Functional Upregulation of Granulocytes Labeled with Technetium-99m-HMPAO and Indium-111-Oxinate

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Indium-111-oxinate-labeled granulocytes have been used in vivo for several years for the detection of abscesses. Technetium-99m-hexa methylpropyleneamine oxime (^{99m}Tc-HMPAO) labeling has more recently been described. **Methods:** The influence of radiolabeling by both radiotracers on adhesion glycoprotein CD11b quantification was studied in quiescent and formyl-methionylleucylphenylalanine (fMLP)-activated neutrophils (PMN). Adhesion was assessed on human umbilical endothelial cells (HUVEC) as well as the repercussion of the granulocyte labeling on HUVEC viability (neutral red) and metabolic activity (MTT). Chemotaxis of PMN was evaluated by measuring migration under agarose with fMLP as chemoattractant. We also measured phagocytosis and the production of hydrogen peroxide induced by staphylococcus aureus. **Results:** Whereas whole functional integrity is maintained after labeling, most of the functions (CD11b expression, adhesion, HUVEC metabolic activity) are up-regulated while chemotaxis is decreased in the presence of both radiotracers. Indium-111-oxinate induces larger alterations than ^{99m}Tc-HMPAO. **Conclusion:** These data were obtained in normal volunteers. In patients, alterations due to the in vitro labeling procedure, in addition to potential functional alterations caused by the underlying pathology, should be taken into account during image interpretation.

Key Words: indium-111-oxinate-labeled granulocytes; technetium-99m-HMPAO; chemotaxis; endothelial cells

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Several scintigraphic methods using labeled leukocytes and particularly labeled granulocytes have been developed in recent years for the detection of inflammatory and septic lesions (*1–4*). Their field of application primarily concerns localization of abdominal abscesses and occult infections of the bones. Neutrophils are the predominant phagocytes of circulating blood; they circulate for only 6 to 10 hr and are the first cells to reach the site of infection (*5*). Two main techniques for leukocyte labeling are presently used in our hospital: the technique developed by Thakur (*6*) using ^{111}In oxinate, which remains the gold standard, and that first described by Peters (*7*) in 1986 using ^{99m}Tc coupled with HMPAO. Both agents are lipophilic radiopharmaceutical drugs which diffuse through the cell membrane and bind intracellularly, being trapped in the cytoplasm of the cell.

Indium-111-oxinate and ^{99m}Tc-HMPAO have been largely compared in the literature for their relative advantages and disadvantages regarding: quality of imaging characteristics; radiation dose; efficiency of granulocyte labeling; and the uptake by the infection site (*8–12*). Among these functions, the interactions between PMN and endothelial cells are important because the endothelium is also intimately involved in the host defense and inflammatory response. Also, leukocyte adherence to endothelium is a prerequisite for subsequent extra-vascular migration and expression of end-functions. It is yet to be accepted that through handling during cell isolation and labeling, some activation and pharmacological alteration of the granulocyte functions may occur, and that they could even have repercussions on the quality of the scintigraphic approach.

We therefore compared partially isolated but unlabeled neutrophils (control neutrophils) with neutrophils labeled by ^{111}In-oxinate and ^{99m}Tc-HMPAO during circulating resting state and activated state: expression of adhesion molecules in response to chemoattractant agents, adhesion to endothelium and consequences on endothelial metabolic activity, migration, ingestion of particles (phagocytosis) and neutrophil activation with generation of reactive oxygen metabolites from molecular oxygen (H_2O_2).

MATERIALS AND METHODS

PMN Isolation and Labeling

Fifty milliliter of blood were obtained from the peripheral veins of ten healthy volunteers (8 women, 2 men) in 7.5 ml acid citrate dextrose (ACD). Spontaneous sedimentation of red blood cells at 37°C makes it possible to obtain leukocyte-rich plasma (LRP). After 90 min of incubation, LRP was deposited on ficoll metrizoate gradient at 20°C. After centrifugation (10 min, 100 g) the cell pellet was obtained and resuspended in 6 ml of RPMI after elimination of platelets and lymphocytes (PMN purity = 92 ± 8% of leukocytes) and without red blood cell lysis.

Each PMN suspension was separated into three parts: one labeled with ^{111}In-oxinate, one with ^{99m}Tc-HMPAO and the third served as an unlabeled control for the various tests.

One vial of ^{111}In-oxinate (1.4 mCi/ml or 51.8 MBq, 0.025 mg oxine) was prepared in 400 μl of Tampon Tris. Two vials of HMPAO were prepared with 24 mCi of ^{99m}Tc-HMPAO or 888 MBq in 8 ml of 9% NaCl. Each mixture was gently shaken for 5 min, centrifugated (5 min–100 g), divided into ten parts and deposited on the 2 ml of each leukocyte suspension (mean cell number = 15.5 ± 3.1 × 10^6). The unlabeled PMN underwent the same procedures, carried out before and after labeling. Labeling efficiency was calculated for each pellet. To insure sterility, isolation and labeling of PMN leukocytes took place in a laminar-flow hood.

Membrane CD_{11b} Quantitated by Immunofluorescence Flow Cytometry

We analyzed CD11b surface expression by exposing 5 × 10^5 cells to buffer or fMLP at 10^{-7} *M* final concentration for 2 min at 37°C. Indirect fluorescence was performed using OKM-1 (mouse monoclonal antibody) and FITC-conjugated goat antimouse IgG

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(GAM-FITC). A FACS-scan analyzer was for cytometry. The use of flow cytometry technique allows easy identification of each cell type. PMN fluorescence was analyzed by gating the fluorescence on the basis of their wide angle light scatter (forward scatter) and low angle scatter (side scatter). Quantification of cell surface binding sites was determined using uniform cell-sized beads bearing various and known Mabs densities accessible to the second-step reagent (GAM-FITC). The beads were stained identically to and in parallel with PMN, allowing the building of a linear calibration curve relating mean fluorescence intensity (MFI, on a linear scale) with the number of Mab molecules.

PMN Adherence to Monolayers of Human Endothelial Cells

Human endothelial cells (HUVEC) were isolated and cultured as previously described (*13,14*) according to the original method of Jaffe (*15*). Endothelial cells were seeded in 96-well microtiter plates and grown until confluency ($35 \pm 2.8 \times 10^3$ cells/well, n = 4). Indium-111-oxinate and ^{99m}Tc-HMPAO labeled human PMN (resuspended in fetal calf serum supplemented RPMI, 10 donors) were added in the wells (13 PMN/1 HUVEC) and incubated for 7, 15, 30 and 60 min at 37°C. Nonadherent PMN were then discarded. Adhered PMN were fixed by addition of glutaraldehyde (2%). After dissolution by 6 N NaOH, supernatants were counted in a gamma counter while an aliquot of the radioactive suspension before seeding was counted under the same geometric conditions (100%).

HUVEC viability was checked by the neutral red assay (*16*), which is based on the uptake of the dye neutral red by viable cells. Viability was quantitated by extracting the dye (after 3 hr incubation) by using acid alcohol (1% acetic acid in 50% v/v ethanol solution) and measuring the resulting color intensity spectrophotometrically at 540 nm. The optical density, proportional to the viability of the cell population, was measured in HUVEC in the absence and presence of unlabeled or labeled PMN. The metabolic activity of the HUVEC was measured in the same conditions by using the MTT assay (*17*). Briefly, the water-soluble tetrazolium dye 3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was metabolized into an insoluble formazan salt by a mitochondrial enzyme of viable cells. The intensity of the blueness of the formazan crystals formed after 3 hr incubation at 37°C and measured at 540 nm is considered to be directly proportional to the metabolic activity of the cells.

Chemotaxis

Chemotaxis was studied using the under-agarose method described by Nelson et al. (*18*). In the assay, three wells were placed in line in a culture plate containing agarose supplemented with fetal calf serum. PMN (5×10^5 cells/5 μl), labeled or unlabeled, were placed in the central well and fMLP-chemoattractant (10^{-7} M) and PBS were placed opposite each other. PMN migrated radially from the central well in an egg-shaped distribution, with the pointed end of the pattern facing the well containing the chemoattractant. After a period of incubation (2 hr at 37°C), the cells were fixed by flooding the plate with 2.5% glutaraldehyde for 15 min at room temperature. Following removal of the agarose, the cells were stained with May-Grünwald-Giemsa dye. Migrating cells were quantified using a digital image analyzer. The quantitation method has been described previously (*19*).

Phagocytosis Measurement and Production of Hydrogen Peroxide

Phagocytosis and generation of hydrogen peroxide were analyzed according to the method described by Hasui et al. (*20*) by flow cytometry. PMN were isolated from citrated whole blood. Hydrogen peroxide was assayed using the stable nonfluorescent cell permeant DCFH-DA 2′,7′-dichlorofluorescein diacetate which

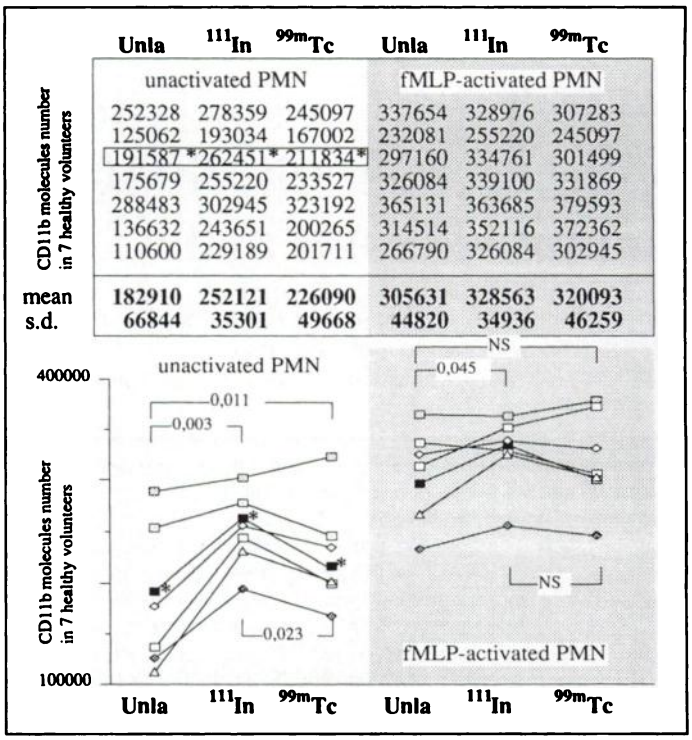

FIGURE 1. Effect of radiolabeling on CD11b molecules number PMN from seven normal volunteers before and after fMLP activation.

diffuses into cells where it is hydrolyzed to nonpermeant DCFH. In the presence of hydrogen peroxide, DCFH is oxidized to the highly fluorescent 2′,7′ dichlorofluorescein (DCF) which emits green fluorescence (*21*).

Phagocytosis was evaluated using a propidium iodide (PI)-labeled suspension of staphylococci. Propidium iodide emits a red fluorescence after excitation at 488 nm. Before the assay, the labeled staphylococci were opsonized by incubation in the presence of 10% homologous serum at 37°C.

The following procedure was used: 0.050 ml of DCFH-DA 0.5 m*M* was incubated for 10 min at 37°C in a shaking water bath with 0.5 ml of heparin-pretreated granulocytes for 15 min. An aliquot of 0.050 ml of PMN was removed and added to 1% paraformaldehyde (PFA): the fluorescence at this point was taken as the zero time value. At the same time, 0.050 ml of PI-labeled staphylococci were added. Then, 15 min after adding the staphylococcal suspension, 0.050 ml of the PMN suspension were removed and added to 1% PFA for fluorescence determination.

Statistical Analysis

Mean ± s.d. of chemotaxis, phagocytosis, hydrogen peroxide release, absorbance units and adhesion percentages were calculated. The difference of the means were compared using paired Student's t-test; $p < 0.05$ was considered statistically significant.

RESULTS

The two labeling efficiencies expressed in percentages of whole radioactivity were similar: 27.1 ± 4.5% with ^{111}In-oxinate and 21.4 ± 5.6% with ^{99m}Tc-HMPAO. The mean ^{99m}Tc/^{111}In radioactivity ratio calculated by dividing the radioactivity obtained for each donor (n = 10) was homogeneous: 19.1 ± 1.9.

Figure 1 summarizes the individual data of the seven volunteers for CD11b expression in unactivated and fMLP-activated PMN. Labeling with ^{111}In-oxinate induced a large and significant increase in the number of CD11b molecules expressed at the cell surface of quiescent PMN ($p < 0.003$): a mean increase of 70,000 molecules representing around 40% of the initial CD11b expression was observed after ^{111}In labeling, while

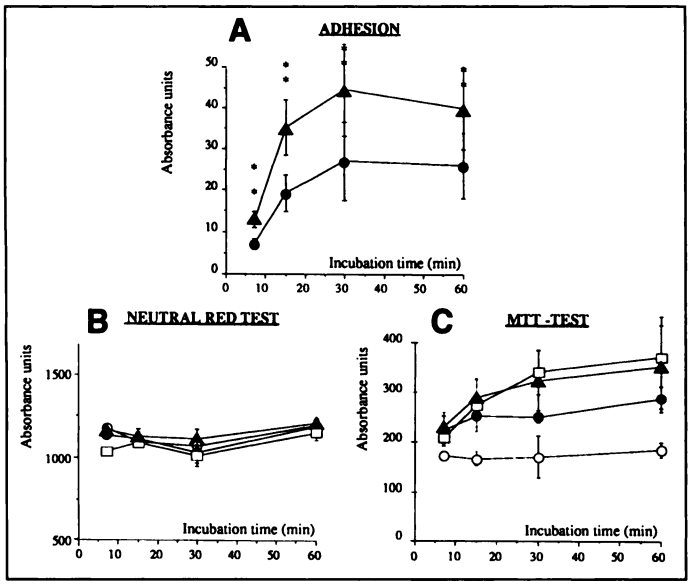

FIGURE 2. PMN labeling influence on HUVEC behavior. (A) Adhesion, (B) viability studied by neutral red assay and (C) metabolic activity studied by MTT test: HUVEC alone (○), unlabeled PMN + HUVEC (□), ^{111}In-oxinate-labeled PMN + HUVEC (●) and ^{99m}Tc-HMPAO-labeled PMN + HUVEC (▲).

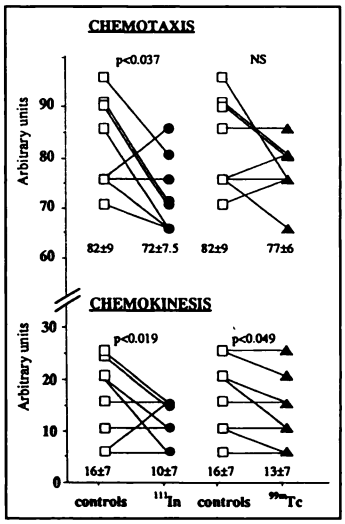

FIGURE 3. Results of quantification of random and directed PMN migration by image analysis after labeling: Unlabeled PMN (□), ^{111}In-oxinate-labeled PMN (●) and ^{99m}Tc-HMPAO-labeled PMN (▲).

fMLP induced an approximate increase of 70% in CD11b molecules (mean increase of 120,000 molecules). The mean increase in CD11b molecules was significant but less pronounced in the presence of ^{99m}Tc-HMPAO ($p < 0.01$), with only around 40,000 new molecules expressed representing an increase of less than 25%. The effect of ^{99m}Tc-HMPAO on the CD11b expression increase in quiescent PMN was thus significantly lower than the effect of ^{111}In-oxinate ($p < 0.02$). When PMN were fMLP-activated after labeling with ^{111}In-oxinate or ^{99m}Tc-HMPAO, the influence of the radiotracer was significant only in the presence of ^{111}In-oxinate, which induced a further increase in CD11b of around 20,000 molecules ($p < 0.045$), thus representing a relative increase of around 7.5% when compared to unlabeled fMLP-activated PMN. The ^{99m}Tc-HMPAO effect on fMLP-activated PMN did not reach significance.

Both radiotracers were therefore able to increase CD11b expression on quiescent PMN, but ^{111}In-oxinate induced a larger increase than ^{99m}Tc-HMPAO. The quantitative increase in the surface expression of CD11b following stimulation by fMLP, a soluble activator, is well-documented (*22*): this increase is further enhanced when PMN are ^{111}In-oxinate-labeled prior to activation.

Adhesion to HUVEC

Figure 2A shows the results of PMN adhesion to HUVEC as measured by radioactivity counting. Only ^{111}In-oxinate and ^{99m}Tc-HMPAO-labeled PMN were thus assessed in this model, where no control group of unlabeled PMN could be included. The adhesion of labeled PMN is expressed as a percentage of retained radioactivity related to the deposited radioactivity per well. Adhesion increased as a function of time and reached a plateau from the 30th minute with both radiotracers, but a significant difference ($p < 0.001$) was observed between the two labeling procedures. Surprisingly, adhesion was lower in the presence of ^{111}In-oxinate-labeled PMN (which was shown above to express increased CD11b expression) than in the presence of ^{99m}Tc-HMPAO-labeled PMN: at 7 min incubation, 12.8 ± 1.9% of deposited ^{99m}Tc-HMPAO-labeled PMN were retained on HUVEC versus 7.2 ± 1.2% of ^{111}In-oxinate-labeled PMN, and at 15 min incubation: 35.2 ± 6.8% of ^{99m}Tc-HMPAO-labeled PMN were retained versus 19.3 ± 4.4% of ^{111}In-oxinate-labeled PMN. There is thus a significant difference in PMN adhesion of ^{111}In-oxinate-labeled PMN and ^{99m}Tc-HMPAO-labeled PMN, but the absence of a control group of unlabeled PMN does not make it possible to interpret these results as increasing or decreasing influences.

The neutral red assay shown in Figure 2B demonstrates that none of the radiotracers used altered the endothelial cell viability. On the other hand, the MTT test (Fig. 2C), reflecting the metabolic activity of the cells, shows that this activity was modified in the presence of PMN. When endothelial cells were in the presence of unlabeled PMN, their metabolic activity increased significantly from 7 to 60 min ($p < 0.001$), reaching steady state at 30 min. Indium-111-oxinate-labeled PMN induced a decreased metabolic activity which was significant at 30 and 60 min ($p < 0.05$) when compared to that of HUVEC in the presence of ^{99m}Tc-HMPAO-labeled and -unlabeled PMN.

Chemotaxis

Results (expressed in arbitrary units) are shown in Figure 3. Mean values are given under the individual data of chemokinesis and chemotaxis. Both labeling techniques induced a slight decrease in cell random migration, but the decrease was larger and more significant with ^{111}In-oxinate than with ^{99m}Tc-HMPAO (control PMN: 16 ± 7, versus ^{111}In-oxinate-labeled PMN: 10 ± 7, $p < 0.02$ and versus ^{99m}Tc-HMPAO-labeled PMN: 13 ± 7, $p < 0.05$). The fMLP-attracted migration was significantly decreased in the presence of ^{111}In-oxinate-labeled PMN (control PMN: 82 ± 9, versus ^{111}In-oxinate-labeled PMN: 72 ± 7.5, $p < 0.04$) but not significantly decreased in ^{99m}Tc-HMPAO-labeled PMN.

Phagocytosis and Burst

Phagocytosis ($n = 10$) was expressed by the mean channel of fluorescence (Fig. 4A). There was no difference between control granulocytes and labeled granulocytes irregardless of the labeling technique used.

The respiratory burst was evaluated from the production of hydrogen peroxide in response to phagocytosis of staphylococci. Results are expressed as the mean channel of fluorescence of the oxidized 2′7′-dichlorofluorescein after 15 min of phagocytosis (Fig. 4B). H_2O_2 was slightly increased after radioactive labeling, but the increase was significant only in ^{111}In-oxinate-labeled PMN ($p < 0.008$).

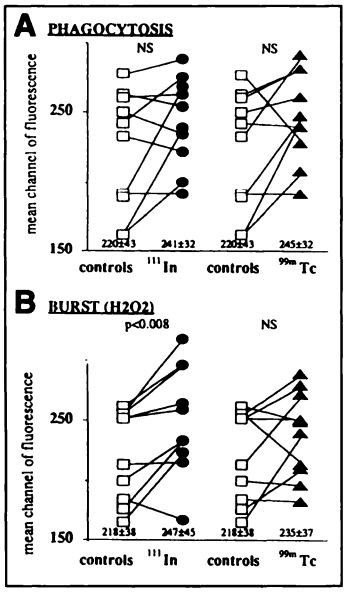

FIGURE 4. Flow cytometric measurement of propidium iodide-labeled staphylococci phagocytosis at 15 min (A) and of hydrogen peroxide production at 15 min (B) expressed as mean fluorescence intensity on a linear scale: Unlabeled PMN (□), ^{111}In-oxinate-labeled PMN (●) and ^{99m}Tc-HMPAO-labeled PMN (▲).

DISCUSSION

Previous Data

Although our data demonstrate that PMN functions are significantly altered by both radiotracers studied, labeled PMN do remain the best tool among the radioactive agents available at the present time to demonstrate foci of inflammation by gamma camera imaging. Mac Affee (*23*) compared PMN labeling in dogs with other agents used in scintigraphic inflammation imaging such as ^{67}Ga citrate, ^{99m}Tc-polyclonal immunoglobuline, serum albumin, rabbit polyclonal IgG, monoclonal antibody TNT-1-F(ab)'2 against nuclear antigens present in necrotic neutrophils or other cells, radiolabeled monoclonal antibodies directed against PMN, ^{57}Co porphyrin and serum albumin nanocolloïds. Most of these techniques have been shown to achieve abscess concentrations far lower than those obtained with labeled leukocytes with an abscess/blood ratio twenty times lower when compared to labeled leukocytes. Cell harvesting and labeling are time consuming but they remain necessary since lipophilic radiotracer complexes label all cell types, and labeling specificity can only be achieved by PMN purification.

PMN functions after radiolabeling by the same procedures as ours have already been studied in the literature. In 1983, Colas-Linhart (*9*) demonstrated a decrease in PMN chemotaxis after ^{111}In-labeling using the under-agarose assay, but no modification of phagocytosis nor oxygen consumption by using ingestion of ^{14}C Klebsiella and nitroblue tetrazolium reduction. In 1988, Kelbaek (*24*) demonstrated no modification of PMN chemotaxis after ^{99m}Tc-HMPAO labeling using the Boyden Chamber. In 1989, Mortelmans did not demonstrate any modification of PMN function after ^{99m}Tc-HMPAO labeling (*12*) when using Cytochrome C reduction for studying superoxide production, under-agarose migration for studying chemotaxis, pourplating technique for studying staphylococcus aureus phagocytosis and adhesion to gelatin-coated plastic surfaces. In 1990, De Labriolle-Vaylet (*25*) demonstrated, after ^{99m}Tc-HMPAO labeling, no modification of superoxide production in response to fMLP but an increase in response to phorbol-myristate-acetate when using Cytochrome C reduction, no effect on chemotaxis and a decrease in adhesion when performed on nylon culture chamber slides.

Strengths of the Study

Our own results confirm most of these data. We confirm the decrease in chemotaxis in the presence of ^{111}In-oxinate which is not found in the presence of ^{99m}Tc-HMPAO. Nevertheless, the demonstration of a slight decrease in chemotaxis in the presence of ^{99m}Tc-HMPAO, formerly undetectable due to the limitations of optical methods, was possible with a digital imaging technique that precisely quantifies the density and surface of the migrating cell carpet. In the same way, assessment of phagocytosis and H_2O_2 production by flow cytometry allows the simultaneous measurement of these two end functions. Interestingly, phagocytosis was unmodified by the radiolabeling, as already mentioned in the literature, but phagocytosis-induced H_2O_2 production, which has not been previously studied in the literature in these conditions, was increased in the presence of ^{111}In-oxinate. This radiotracer, thus, behaves as a "priming agent" for H_2O_2 production while impeding cell motion as shown by the decrease in chemotaxis.

Moreover, ^{111}In-oxine also decreases PMN adhesion to endothelial cells when compared to ^{99m}Tc-HMPAO. Adhesion is indeed the initial step in chemotaxis which results from complex interactions between granular and endothelial receptors. Among several granular receptors, CD11b is the prominent glycoprotein involved in the adhesion process. Moreover, this integrin is the most often used marker for studies on granulocyte activation. Activated granulocytes express far more CD11b than quiescent PMN. In response to exposure to stimuli inducing total degranulation (such as calcium ionophore A23187), the amount of surface CD11b present on resting normal granulocytes increases 3-to-10 fold. This increase results from exocytosis of a subset of secondary granules containing CD11b in their perigranular membranes. These granules are translocated toward the cell membrane from an intracellular pool. It was recently shown that whereas integrins are involved in almost every aspect of cell adhesion, increased surface expression of CD11b is not always required for stimulated neutrophil adherence to cultured endothelium (*26*). Integrins require activation to bind to their immunoglobulin superfamily ligands and to carry out the second stage of adherence occurring after the initial step of rolling promoted by the selectins.

The surface expression of CD11b is thus thought to reflect the activation state of PMN rather than their adhesive function. The observed up-regulation of CD11b expression in the presence of ^{111}In-oxinate is in fact associated with a relative decrease in adhesion of PMN on endothelial cells when compared to ^{99m}Tc-HMPAO-labeled PMN. Even though, adhering to a lesser extent, ^{111}In-oxinate-labeled PMN demonstrate a potential deleterious effect upon endothelial cells revealed by a decrease in their metabolic activity. The hypothesis of an up-regulation towards an armed state of PMN in the presence of this radiotracer is enforced by the observation of an increased hydrogen peroxide production in response to phagocytosis of staphylococci. This increase is highly significant and attests to the primed state of the PMN due to ^{111}In-oxinate labeling prior to the activation by the particular stimulus. The significantly decreased chemokinesis and chemotaxis of ^{111}In-oxinate-labeled PMN in response to fMLP is in accordance with the fact that saturating concentrations of stimulus can inhibit neutrophil locomotion in spite of a large and sustained increase in CD11b expression (*26*).

The cellular effects described in the presence of ^{111}In-oxinate or of ^{99m}Tc-HMPAO may result from an effect of the radionucleide or of the lipophilic carrier. The exact repartition of the radiotracers between the different subcellular compartments is incompletely known. The lipophilic component of the radio-

tracers can penetrate and disturb the membrane bilayer and consequently, the membrane bound glycoproteins. Oxinate has already been shown to present a cellular toxicity: Segal (*27*) demonstrated that chemotaxis was reduced to half its value by adding an oxine concentration of 100 $\mu g/10^8$ cells, a concentration six times that used in our study (2.5 $\mu g/15 \times 10^6$ cells). The work of Mortelmans (*10*) seems to indicate that both radionucleide and carrier may be involved in the inhibition of migration observed in the presence of high concentrations of ^{111}In-oxinate. He reported a progressive inhibition in the presence of 5 to 10 μg/ml cold oxine which increased dramatically in the presence of ^{111}In. This additional effect of ^{111}In is not observed in the presence of other chelating agents (tropolone and mercaptopyridine) and could be attributed to the higher specific activity of ^{111}In-oxinate.

Clinical Relevance

Extrapolating possible in vivo repercussions from our in vitro data is complex. This complexity is due to the many steps involved in their activation pathway resulting in heterogeneous subpopulations co-existing in various stages from quiescent to primed and to fully activated cells. Moreover, their activities are regulated by the imbalance of complex stimulatory and inhibitory pathways responding to a plethora of locally and systemically produced mediators through multiple signal transduction processes.

Like spies betraying their country, in vitro radiolabeled autologous PMN are supposed to melt into the circulating PMN population and behave as do the other ones. The only difference is that they can inform us by sending gamma radiations. Their capacity to join a potential inflammation site is the prominent function that they are intented to fulfill: under the influence of a chemoattractive gradient generated from the infection site, they must accumulate onto the endothelium of vessels surrounding the abscess, penetrate the endothelial layer and migrate to connective tissues at the site of infection, where they finally must congregate and adhere to extracellular matrix components. Thus, all the activation steps are very localized in the immediate proximity of the inflammation site and do not occur systematically under physiological conditions. Whether radiolabeled PMN behave similarly to unlabeled PMN in fighting the infection through their "late functions" (oxidation, degranulation, phagocytosis and wound healing) is not very critical. Our results on radiotracer-labeled PMN represent a modest contribution to an extensive literature struggling to adapt experimental findings to actual pathologic situations. Pathologies involving abnormal PMN activation with CD11b upregulation result in abnormal PMN accumulation in the vessels, as seen in the paradigm pathology for systemic PMN activation, the adult respiratory distress syndrome (*28*). Our results suggest that the radiolabeling per se may constitute a mild stimulus similar to the systemic stimuli observed in such pathologies. The observed alterations in the motility of PMN may thus help advance our understanding of differences observed in the sensitivity (ability to join the potential abscess) and specificity (selectivity of adherence and diapedesis for the endothelial line surrounding the abscess rather than elsewhere) between radiotracers. In this way, the dayly observed higher sensitivity of ^{99m}Tc-HMPAO-labeled PMN may be related to a better preserved motility when compared to ^{111}In-oxine-labeled PMN. Our data obtained from normal control PMN should be completed by further studies on PMN withdrawn from the patients explored for abscess detection (for example, by studying PMN chemotaxis before and after labeling to analyze the influence of both pathology and labeling on PMN) taking into account their underlying pathologies, which can impair PMN functions.

CONCLUSION

In clinical practice, PMN labeling allows abscess targeting but the final image is often the only criterium of PMN functional quality. A re-appraisal of quality control of PMN functions after radiolabeling seems necessary to progress towards more efficient detection of inflammation or infection. Some failures of this tool to detect abscesses or acute inflammation in certain circumstances are well known, but remain unexplained (*29*). Alteration of PMN functions are highly probable in such circumstances. It must be kept in mind that this diagnostic tool is directly linked to the patient, as PMN are autologous, and the underlying pathologies can alter this probe. As PMN constitute both the target and the probe, an overview of the complete system should improve the diagnostic approach to PMN collections.

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REFERENCES

1. Thakur ML, Lavender JP, Arnot RN, Silvester DJ, Segal AW. Indium-111-labeled autologous leukocytes in man. *J Nucl Med* 1977;18:1014–1021.
2. Becker W, Düsel W, Berger P, Spiegel W. The ^{111}In granulocyte scan in prosthetic vascular graft infections: imaging technique and results. *Eur J Nucl Med* 1987;13: 225–229.
3. Moisan A, Le Cloirec J, Bretagne JF, Loreal O, Raoul JL, Herry JY. Imaging of inflammatory bowel disease and scintigraphic assessment of resected colon: comparison of ^{111}In-oxine and ^{99m}Tc-HMPAO-leukocyte labeling. *Progress Clin Biochem Res* 1990;355:159–164.
4. Moragas M, Lomena F, Herranz R, et al. Technetium-99m-HMPAO-leukocyte scintigraphy in the diagnosis of bone infection. *Nucl Med Commun* 1991;12:417–427.
5. Borregaard N. The human neutrophil. Function and dysfunction. *Eur J Haematol* 1988;41:401–413.
6. Thakur ML, Coleman RE, Welch MJ. Indium-111-labeled leukocytes for localization of abscesses: preparation, analysis tissue distribution and comparison with gallium 67 in dogs. *J Lab Clin Med* 1977;89:217–228.
7. Peters AM, Osman S, Henderson BL, et al. Clinical experience with ^{99m}Tc-hexamethyl propylamine oxine for labeling leukocytes and imaging inflammation. Preliminary communication. *Lancet* 1986:946–949.
8. Thakur ML, Seifert CL, Madsen MT, Mc Kenney SM, Desai AG, Park CH. Neutrophil labeling. Problems and Pitfalls. *Semin Nucl Med* 1984;14:107–117.
9. Colas-Linhart N, Barbu H, Gougerot MA, Bok B. Five leukocytes labeling techniques: a comparative in vitro study. *Br J Haematol* 1986;5:31–41.
10. Mortelmans L, Verbruggen A, Malbrain S, et al. Evaluation of ^{111}In-labeled white blood cells by in vitro functional tests and electron microscopy. *Eur J Nucl Med* 1988;14:159–164.
11. Peters AM, Roodie ME, Danpure HJ, et al. Technetium-99m-HMPAO-labeled leukocytes: comparison with ^{111}In tropolonate-labeled granulocytes. *Nucl Med Commun* 1988;9:449–463.
12. Mortelmans L, Malbrain S, Stuyck J, et al. In vitro and in vivo evaluation of granulocyte labeling with [^{99m}Tc] d, l-HMPAO. *J Nucl Med* 1989;30:2022–2028.
13. Bordenave L, Bareille R, Rouais F, Lefebvre F, Baquey Ch, Rabaud M. New artificial connective matrix-like structures made of elastin solubilized peptides and collagens. II. Thrombogenicity and use as endothelial cell culture support. *Biomaterials* 1992;13:439–447.
14. Bordenave L, Baquey Ch, Bareille R, et al. Endothelial cell compatibility testing of three different Pellethanes®. *J Biomed Mater Res* 1993;27:1367–1381.
15. Jaffe EA, Nachman RL, Becher CG, Minick CR. Culture of human endothelial cells derived from umbilical veins. *J Clin Invest* 1973;52:2745–2756.
16. Parish CR, Mullbacher A. Automated colorimetric assay for T cell cytotoxicity. *J Immunol Meth* 1983;58:225–237.
17. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Meth* 1983;65:55–63.
18. Nelson RD, Quie PG, Simmons RL. Chemotaxis under agarose: a new and simple method for measuring chemotaxis and spontaneous migration of PMN and monocytes. *J Immunol* 1975;115:1650–1656.
19. Labrouche S, Freyburger G, Belloc F, Boisseau MR. Influence of selected heparins on human neutrophils functions in vitro. *Thromb Haemost* 1992;68:556–562.
20. Hasui M, Hirabayashi Y, Kobayashi Y. Simultaneous measurement by flow cytometry of phagocytosis and hydrogen peroxide production of neutrophils in whole blood. *Immunol Meth* 1989;117:53–58.
21. Weening RS, Wever R, Roos D. Quantitative aspects of the production of superoxide radicals by phagocytizing human granulocytes. *J Lab Clin Med* 1975;85:245–252.
22. Sengelov H, Kjeldsen L, Diamond MS, Springer TA, Borregaard N. Subcellular localization and dynamics on Mac 1 (am β2) in human neutrophils. *J Clin Invest* 1993;92:1467–1476.
23. Mc Afee JG, Gagne G, Subramanian G, Schneider RF. The localization of ^{111}In-leukocytes, gallium-6 polyclonal IgG and other radioactive agents in acute focal inflammatory lesions. *J Nucl Med* 1991;32:2126–2131.

24. Kelback H, Linde J, Nielson SL. Evaluation of a new leukocyte labeling procedure with ^{99m}Tc-HMPAO. *Eur J Nucl Med* 1988;14:621–623.
25. De Labiolle-Vaylet C, Colas Lihnart N, Petiet A, Bok B. Morphological and functional status of leukocytes labeled with ^{99m}Tc-HMPAO. In: Sinzinger H, ed. *Radiolabelled cellular blood elements*. New York: Liss; 1990:119–129.
26. Vedder NB, Harlan J. Increased surface expression of CD11b/CD18 is not required for stimulated neutrophil adherence to cultured endothelium. *J Clin Invest* 1988;81:676–682.
27. Segal AW, Deteix P, Garcia R, Tooth P, Zanelli GD, Allison AC. Indium-111-labeling of leukocytes: a detrimental effect on neutrophil and lymphocyte function and an improved method of cell labeling. *J Nucl Med* 1978;19:1238–1244.
28. Laurent T, Markert M, Von Fliedner V, et al. CD11b/CD18 expression, adherence and chemotaxis of granulocytes in adult respiratory distress syndrome. *Am J Respir Crit Care Med* 1994;149:1534–1538.
29. Jacobson AF, Gilles CD, Cerqueira MD. Photopenic defects in marrow-containing skeleton on ^{111}In leukocyte scintigraphy prevalence at sites suspected of osteomyelitis and as an incidental finding. *Eur J Nucl Med* 1992;19:858–864.

Technetium-99m Radiolabeling Using a Phage-Derived Single-Chain Fv with a C-Terminal Cysteine

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Single-chain Fv (scFv) antibody fragments have potential for clinical imaging studies because of their rapid tumor penetration and high tumor-to-tissue ratios at early time points. ScFvs clear rapidly from the circulation so radiolabels such as ^{99m}Tc which have short half-lives are desirable, but the free thiol groups necessary for labeling with ^{99m}Tc are not normally found on these molecules. **Methods:** We constructed a vector which enabled a free cysteine to be linked to the C-terminus of scFvs. MFE-23, a scFv directed against carcinoembryonic antigen (CEA), was cloned into this vector and cys-tagged MFE-23 was labeled with ^{99m}Tc using a D-glucarate transfer method. **Results:** The radiolabeled product was stable in vivo and in vitro and showed favorable tumor-to-blood ratios in vivo at early time points (4:1 at 24 hr and 8:1 at 48 hr), although high kidney levels were also detected. **Conclusion:** Our study demonstrates an effective method to enable scFvs radiolabeling with ^{99m}Tc and also shows the potential of using a ^{99m}Tc-labeled scFv for clinical imaging studies.

Key Words: single-chain Fv antibody fragments; technetium-99m

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Small antibody fragments have potential for good tumor targeting since they penetrate rapidly and give high tumor-to-background ratios at early time points (*1–3*). Single chain Fvs (scFvs) consist of a variable heavy (VH) and a variable light (VL) chain of an immunoglobulin tethered together with a flexible linker (*4*) and as such these are the smallest antibody fragments (mol wt 27 kD) which maintain full antibody binding capacity. In accordance with their low molecular weight, scFvs are cleared rapidly from the circulation and, when combined with their rapid tumor penetration, allows patient imaging within 24 hr (*5*). MFE-23 used for this study is a high affinity, high specificity scFv directed against carcinoembryonic antigen (CEA). It was produced by phage technology, which made it possible to select for high affinity, and it was expressed in *E. coli* with a high yield. MFE-23 has already shown favorable biodistribution in an animal model, when radiolabeled with ^{125}I (*6*).

As for radiolabel choice, a half-life appropriate to the rate of antibody uptake by the tumor is desirable (*7*). Moreover, with the rapid tumor uptake of scFvs, radiolabels with short half-lives such as ^{99m}Tc and ^{123}I are most suitable. Radiolabeling scFvs with ^{123}I has some disadvantages. First, if tyrosine residues are situated in the antigen-binding site, radiolabeling these residues may lower the antigen-binding capacity of the scFv. Further, ^{123}I is very expensive and thyroid blocking agents are necessary to prevent iodine uptake by the thyroid. These disadvantages may be overcome by using ^{99m}Tc, but for radiolabeling with ^{99m}Tc, free sulphydryl groups are shown to be essential (*8*). These groups are not available on scFvs unless specifically added, for example by chemically modifying the scFv (*9*). We achieved this by engineering a free cysteine to the C-terminal amino acid of MFE-23 and, using this as a labeling attachment for ^{99m}Tc, developed a simple technique for inserting free thiol groups in genetically engineered scFvs. The cys-tagged product was called MFE-23-cys.

MATERIALS AND METHODS

Expression Vector and Cloning of MFE-23-cys

To create a new expression vector with a cysteine in the C-terminal tail, inverse PCR site-directed mutagenesis (*10*) was used to replace a histidine in the previously described (*11*) pUC119-based expression vector containing a C-terminal hexahistidine tag. Modification was achieved using 25 cycles of PCR with the oligonucleotides Cys-His-For (5′-TGGTGATGACATGCGGCCGCC CGTTTGAT-3′) and His6-Back (5′-TCATCACTAATAAGAATTCACTGGCCG-3′) followed by self-ligation. Clones containing the required sequence (Fig. 1) were identified by DNA sequencing. MFE-23 (*6*) was subcloned into this vector as an NcoI/NotI fragment.

Expression of MFE-23-cys in *E. coli*

E. coli 'Sure' cells were transformed with the plasmid construct shown in Figure 1. Cells were shaken at 37°C in 2X TY medium with 100 μg/ml ampicillin and 0.1% glucose until an optical density of 0.9 at 600 nm was obtained. Protein expression was induced by adding 1 m*M* isopropyl beta-D thiogalactoside overnight at 30°C. The cells were then pelleted and the supernatant containing MFE-23-cys decanted and stored at 4°C.

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