulocyte cell after labeling is shown in Figure 4.

Figure 5 shows the time activity curves during the first hour in the region of the lungs, the spleen and the liver of one of the patient studies. A clinical example of uptake of ^{99m}Tc-labeled WBC in an infected fracture of the left ankle of a patient is illustrated in Figure 6. Eleven patients had the highest score; two patients were scored as highly suspect; three were interpreted as possibly infected; and one had a negative scan.

DISCUSSION

The most important reasons for investigation of a labeling technique with ^{99m}Tc are the better physical characteristics of the tracer, the lower radiation dose,

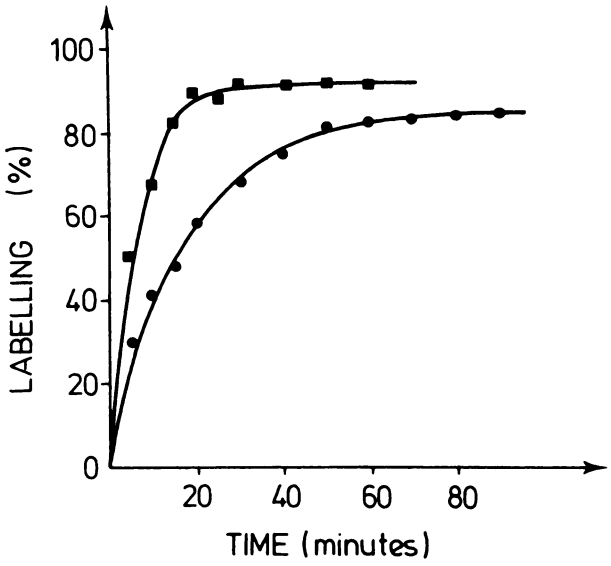

FIGURE 2
Incorporation of [^{99m}Tc]HM-PAO in the leukocytes (%) versus time for two different incubation volumes. "●": The granulocytes (60×10^6) are resuspended in 1 ml of 0.9% NaCl and 1 ml [^{99m}Tc]HM-PAO (54 μg HM-PAO; 11.1 mCi of ^{99m}Tc). "■": The granulocytes (60×10^6) are resuspended in 1 ml [^{99m}Tc]HM-PAO (54 μg HM-PAO; 12.7 mCi of ^{99m}Tc).

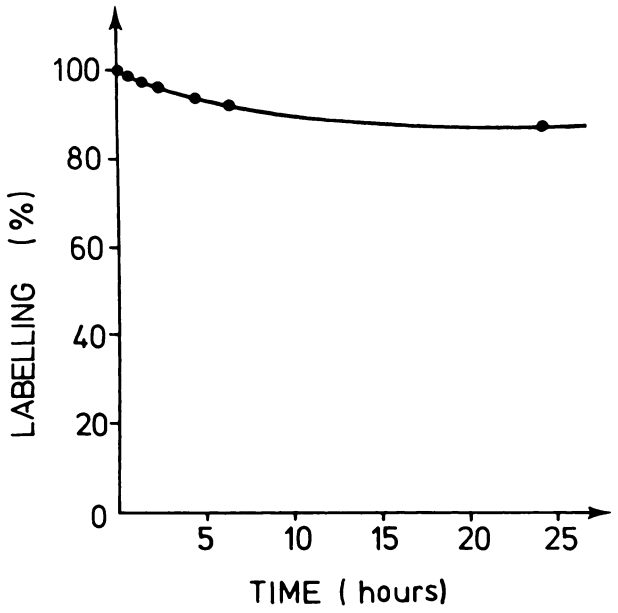

FIGURE 3
Percentage of the radioactivity retained in the cells versus time. After labeling (cfr. Figure 2; method 2), the granulocytes were resuspended in plasma and kept at 37°C for 24 hr. Sequentially the radioactivity remaining in the cells was measured.

the lower cost, and the ready availability of a ^{99}Mo-^{99m}Tc generator. Peters et al. (*9*) recently introduced [^{99m}Tc]d,l-HM-PAO labeling of WBC as a new alternative to currently used methods of labeling with ^{111}In or ^{99m}Tc. Many papers have since described the use of this technique to replace ^{111}In labeling of granulocytes (*12–22*). The success and usefulness of this labeling method will greatly depend on the functional integrity of the white blood cells after labeling, which is necessary for their localization at the inflammation site.

As illustrated by a previous study, it is essential to perform granulocyte functional tests in vitro on the purest possible cell population because contaminating cells (platelets, monocytes) definitely influence the results of the very sensitive granulocyte tests to a varying extent (*23*). For this reason, further steps like hypotonic lysis and percoll gradient were added to the separation procedure. Although it is well known that some activation of cells certainly occurs and although there may be some phagocytosis of silica particles present, sufficient granulocyte functional capacity is preserved to perform experiments in controlled conditions. Phagocytosis, in particular, is still sufficient, as is illustrated by its control value of 93% and 86% (Tables 1 and 2). The labeling of RBC fragments by lipid soluble tracers after the hypotonic lysis is prevented by three extra separation steps: (a) centrifugation of the isotonic mixture, (b) gently pipetting and aspiration of 1 ml of HBSS for the elimination of the debris of the RBC, and (c) further purification by discontinuous gradient centrifugation. Labeling of the isolated cells in saline was performed to enhance the labeling yield since the results of functional and morphologic tests could not demonstrate a deleterious influence of short incubation of the granulocytes in a medium without plasma.

As published previously (*24*), the separation technique developed in our laboratory delivers functionally and morphologically intact cells. In this study, normal values of lung transit time and spleen and liver uptake were measured, which is an argument that the leukocytes were not affected by the labeling procedure (Fig. 5).

TABLE 1
Functional and Enzymatic Test of Leukocytes Labeled with Different HM-PAO Concentrations

Test	Blanc	HM-PAO 0.05 mg/ml	HM-PAO 0.25 mg/ml	HM-PAO 0.5 mg/ml
MPO*	11.9 ± 1.3	11.2 ± 2.0	11.9 ± 1.8	11.7 ± 1.5
S.O.†	27.1 ± 0.7	26.7 ± 1.5	27.1 ± 1.7	26.7 ± 2.9
Phagocytosis (%)	93.3 ± 1.8	84.7 ± 9.3	89.2 ± 1.7	83.2 ± 10.5
Killing (%)	83.6 ± 4.9	69.4 ± 14.7	60.6 ± 17.4	61.7 ± 22.8
K/F-Index	0.90 ± 0.04	0.81 ± 0.09	0.68 ± 0.19	0.73 ± 0.19
Chemotaxis (μm)				
FMLP	1930 ± 352	2040 ± 393	2080 ± 335	2060 ± 330
ZAS	1650 ± 260	1440 ± 453	1780 ± 195	1720 ± 200
RM	600 ± 165	670 ± 165	660 ± 137	627 ± 139
Adhesion				
Basal	27.4	16.1 ± 7.5	20.5 ± 8.4	28.9 ± 7.2
FMLP	60.7	56.4 ± 7.9	50.0 ± 5.2	53.7 ± 9.0

* Myeloperoxidase content (units MPO/2.10^6 PMN).
† Superoxide generation (nmol of cytochrome C reduced per 4.10^6 PMN in 15 min.
‡ Formyl methionyl leucyl phenylaline.
§ Zymosan activated serum.
¶ Random migration.

TABLE 2
Functional and Enzymatic Test of Leukocytes Labeled with a Constant Amount of HM-PAO and Increasing ^{99m}Tc Activities*

Test	Blanc	mCi ^{99m}Tc 0	1	5	15
S.O.		20.5	25.0	25.6	25.0
Chemotaxis (μm)					
FMLP	1790 ± 288	1598	1875	1643 ± 169	1640 ± 148
ZAS	1517 ± 90	1453	1500	1430 ± 62	1437 ± 76
RM	375 ± 93	345	440	350 ± 46	367 ± 38
Phagocytosis (%)	86.5 ± 9.2	85.0	96.2	86.9 ± 8.2	89.2 ± 6.1
Killing (%)	75.1 ± 15.6	70.9	85.2	75.0 ± 11.0	79.2 ± 17.4
K/F-Index	0.86 ± 0.10	0.83	0.88	0.86 ± 0.09	0.85 ± 0.10
Adhesion (%)					
Basal	42.6		43.0	37.8 ± 4.5	38.6 ± 6.0
FMLP	61.2		66.0	60.0 ± 5.6	58.4 ± 7.8

* For definitions of abbreviations, see Table 1.

From the results in Tables 1 and 2, it is clear that random migration, chemotaxis (FMLP and ZAS), and adhesion of the labeled granulocytes are not influenced by labeling with different amounts of tracer and/or HM-PAO. The results in Table 1 suggest that phagocytosis and killing are possibly slightly affected, but there is no clear correlation with the HM-PAO concentration. To exclude the potential influence of the amount of radioactivity, the same tests were repeated after labeling with a constant amount of HM-PAO but with increasing activities of ^{99m}Tc. The normal results of these tests indicate that the results of the first experiments were probably within the limits of experimental error. Normal in vitro results have also been reported by other authors (*12,15,18*).

Figure 2 shows that during the labeling step an equilibrium is reached 20 min after resuspending the white blood cell pellet in 1 ml of the [^{99m}Tc]d,l-HM-PAO solution. Resuspension of the white blood cells in 1 ml of physiologic saline before addition of the ^{99m}Tc complex results in a slower and less efficient tracer uptake, probably due to the dilution effect. The mean separation efficiency expressed as a percentage of the number of leukocytes present in the patients' blood was 40%

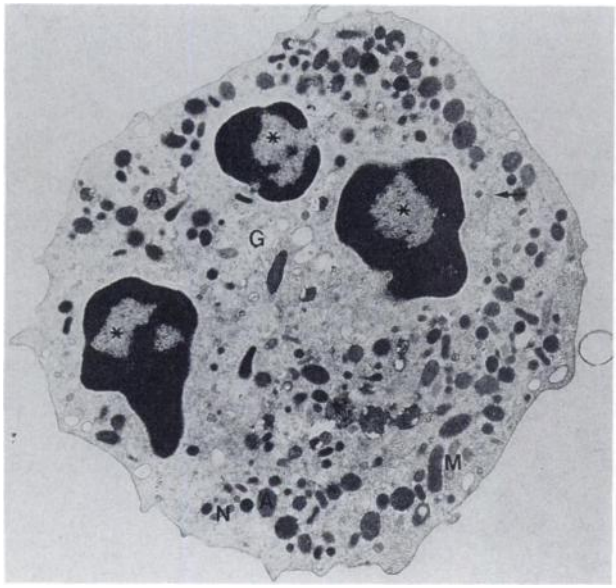

FIGURE 4
Electron micrograph of a ^{99m}Tc-labeled neutrophil granulocyte. The cell profile has a slightly irregular outline. Azurophil (A) and neutrophil (N) granules, mitochondria (M), and polyribosomes (arrow) are dispersed in the cytoplasm. Three nuclear profiles (*) are visible. The Golgi apparatus (G) is localized in the nuclear region. The shape, organization, and ultrastructure of the labeled cells do not appear to differ from that of the control cells (× 20,000).

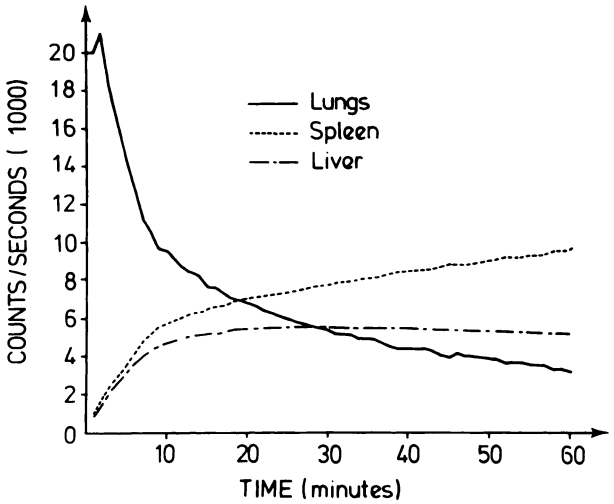

FIGURE 5
Time-activity curves in the region of the lungs, spleen, and liver during the first hour after injection of 15 mCi of autologous ^{99m}Tc-labeled granulocytes.

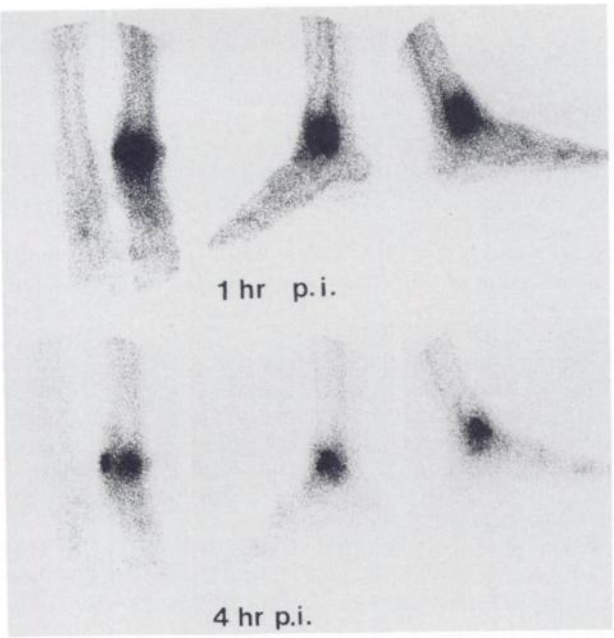

FIGURE 6
WBC scan is shown of a patient with a left bimalleolar ankle fracture. He was treated 6 mo ago with an open reduction and was admitted to the hospital with complaints of pain and inflammation. On the upper ray: the anterior projection of both ankles and the left lateral and medial projection of the left ankle 1 hr postinjection are shown. There is a clear uptake of labeled cells in the distal tibia and the left ankle. The same projections 3 hr postinjection are shown on the lower ray. A more focal accumulation in the medial malleolus, distal tibia and ankle joint is shown. The three-phase skeletal scintigraphy was positive in all phases. The x-rays of the left ankle were compatible with a septic arthritis. The cultures of the exudate showed the presence of *Staphylococus aureus*.

(range 18–78). This means that, with an average leukocyte count of 7000 cells per μl, 8 to 26 $\times$ 10^7 cells were used for labeling. The average labeling efficiency in the case of the 17 patients was 69.3% (range 50–81), thus demonstrating the effectiveness of our labeling method as compared to the lower results of other authors (*19*). As shown in Figure 3 the labeling is quite stable, and only 20% of the bound tracer activity is eluted from the cells during the first 24 hr. It is not known in which chemical form ^{99m}Tc is released from the cells, but free pertechnetate is excluded because no thyroid uptake has been demonstrated.

Some authors (*9,19*) have reported that in vitro the activity elutes less from granulocytes (6.1% over 2 hr) than from mononuclear cells (30% over 2 hr). Based on these observations, one may conclude that the white blood cell suspension obtained after the hemolysis step (Fig. 1) does not have to be purified any further by supplementary steps (gradient centrifugation, etc.). As only one-fifth of the [^{99m}Tc]d,l-HM-PAO preparation is used during the labeling step, the granulocytes were incubated with no more than 0.1 mg of HM-PAO and 1 μg of $SnCl_2 \cdot 2H_2O$. The addition of such small amounts of these substances undoubtedly reduces the risk of alteration in the cell structure (Fig. 4).

Transmission electron microscopy (TEM) is a valuable tool in the evaluation of labeled cells, as was demonstrated in our previous study on the ^{111}In labeling of white blood cells with three different chelators (*25*). In this article, the deleterious effect of oxine and Merc on the morphology of cells was clearly demonstrated by TEM. As shown in Figure 4, the TEM image of the technetium-labeled white blood cells was completely normal, which is another argument in favor of this method.

The scintigraphic images of the patient studies have some special features. The bladder is visualized early whereas the thyroid gland was never seen. Bone marrow uptake is prominent, and there is some bowel visualization without previous hepatic secretion after 4 to 6 hr. In case of bowel disease, the early timing of the images and the intensity of the tracer uptake can be helpful in the final diagnosis (*9,17*). A 24-hr study with regular acquisition of the abdomen of normal persons could provide more information of the normal sequence of the intestine uptake.

The scintigraphic score was 2 (suspect) or 3 (positive) in 13 of the 17 patients. These lesions were already clearly visible at 3 hr and no extra lesion was found at 24 hr (Fig. 6). Some authors mention that most lesions become positive already 30–60 min after injection (*9,10,13–15*). This finding is also demonstrated in the clinical example showing a clear hot zone in the distal tibia. A more localized uptake is found on the images 3 hr p.i. There was one false-negative result with a patient with an infected fracture of the tibia. The lung transit of this study was prolonged, so this negative result could be explained by an improper separation or labeling step.

The images are as good or better than those obtained with indium-labeled WBC. This is in agreement with the findings of other authors who already used HM-PAO labeling (*26,27*). Comparing the images of simultaneously injected [^{99m}Tc]d,l-HM-PAO and [^{111}In]oxinate labeled white cells in 12 patients, Lui found complete agreement in lesion detectability but a superior quality (better spatial and contrast resolution) when using the ^{99m}Tc-labeled agent (*15*). An additional important advantage of the ^{99m}Tc-labeled granulocytes is the lower radiation dose (*9*), which permits the use of higher activities.

It is concluded that labeling of leukocytes with [^{99m}Tc]d,l-HM-PAO results in functionally and morphologically intact cells. The cost, availability, and physical characteristics of the tracer and the final clinical results justify this cell-labeling technique as a valuable alternative for ^{111}In labeling.

ACKNOWLEDGMENTS

The authors thank M. J. Vangoetsenhoven for her skillful secretarial work and W. Scheers for performing all of the functional tests.

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