Chemotaxis, Viability, and Labeling Stability of Leukocytes Labeled with ^{99m}Tc-Exametazime Stabilized with Methylene Blue

Manuel Roca, Fernando Iglesias, Víctor García, Félix Armero, and Mª Carmen Díaz

Radiopharmacy Unit, Nuclear Medicine Department, L'Hospitalet de Llobregat, Ciutat Sanitària Universitària de Bellvitge, Barcelona, Spain

The in vitro viability, chemotaxis, and labeling stability of leukocytes labeled using ^{99m}Tc-exametazime stabilized with methylene blue were evaluated and compared with those obtained using nonstabilized ^{99m}Tc-exametazime. **Methods:** Two identical leukocyte populations, from 30 healthy donors, were labeled simultaneously using freshly prepared and 2-h-old stabilized ^{99m}Tc-exametazime. The following quality control techniques were performed on each labeled leukocyte sample: eosin Y staining, chemotaxis radioassay, and labeling stability at 2 h after labeling. **Results:** Eosin Y staining showed a cell viability of at least 98% in all samples, without a significant statistical difference between the populations. Chemotactic indices obtained with leukocytes labeled with freshly prepared, unstabilized ^{99m}Tc-exametazime were statistically greater than those obtained using ^{99m}Tc-exametazime stabilized with methylene blue ($z = 2.41$; $P < 0.02$). Labeling stability at 2 h after labeling was the same for both populations. **Conclusion:** The use of ^{99m}Tc-exametazime stabilized with methylene blue for leukocyte radiolabeling does not affect either cell membrane integrity or labeling stability but can cause a decrease in the cell chemotactic capacity that discourages its clinical use.

Key Words: leukocytes; ^{99m}Tc-exametazime; radiopharmaceutical stabilization; methylene blue; chemotaxis

J Nucl Med 2001; 42:505–508

A mixture of methylene blue and phosphate buffer was approved by the Food and Drug Administration as a stabilizing agent for ^{99m}Tc-exametazime in 1995. However, in the Ceretec (Amersham International, Buckinghamshire, U.K.) package insert, ^{99m}Tc-exametazime stabilized with methylene blue and phosphate buffer is indicated only for brain imaging studies and not for labeling white blood cells (WBCs) (*1*). The reason is probably the dark blue appearance of the mixture of stabilized ^{99m}Tc-exametazime–methylene blue and isolated leukocytes, which makes separation of the free ^{99m}Tc-exametazime from the labeled cells difficult or impossible. Moreover, other possible disadvantages are a decrease in the viability of WBCs and a negative effect on their function.

Recently, Hung et al. (*2*) described a method for labeling WBCs with ^{99m}Tc-exametazime stabilized with methylene blue and phosphate buffer. Hung et al. lightened the dark blue color of the dye by diluting the labeling medium with 12.6% acid citrate dextrose mixed with 0.8% NaCl, volume in volume. They also showed that WBCs can be labeled with a high labeling efficiency (LE), a long-lasting in vitro stability, and a preserved in vitro viability (trypan blue technique). We developed a WBC labeling method with stabilized ^{99m}Tc-exametazime and saline but using 18.75 μg methylene blue instead of the 250–500 μg used by Hung et al. and using a labeling medium with 50% leukocyte-poor plasma. We also achieved a reasonable LE and in vitro viability (eosin Y technique) (*3,4*). However, both studies have two serious drawbacks to recommending the use of methylene blue in WBC labeling: the limited number of experiments performed (6 in the series of Hung et al.; 8 and 10 in our series) and the lack of testing for WBC function.

The purpose of our study was to test the usefulness of ^{99m}Tc-exametazime stabilized with methylene blue in the labeling of leukocytes, using our own method and a large series of blood samples, and to study the chemotactic capacity of the labeled WBCs.

MATERIALS AND METHODS

Preparation of Stabilized and Nonstabilized ^{99m}Tc-Exametazime

Exametazime fractions were previously prepared as follows: each vial of Ceretec was reconstituted with 2.1 mL N_2-purged 0.9% NaCl sterile solution and split into 0.5-mL portions in separate N_2-filled sterile vials. These were stored at −40°C, for a maximum of 1 mo, until their use. Nonstabilized ^{99m}Tc-exametazime was obtained by adding 1.1 mL fresh ^{99m}Tc-pertechnetate to each thawed exametazime fraction. ^{99m}Tc-pertechnetate was obtained from a generator that had been eluted within the previous 24 h. Radioactivity was always approximately 1,110 MBq.

Stabilized ^{99m}Tc-exametazime was obtained from ^{99m}Tc-exametazime prepared in the same way but using 1.0 mL ^{99m}Tc-pertechnetate and approximately 1,400 MBq. Two minutes later,

Received May 10, 2000; revision accepted Nov. 7, 2000.

For correspondence or reprints contact: Manuel Roca, PhD, Medicina Nuclear, Ciutat Sanitària Universitària de Bellvitge, c/Feixa Llarga s/n, 08907-L'Hospitalet de Llobregat, Barcelona, Spain.

0.1 mL methylene blue (0.6 mg/mL in saline) was added to the vial.

The osmolarity and pH of six samples of both stabilized and nonstabilized ^{99m}Tc-exametazime were analyzed using an osmometer (Krioscop 800; Slamed, Frankfurt, Germany) and a pH meter (model 125; Corning, Medfield, MA). Therefore, we analyzed the osmolarity and pH of six samples of nonstabilized ^{99m}Tc-exametazime, following the manufacturer's instructions, from an entire vial of Ceretec. The lipophilic fraction of ^{99m}Tc-exametazime was analyzed in all cases using the chloroform extraction technique (*5*).

Blood Samples

Two 45-mL samples of venous blood collected over 6 mL acid citrate dextrose were obtained from 30 healthy blood donors. Written informed consent was obtained from all donors. The leukocyte-rich pellet was obtained from each blood–acid citrate dextrose sample using the hydroxy-ethyl-starch sedimentation technique according to the consensus protocol of the International Society of Radiolabelled Blood Elements (*6*).

Radiolabeling of Mixed Leukocytes with Stabilized and Nonstabilized ^{99m}Tc-Exametazime

Each leukocyte-rich pellet was gently resuspended in 0.5 mL cell-free plasma using a polypropylene Pasteur-type pipette. Afterward, 0.5 mL ^{99m}Tc-exametazime (~340 MBq, freshly prepared or stabilized 2 h before) was added to each sample. The incubation medium was mixed by swirling each test tube and was incubated for 15 min at room temperature.

Immediately after incubation, 4 mL cell-free plasma were added to each test tube. Afterward, both tubes were centrifuged at 150*g* for 5 min. The pellet containing the leukocytes labeled with methylene blue and ^{99m}Tc-exametazime was clearly seen because the plasma was light blue. Therefore, the plasma supernatant containing the unbound ^{99m}Tc-exametazime was easily removed with the aid of a polypropylene Pasteur-type pipette. Finally, both ^{99m}Tc-labeled leukocyte samples were suspended in sufficient Hank's solution (GIBCO, Life Technologies Ltd., Paisley, U.K.) to yield a cell suspension of 3×10^7 leukocytes per milliliter.

Quality Control of the Labeled Leukocytes

LE. The LE was calculated by the equation:

$$\text{LE (\%)} = \frac{\text{Radioactivity bound to cells}}{\text{Total radioactivity added}} \times 100.$$

In Vitro Viability Test. Eosin Y staining was performed on each labeled leukocyte suspension.

Chemotaxis Radioassay. A chemotaxis radioassay was performed by modifying the technique of Gallin et al. (*7*), using conventional Boyden chambers (Nucleopore Corp., Pleasanton, CA). The chemotactic factor was generated in two steps. First, 60 μg endotoxin (*Escherichia coli* 055:B5, L6529; Sigma, St. Louis, MO) dissolved in 20 μL saline were added to 100 μL donor-cell–free plasma. Second, 50 μL of this endotoxin-activated plasma were added to 1,000 μL Hank's solution.

The lower compartment of the Boyden chamber was filled with chemotactic factor (~200 μL). Two 3-μm polycarbonate filters (Millipore Corp., Molsheim, France) were placed so as to separate the lower compartment from the upper. The upper compartment was filled with 200 μL cell suspension. After exactly 3 h of incubation at 37°C, the cells of the upper compartment were gently suspended with the aid of a Pasteur-type glass pipette, and all the fluid was immediately removed. Both filters and the fluid from the lower chamber were also removed. Each filter and the upper and lower liquids were put into test tubes and counted in a γ counter (LKB-Wallac 1282 CompuGamma; Wallac, Turku, Finland) 48 h later.

The chemotactic index (CI) described by English and Clanton (*8*) was calculated as follows:

$$\text{CI} = \frac{\text{Radioactivity bound to lower filter}}{\text{Total radioactivity placed in chamber}} \times 10^6.$$

In all cases, chambers containing Hank's solution, instead of chemotactic factor, were used to evaluate the random chemotaxis. The random index (RI) was calculated as described above. The CI/RI ratio was used to compare the chemotactic capacity of the leukocytes labeled with unstabilized ^{99m}Tc-exametazime and the leukocytes labeled with methylene blue–stabilized ^{99m}Tc-exametazime.

In Vitro Stability. The remaining labeled leukocyte samples, suspended in Hank's solution, were stored at 37°C for 2 h. The in vitro cell-labeling stability at this time was determined by centrifuging both unstabilized and stabilized ^{99m}Tc-exametazime–labeled leukocytes and measuring the amount of ^{99m}Tc activity bound to the cells.

Statistical Analysis

A paired-sample statistical test was used to analyze the results.

RESULTS

Radiochemical Purity of ^{99m}Tc-Exametazime Preparations

The ^{99m}Tc-exametazime prepared without a stabilizing agent ($n = 30$) had a mean radiochemical purity of 95.5% (SD, 3.3%; range, 92.7%–98.2%). The 2-h-old methylene blue–stabilized ^{99m}Tc-exametazime had a mean radiochemical purity of 86.7% (SD, 3.2%; range, 77.1%–91.0%).

Osmolarity and pH of Stabilized and Nonstabilized ^{99m}Tc-Exametazime Preparations

The osmolarity of stabilized ^{99m}Tc-exametazime preparations ranged from 305 to 309 mOsm/L (mean, 307 mOsm/L; SD, 1.4 mOsm/L; $n = 6$), and the pH ranged from 7.0 to 7.6 (mean, 7.3; SD, 0.2; $n = 6$). The osmolarity of unstabilized ^{99m}Tc-exametazime preparations ranged from 309 to 313 mOsm/L (mean, 311 mOsm/L; SD, 1.9 mOsm/L; $n = 6$), and the pH ranged from 7.0 to 8.0 (mean, 7.5; SD, 0.3; $n = 6$). The osmolarity of ^{99m}Tc-exametazime obtained from an original unfractionated Ceretec vial ranged from 313 to 317 mOsm/L (mean, 315 mOsm/L; SD, 1.5 mOsm/L; $n = 6$), and the pH ranged from 8.2 to 9.0 (mean, 8.7; SD, 0.2; $n = 6$).

Quality Control of ^{99m}Tc-Exametazime–Labeled Leukocytes

LE. Leukocytes were labeled with unstabilized ^{99m}Tc-exametazime with a mean LE of 63.5% (SD, 13.6%; range, 40.1%–83.9%; $n = 30$). Leukocytes from the same donors were labeled with 2-h-old methylene blue–stabilized ^{99m}Tc-exametazime with a mean LE of 58.7% (SD, 12.6%; range, 36.3%–79.7%; $n = 30$).

In Vitro Stability. The radioactivity that remained bound to the leukocytes labeled with unstabilized ^{99m}Tc-exametazime, after 2 h stored at 37°C, ranged from 92.5% to 98.4% (mean, 96.0%; SD, 1.5%; $n = 30$). The activity that remained bound to the leukocytes labeled with 2-h-old methylene blue–stabilized ^{99m}Tc-exametazime, after 2 h stored at 37°C, ranged from 92.9% to 98.5% (mean, 96.0%; SD, 1.4%; $n = 30$). The results for the two populations did not statistically differ.

Eosin Y Staining. At least 98% of the mixed leukocyte samples, labeled with either stabilized or unstabilized ^{99m}Tc-exametazime, did not concentrate the dye.

Chemotaxis Radioassay. Leukocytes labeled with unstabilized ^{99m}Tc-exametazime preparations ($n = 30$) had a mean CI/RI ratio of 10.4 (SD, 8.8; range, 1.4–38.4). Leukocytes from the same donors, labeled with 2-h-old stabilized ^{99m}Tc-exametazime, had a mean CI/RI ratio of 8.0 (SD, 7.3; range, 1.3–32.2). The CI/RI ratios from the two populations were statistically different ($z = 2.41$; $P < 0.02$). Variations in CI/RI ratios were derived from variations in the CI values (Table 1).

DISCUSSION

Many agents have been described as stabilizing ^{99m}Tc-exametazime (*9–13*). But only methylene blue and cobalt chloride have been described with regard to their usefulness for leukocyte labeling (*2,14–15*). However, none has been approved for labeling WBCs in either the United States (*1*) or Europe (*16*).

We performed this study to clarify the possibility of using methylene blue as a stabilizing agent for labeling WBCs. Using 18.75 μg methylene blue in each labeling medium instead of the 250–500 μg used by Hung et al. (*2*), we avoided the problems associated with the dark blue color of the stabilizer; therefore, we can perform our labeling method with or without methylene blue. Moreover, using this amount of methylene blue achieved a fair stabilization: 2 h after the ^{99m}Tc-exametazime stabilization, we obtained a mean lipophilic fraction of 86.7%. The pH and osmolarity of this stabilized solution were in the physiologic range.

In our department, we label a maximum of two different leukocyte samples simultaneously, to avoid undesirable

TABLE 1
Chemotaxis Radioassay

Sample no.	Leukocytes labeled with unstabilized ^{99m}Tc-HMPAO			Leukocytes labeled with stabilized ^{99m}Tc-HMPAO		
	CI	RI	CI/RI	CI	RI	CI/RI
1	1.3	0.9	1.4	1.2	0.9	1.3
2	3.4	2.4	1.4	2.7	2.1	1.3
3	1.0	0.7	1.4	5.4	1.0	5.4
4	4.2	1.9	2.2	2.9	1.5	1.9
5	1.6	0.7	2.3	0.7	0.5	1.3
6	3.5	0.8	4.4	2.3	0.7	3.3
7	3.8	0.8	4.7	5.1	0.9	5.7
8	12.5	2.4	5.2	9.9	2.9	3.4
9	7.0	1.3	5.4	3.6	0.7	5.2
10	3.8	0.6	6.4	3.5	0.6	5.9
11	5.2	0.8	6.5	4.7	0.8	5.9
12	3.4	0.5	6.8	2.8	0.4	7.1
13	30.4	4.4	6.9	36.0	4.5	8.0
14	4.8	0.7	6.9	2.5	0.7	3.5
15	8.5	1.0	8.5	1.9	0.9	2.1
16	4.3	0.5	8.6	6.4	0.6	10.7
17	13.2	1.5	8.8	11.8	1.9	6.2
18	7.5	0.8	9.4	7.8	0.7	11.1
19	9.5	1.0	9.5	3.1	0.9	3.4
20	8.1	0.8	10.1	3.0	1.1	2.7
21	7.3	0.7	10.4	8.5	0.9	9.4
22	12.8	1.1	11.6	5.5	1.2	4.6
23	11.6	1.0	11.6	4.5	0.8	5.6
24	6.8	0.5	13.5	5.5	0.6	9.1
25	12.2	0.9	13.6	18.8	0.8	23.5
26	26.2	1.6	16.4	19.4	1.7	11.4
27	35.6	1.4	25.4	14.4	1.5	9.6
28	23.9	0.9	26.6	25.8	0.8	32.2
29	35.0	1.2	29.2	18.5	1.0	18.5
30	19.2	0.5	38.4	15.2	0.7	21.7

CI = chemotactic index; RI = random index.

cross-contaminations. Two hours later, another two leukocyte samples can be labeled. This is the reason we chose 2 h as a practical time for investigating the usefulness of the stabilized ^{99m}Tc-exametazime.

Leukocytes are labeled in a 50% plasma medium. Other authors who use only a saline medium have obtained an LE approximately 20%–25% higher than ours (mean LE, 58.7%) (*2*,*14*). Nevertheless, we prefer to maintain the leukocytes in a more physiologic medium during the incubation step instead of increasing the LE. The LE that we achieved using 2-h-old stabilized ^{99m}Tc-exametazime was as we expected: approximately 90% of that obtained with unstabilized ^{99m}Tc-exametazime.

The in vitro stability of ^{99m}Tc-exametazime-labeled leukocytes was the same for both leukocyte populations. Our results were similar to those obtained by authors who labeled leukocytes in saline medium (*17*–*19*) or in 50% plasma–saline medium (*20*).

The results of the eosin Y test showed suitable cell viability for both leukocyte populations. The in vitro stability and the results of the eosin Y test were as expected, because we used significantly less methylene blue (18.75 μg instead of 250–500 μg) than did other authors (*2*), with a similar number of leukocytes. However, the chemotaxis radioassay showed that the leukocytes labeled with unstabilized ^{99m}Tc-exametazime had a better preserved chemotactic response than did those labeled with stabilized ^{99m}Tc-exametazime. The value of our results is based on our simultaneously performing the labeling method on two equal leukocyte samples belonging to the same donor and on our inclusion of a large enough number of donors (n = 30) to achieve valuable statistical results.

The presence of methylene blue in the labeling medium acts on the capacity of the leukocytes to move in response to a chemotactic stimulus. However, the magnitude of individual-to-individual variations in CI/RI ratio is large, compared with the magnitude of the stabilized-versus-nonstabilized differences for leukocytes from individual subjects. For this reason, we doubt whether the observed stabilized-versus-nonstabilized difference in CI/RI ratio has clinical significance. However, the existence of this possibility, based on our study using much less methylene blue than the manufacturers recommend to stabilize ^{99m}Tc-exametazime, discourages us from advising that methylene blue–stabilized ^{99m}Tc-exametazime be used for labeling leukocytes.

CONCLUSION

The use of ^{99m}Tc-HMPAO stabilized with methylene blue in the labeling of leukocytes does not affect either cell viability or labeling stability but can cause a decrease in the cell chemotactic capacity that discourages its clinical use.

ACKNOWLEDGMENTS

This study was supported by grant FIS 98/0582 from the Spanish Ministry of Health. This study was presented in part at the ninth congress of the International Society of Radiolabeled Blood Elements, October 20–23, 1999, and was published as an abstract (*Nucl Med Commun.* 1999;20:967).

REFERENCES

1. Ceretec [package insert]. Arlington Heights, IL: Amersham Corp.; 1990.
2. Hung JC, Chowdhury S, Mullan BP. Use of stabilized technetium-99m-exametazime for radiolabeling leukocytes. *J Nucl Med.* 1998;39:912–917.
3. Roca M, Garcia V, Armero F, Vallejos V, Castell M, Martín-Comín J. Radiolabeling leucocytes with ^{99m}Tc-HM-PAO stabilized with methylene blue [abstract]. *Eur J Nucl Med.* 1997;24:1041.
4. Garcia V, Roca M, Armero F, Iglesias F, Gomes V, Martín-Comín J. Stabilization of ^{99m}Tc-exametazime with methylene blue: its use in the labeling of leukocytes [in Spanish]. *Rev Esp Med Nuclear.* 1998;17:261–264.
5. Ballinger JR, Gulenchyn KY, Reid RH. Radiopharmaceutical factors in the variable quality of (^{99m}Tc)HM-PAO images of the brain. *J Nucl Med.* 1990;31:118–122.
6. Roca M, Martín-Comín J, Becker W, et al. A consensus protocol for white blood cells labelling with ^{99m}Tc hexamethylpropylene amine oxime. *Eur J Nucl Med.* 1998;25:797–799.
7. Gallin JI, Clark RA, Kimball HR. Granulocyte chemotaxis: an improved in vitro assay employing ^{51}Cr-labeled granulocytes. *J Immunol.* 1973;110:233–240.
8. English D, Clanton JA. Evaluation of neutrophil labeling techniques using the chemotaxis radioassay. *J Nucl Med.* 1984;25:913–916.
9. Hung JC, Volkert WA, Holmes RA. Stabilization of technetium-99m-d,l-hexamethylpropyleneamineoxime (^{99m}Tc-d,l-HMPAO) using gentisic acid. *Nucl Med Biol.* 1989;16:675–680.
10. Sampson CB, Solanki C. Stabilization of ^{99m}Tc-exametazime using ethanol and storage at low temperature [abstract]. *Eur J Nucl Med.* 1991;18:532.
11. Millar AM. A routine method for using sodium iodide to stabilize sodium pertechnetate-^{99m}Tc dispensed for the preparation of ^{99}Tcm-exametazime. *Nucl Med Commun.* 1992;13:306–311.
12. Weisner PS, Bower GR, Dollimore LA, Forster AM, Higley B, Storey AE. A method for stabilizing ^{99m}Tc-exametazime prepared from a commercial kit. *Eur J Nucl Med.* 1993;20:661–666.
13. Mang'era KO, Vanbilloen HP, Schiepers CW, Verbruggen AM. Stabilisation of high-activity ^{99m}Tc-*d,l*-HMPAO preparations with cobalt chloride and their biological behaviour. *Eur J Nucl Med.* 1995;22:1163–1172.
14. Hung JC, Chowdhury S, Mahoney DW, Mullan BP. Radiolabelled mixed leukocytes and pure granulocytes with stabilized ^{99}Tcm-exametazime. *Nucl Med Commun.* 1998;19:981–987.
15. Prangere T, Bowden AD, Beauchat V, et al. A study of the behaviour of cobalt chloride during the labelling of leukocytes with ^{99}Tcm-HMPAO stabilised *in vitro* by the addition of cobalt chloride solution. *Nucl Med Commun.* 1997;18:258–261.
16. Ceretec [package insert]. Arlington Heights, IL: Amersham Healthcare; 1995.
17. Peters AM, Osman S, Henderson DL, et al. Clinical experience with ^{99m}Tc-hexamethylpropylene-amineoxime for labelling leukocytes and imaging inflammation. *Lancet.* 1986;25:946–949.
18. Danpure HJ, Osman S, Carroll MJ, Roddie ME, Peters AM, Lavender JP. In vitro studies to develop a clinical protocol for radiolabelling mixed leukocytes with Tc-99m HMPAO [abstract]. *J Nucl Med.* 1987;28:694.
19. Mortelmans L, Malbrain S, Stuyck J, et al. In vitro and in vivo evaluation of granulocyte labeling with [^{99m}Tc]d,l-HM-PAO. *J Nucl Med.* 1989;30:2022–2028.
20. Moragas M, Piera C, Muxi A, et al. Leukocytes labeled with ^{99m}Tc-HM-PAO: labeling technique, quality control and preliminary results in vivo [in Spanish]. *Rev Esp Med Nuclear.* 1989;8:9–18.