Cellular Basis of ECD Brain Retention

M.R. Jacquier-Sarlin, B.S. Polla and D.O. Slosman

Nuclear Medicine Division, Department of Radiology, and Environment and Health Program, Geneva University Hospital, Geneva, Switzerland; Laboratory of Respiratory Physiology, UFR Cochin, Paris, France

Clinical observations have shown discrepancies between ECD and HMPAO regional cerebral perfusion, particularly in brain tumors and during stroke recovery. We investigated the nature of the process(es) involved in ECD accumulation in vitro at the cellular level. **Methods:** Time course incorporation of ECD was studied in a fast-growing human premonocytic line, U937, in a human astrocytic-derived cell line, U373, and a human hybridized endothelial cell line, EaHy926. Cells were further used in experiments aiming to correlate esterase activity and ECD retention. **Results:** Significant differences in ECD retention between these cell lines were observed: $\%U_{ECD}$ (cpm cells/cpm standard of injected) plateaued within 2 hr in all cases but $\%U_{ECD}$ was significantly higher in U937 cells (25.1 ± 3.9% at 120 min) than in the other cell lines (6.1 ± 0.7% and 8.2 ± 2.0% at 120 min for U373 and EaHy926, respectively). Contrary to what we expected, total cellular esterase activity (EA_{TOT}) was inversely correlated to $\%U_{ECD}$. EA_{TOT} was 5-fold lower in U937 cells than in U373 and 20-fold lower than in EaHy926. Thus, we compared the membranar to the cytosolic esterase activity of U937 and analyzed the influence of temperature and diisopropylfluorophosphate (DFP, an inhibitor of cytosolic esterase activity) on both ECD retention and enzymatic activities. When cells were exposed to DFP, $\%U_{ECD}$ was reduced by 80%; while when cells were maintained at 4°C, $\%U_{ECD}$ continuously increased, corresponding to a passive diffusion since both cytosolic and membranar esterase activities were inhibited. **Conclusion:** For optimal uptake of ECD, the membranar fraction of the esterase activity has to be low, while, in contrast, the cytosolic fraction of the esterase activity plays an important role in ECD cell retention. ECD-SPECT is likely able to reflect regional cerebral blood flow in normal and pathological states accurately, but in the event of unusual observations, the membranar esterase activity should be considered to explain reduced ECD retention.

Key Words: brain perfusion; technetium-99m-ECD; technetium-99m-HMPAO; cellular uptake

J Nucl Med 1996; 37:1694-1697

It is well recognized that noninvasive, quantitative measurement of regional cerebral blood flow (rCBF) is one of the leading components in nuclear medicine. The growing interest in functional neuroimaging using SPECT correlates more with the introduction of a new class of brain imaging agents labeled with ^{99m}Tc (*1*) than with the improvement in image quality (triple-headed gamma cameras and fan beam collimators). HMPAO was the first ^{99m}Tc-labeled marker to be introduced in a clinical arena (*2–4*), based on the hypothesis that this molecule crosses the blood-brain barrier through cell membranes and is retained after intracellular conversion in a hydrophilic form (*5*). HMPAO intracellular retention has been related to the cellular glutathione (*6*) content and HMPAO brain SPECT imaging has been shown to correlate with rCBF (*7*). A newer radiopharmaceutical, ethyl cysteinate dimer (ECD), has been introduced because of its successful in vitro stability after the labeling procedure. Its brain retention also has been related to a metabolic pathway, i.e., retention of ECD in cells after intracellular conversion into hydrophilic forms (ECM and EC) being a function of cellular esterase activity (*8–10*).

Both HMPAO and ECD have been used clinically in numerous conditions, particularly in patients with brain tumors, with epilepsy or after strokes. Investigators have identified the presence of discordant results between ECD and other rCBF agents (*11–14*). Their observations led us to study in vitro the cellular mechanisms involved in the cell retention of these ^{99m}Tc-labeled radiopharmaceuticals. We previously studied the mechanisms involved in cellular retention of HMPAO and found that the cellular glutathione content was not the major determinant of HMPAO uptake. The tissue redox activity also controls the extracellular amount of lipophilic substrate that can enter into cells (*15*). Therefore, our aim was to investigate the nature of the process(es) involved in the accumulation of ECD at the cellular level.

MATERIALS AND METHODS

Determination of cellular incorporation of ^{99m}Tc-ECD was performed simultaneously with that of ^{111}In-chloride. Cellular retention of ^{111}In-chloride corresponds to the quality control of the experiments. This is based on the fact that the ^{111}In-chloride remains extracellular and, therefore, that ^{111}In-chloride cellular content reflects nonspecific cellular uptake and/or alterations of cell membranes. Experiments were also conducted with $^{99m}TcO_4$ to evaluate the possible effects of free technetium in the medium on the measurement of ^{99m}Tc-labeled ECD cell contents and were compared to ^{111}In-chloride.

Cells

Because it is as yet unknown which cells are involved in brain retention of HMPAO or ECD, we hypothesized that it could be endothelial cells (when the markers pass through the brain-blood barrier), astrocytes (that correspond to the other side of the brain-blood barrier) or the neurons themselves. We therefore chose a human astrocytic-derived cell line (U373) and a human-hybridized endothelial cell line (EaHy926). Finally, because inflammatory processes may be involved and could explain uptake discrepancies between HMPAO and ECD, the premonocytic cell line U937 was included. The choice of these cell lines also was based on our previous experiments, thus enabling multiple comparisons (*15–17*).

Radiopharmaceuticals and Media

Technetium-99m-ECD and ^{111}In-chloride were obtained commercially. The U937 and U373 were maintained in stationary suspension in RPMI-1640 medium supplemented with fetal calf serum (10%), glutamine (1%) and HEPES (10 m*M* for U373 cells only). The EaHy926 cells were maintained in DMEM medium supplemented with fetal calf serum (10%), glutamine (1%), HEPES (10 m*M*, penicillin-streptomycin (50 U/ml), HAT (1%). In all experiments, fresh stock solutions of ECD were prepared with a specific activity of 1 mCi/ml. The pertechnetate solution was prepared with a specific activity of 1 mCi/ml; ^{111}In-chloride solution was prepared with a specific activity of 0.2 mCi/ml.

Received Sept. 1, 1995; revision accepted Jan. 28, 1996.

For correspondence or reprints contact: Daniel Slosman MD, Nuclear Medicine Division, Geneva University Hospital, 1211 Geneva 14, Switzerland.

Radiopharmaceutical Uptake Studies

First, the time course of incorporation of the markers was studied in the three cell lines. To study the cellular retention of ECD, 1×10^6 cells/ml of medium were placed in conic tubes and exposed to ^{111}In-chloride and ^{99m}Tc-ECD (50 μl of stock solutions) for specific intervals of time from 0 to 360 min. Control experiments were also conducted replacing labeled ECD with $^{99m}Tc\text{-}O_4$. Then, cells were centrifuged at 4°C and washed twice with PBS. Cell pellets were resuspended in 150 μl PBS and then counted in a gamma counter. Fifty microliters of 1/100 stock solutions of ^{99m}Tc-ECD, pertechnetate or ^{111}In-chloride were also counted separately and were defined as the standards of administrated cell dose. The results were expressed as the percentage of counts measured in standards (%U^{99m}Tc, %U^{111}In, %U_{ECD}).

The influence of both the temperature and the esterase inhibitor, diisopropylfluorophosphate (DFP, 5 m*M*) was analyzed on ECD cellular retention. In these studies, the cells either were preincubated for 1 hr at 4°C before adding ^{99m}Tc-ECD and maintained at this temperature throughout the kinetic study, or the cells were incubated overnight with DFP, then washed and treated as already described for ECD incorporation.

In all cases, the experiments correspond to the time course studies of cellular retention for 0 to 360 min of exposure repeated 5 to 10 times.

Cell Fractionation

U937 subcellular fractionation was performed as described by Darte and Beaufay (*18*). Briefly, the cells were collected by centrifugation, washed twice in PBS and resuspended at 4°C in 0.25 M sucrose, 1 m*M* EGTA, 3 m*M* imidazole pH 7.4 at 8×10^7 cells/ml. The cells were disrupted by 40 runs in a cell-cracker (8.02 EMBL: 8.0006-mm diameter ball), leading to more than 90% lysis and leaving the nuclei intact. After elimination of the nuclei by centrifugation at 4°C for 15 min at 1200 g, total cellular particles (including lysosomes, mitochondria, plasma membranes, endosomes and microsomes) were sedimented at 4°C at 33,000 g for 1.5 hr in a centrifuge (JA21 rotor). The supernatant was denoted as the cytosol and stored at −20°C.

Measurement of Cellular and Cytosolic Esterase Activity

Cells were washed, counted and adjusted to 5×10^6 cells/ml and distributed in 96 well plates at a concentration of 1×10^6 cells/well. The substrate N-succinyl-Ala-Ala-Pro-Phe-P-nitroanilide was added at a final concentration of 10 m*M*. After incubation for different periods of time ranging from 0 to 360 min, plates were centrifuged (1200 rpm, 10 min), and the supernatant was collected and read at 405 nm by optical spectrophotometry. The influence of temperature and DFP on esterase activity was determined by preincubating cells for 30 min at 4°C or with 5 m*M* DFP before adding the esterase substrate.

Statistical Analysis

The results are presented as the mean ± s.e.m. (X ± s.e.m.) unless otherwise indicated. Analysis of variance (one-way ANOVA) was used to assess the influence of time on the cellular retention of the different markers tested and to test the significance of differences in retention between the three cell lines tested. To assess the influence of time and the various experimental conditions under which cells were exposed (DFP, T°C), two-way analysis of variance was performed. The Scheffe F-test was used to determine the level of significance between the parameters tested.

RESULTS

Time Course Incorporation of ECD

Cellular retention of ^{99m}Tc or ^{99m}Tc-ECD was measured as a function of time of exposure in parallel to ^{111}In-chloride. In control experiments, %U^{111}In remained lower than 0.7% up to 360 min of incubation and was not significantly different as a function of cell type. In control experiments, %U^{99m}Tc was never significantly different from %U^{111}In.

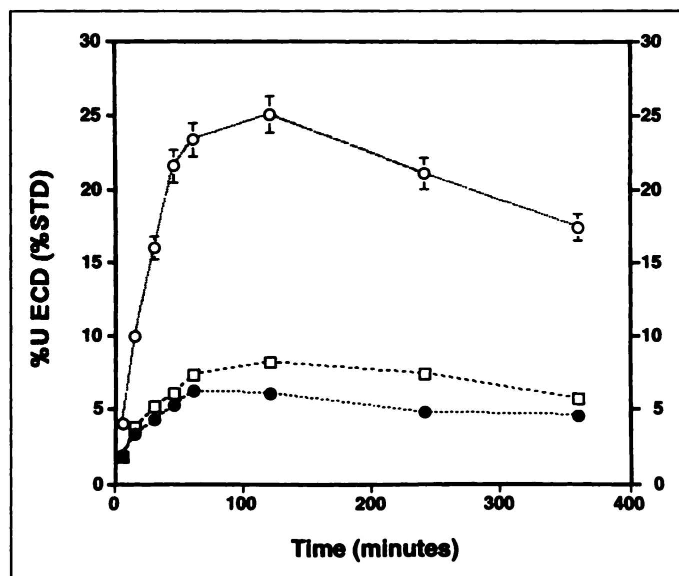

FIGURE 1. Measurements of cellular retention of ^{99m}Tc-ECD (%U ECD) in U937 (-○-), EaHy926 (-□-) and U373 (-●-) cell lines as a function of time (n = 202). Significant differences were observed (two-way ANOVA) as a function of time (partial-F = 9.13, $p < 0.0001$) and cell lines (partial-F = 2.5, $p < 0.005$).

When cells were exposed to ^{99m}Tc-ECD, different time course profiles were obtained. In all cell lines, we noticed a significant increase in %U_{ECD}, but there was a significant difference between time course profiles of the U937 cell line and the other U373 cells and EaHy926 cells (two-way ANOVA, partial-F = 9.13, $p < 0.0001$, n = 202). Overall, U937 cells showed higher ECD retention (two-way ANOVA, partial-F = 2.5, $p < 0.005$, n = 202) (Fig. 1). After a 120-min exposure to ECD, cellular retention of ECD reached a plateau at which point %U_{ECD} was 25.1 ± 3.9% for U937 cells, 6.1 ± 0.7% for U373 cells and 8.2 ± 2.0% for EaHy926 cell lines. Then %U_{ECD} decreased for all cell lines.

Comparison of the Cell Surface Esterase Activity between U937, U373 and EaHy926 Cells

As ECD cellular retention has been attributed to cellular esterase activity, we measured the esterase activity of intact U937, U373 and EaHy926 cells (Fig. 2). As the esterase substrate remains extracellular and as plasma membranes are not altered during the time of incubation (confirmed by MTT experiments, results not shown), the esterase activity denoted on intact cells can be attributed to membranar esterases. We found that the esterase activity was significantly lower for the U937 cells (0.12 ± 0.06) than for the U373 cells (0.62 ± 0.16), as well as for the EaHy926 (7.14 ± 0.15) (n = 63, two-way ANOVA, partial-F = 24.41 $p < 0.0001$). As shown in Figure 2, the esterase activity was time-dependent. The increased activity of membranar esterase as a function of time was in contrast to %U_{ECD} which reached a plateau at 120 min and then decreased (Fig. 1). Contrary to what we expected, cellular esterase activity (the mechanism by which ECD becomes hydrophilic) was inversely correlated to ECD retention (%U_{ECD}).

Effects of Temperature and DFP on Technetium-99m-ECD Incorporation in U937 Cells

To further investigate the cellular mechanism of ^{99m}Tc-ECD incorporation, we used U937 cells. We modulated

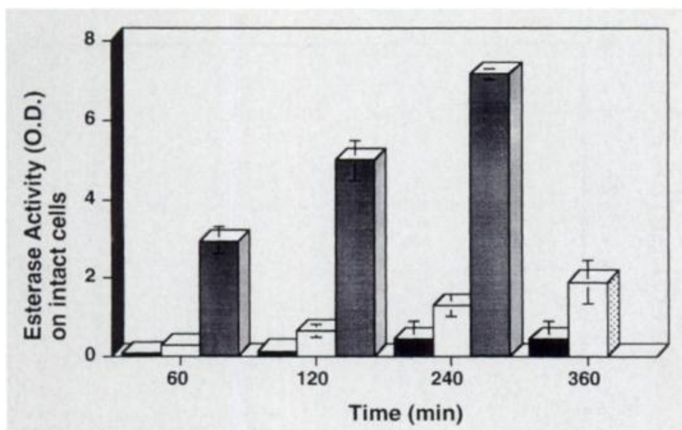

FIGURE 2. Measurements of esterase activity in U937 (■), U373 (□) and EaHy926 (■) cell lines (intact cells) as a function of time (n = 63). Significant differences were observed (two-way ANOVA) as a function of time (partial-F = 31.75, $p < 0.0001$) and cell lines (partial-F = 24.41, $p < 0.0001$).

the enzymatic activity by exposure to cold (4°C) and DFP (5 m*M* (Fig. 3). While at 37°C, intracellular retention of ECD gradually increased, reached a plateau during the first 120 min (23.3 ± 0.9%) and then declined, at 4°C, the intracellular retention of ECD continuously increased as a function of time reaching 30.5 ± 9.0% at 360 min. In the presence of DFP, the intracellular retention of ECD was dramatically reduced by 50% to 80% at 37°C as well as at 4°C (3.6 ± 0.5% and 10.8 ± 1.8% after 360 min of exposure to 37°C and 4°C, respectively).

Comparison of Cellular and Cytosolic Esterase Activity on U937 Cells; Effects of Temperature and DFP

If, indeed, membranar esterase activity is involved in ECD retention, the presence of cytosolic esterase has to be efficient to convert ECD into hydrophilic forms which are retained in cells. Using subcellular fractionation, we isolated the cytosolic fraction of U937 cells and compared the respective esterase activity of intact cells (membranar esterase) and of cytosol. As for membranar esterase activity (Fig. 2), the cytosolic esterase activity was time-dependent with a maximum at 360 min (data not shown). In contrast to the membranar esterase activity, however, the cytosolic esterase activity was more important over time and achieved 2.784 ± 0.139 at 360 min, as compared to 0.441 ± 0.168 (Table 1). As temperature and DFP appeared to modulate $\%U_{ECD}$, we analyzed the influence of these two parameters on both esterase activities (cytosolic and membranar) (Table 1). While both esterase activities were significantly inhibited at 4°C (reduction of 69.2% and 60.4% from control values for the membranar and the cytosolic esterase activities, respectively), only the cytosolic esterase activity was significantly inhibited by DFP at 37°C (reduction of 64.2% from control values). These data suggest that different esterases are implicated in the conversion of ECD to hydrophilic forms at the plasma membrane and in the cytosol.

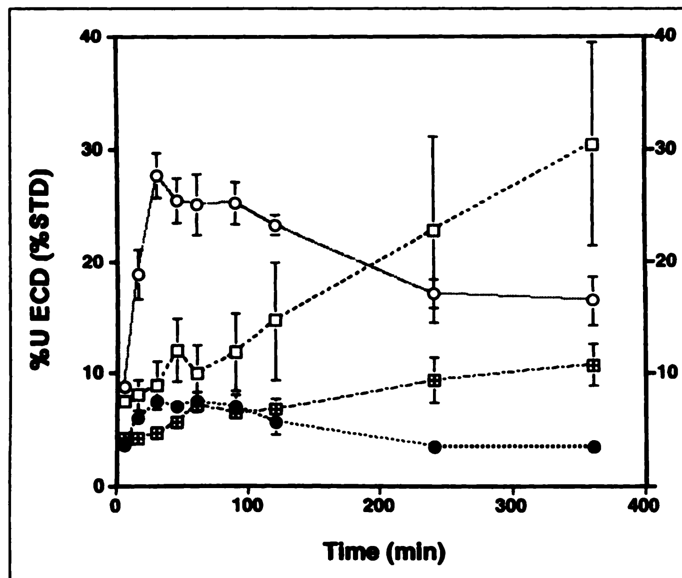

FIGURE 3. Measurements of cellular retention of ^{99m}Tc-ECD (%U ECD) in U937 cells (intact cells) as a function of time and under control conditions (37°C) (○), when cells were exposed to 4°C (□), to DFP at 37°C (●) and to DFP at 4°C (⊞). Significant differences were observed (two-way ANOVA, n = 234) between baseline studies (time-activity at 37°C) and 4°C as well as DFP at 37°C or at 4°C (partial-F = 3.979, $p < 0.001$). Significant differences were also observed between time-activity at 4°C with and without DFP or with DFP at 37°C and 4°C ($p < 0.001$).

TABLE 1
Comparison of Membranar and Cytosolic Esterase Activity in U937 Cell at 360 min under Control Conditions and after Exposure to 4°C or to DFP

Experimental conditions	Membranar esterase activity	Cytosolic esterase activity
Control (37°C)	0.441 ± 0.168	2.784 ± 0.139
Cold (4°C)	0.136 ± 0.060*	1.102 ± 0.107*
DFP (37°C)	0.313 ± 0.160	0.997 ± 0.118*
DFP (4°C)	0.107 ± 0.025*	0.848 ± 0.193*

*$p < 0.001$ from control.

DISCUSSION

Our data provide in vitro evidence to support the view that the esterase activity is the major determinant of the overall retention of ECD, but the presence of membranar esterase enzymes plays an inverse role by inhibiting the transmembranar passage of ECD. As a consequence, we can expect reduction of ECD uptake in the presence of cells that express higher esterase sites at their membrane surface.

Hypothesis. HMPAO, the first ^{99m}Tc-labeled marker introduced in the clinical setting correlates with rCBF (*3,7*). It is based on the hypothesis that this molecule crosses the blood-brain barrier, goes through cell membranes and is retained intracellularly after conversion to a hydrophilic form by glutathione (*6*). However, we recently reported that the major determinant of cell retention of HMPAO is the redox equilibrium of the cell environment and not the intracellular content of glutathione (*15*). Based on the hypothesis that ECD may show a similar pattern and based on the clinical discrepancies observed between ECD and other rCBF agents (*11–14*), our goal was to investigate the cellular mechanism(s) involved in ECD cell retention. To date, it has been shown that ECD crosses the blood-brain barrier, goes through cell membranes because of its lipophilic nature and is retained intracellularly after its conversion to hydrophilic forms by esterase activity (*8–10*). In a clinical setting, ECD brain retention is able to reflect rCBF with minimal limitations (*9,10,12,19–22*). Similar to what has been done with HMPAO, we measured in vitro the kinetic uptake of ECD in three cell lines in order to detect possible differences between endothelial cells, astrocytes and inflammatory cells as we noticed in the HMPAO study (*15*).

Experimental Observations. We measured membranar esterase activity to correlate esterase activity with ECD uptake. We surprisingly found an inverse correlation: ECD uptake was higher in cells with less membranar esterase activity. To

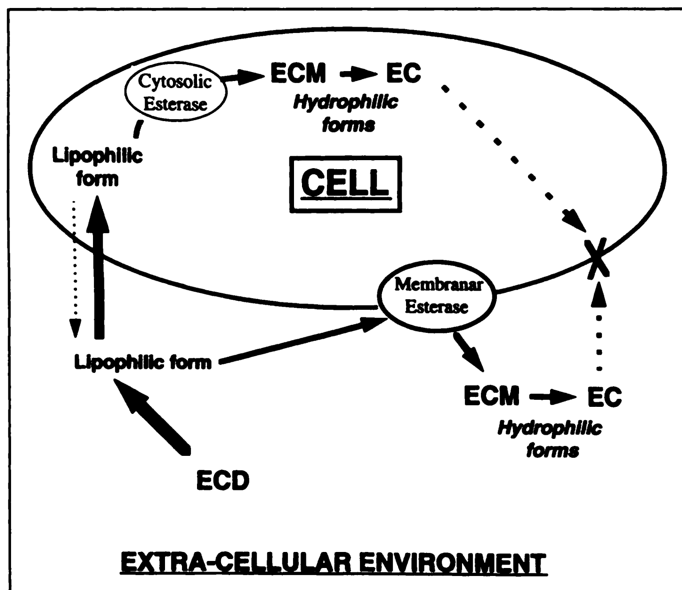

FIGURE 4. Mechanisms involved in ECD cell retention.

demonstrate the involvement of an enzymatic process (in particular esterases) for ECD retention, we exposed cells to 4°C or to the esterase inhibitor, DFP. At 4°C, we observed an unexpected profile of ECD retention with an early reduction of ECD uptake during the first 2 hr followed by a constant increase. In contrast with DFP, we obtained a major reduction of ECD retention that could confirm the involvement of this enzymatic process. These results suggest that if cellular esterase activity has to be limited to favor ECD retention, a cytosolic esterase activity has to be efficient to convert ECD to the hydrophilic forms which are retained in cells. Then we compared the cellular and the cytosolic esterase activity and found that a significant esterase activity was present in the membranes of U937 cells, but this activity was slower compared to the cytosolic activity. Based on these results we hypothesized that, if the lipophilic-hydrophilic reaction is induced outside the cell, the product will not have a chance to enter the cell, in a manner similar to that of HMPAO. Therefore, a high membranar esterase activity will result in a small intracellular ECD content. In contrast, important cytosolic esterase activity will favor ECD retention. This model fails, however, to explain the profile of the kinetic of ECD uptake when cells are exposed to 4°C. Therefore, additional experiments were performed with a simultaneous exposure of the cells to 4°C and DFP (at 37°C or 4°C). The measurement of the influence of temperature and DFP on both membranar and cytosolic esterase activities indicated that, while both esterase activities were inhibited by temperature, DFP exclusively inhibited the cytosolic fraction.

Taking all these observations into account, we propose the following model (Fig. 4). When cells are exposed to ECD, if the membranar esterase activity is important, most of it will follow a lipophilic-hydrophilic reaction in the extracellular environment and a minimal amount of remaining lipophilic compound can pass through the cell membrane. If the membranar esterase activity is minimal, most of the compound can diffuse freely through the cell membrane and the cytosolic activity can induce the lipophilic-hydrophilic reaction and, therefore, ECD can be retained over time. Thus, the ECD incorporation in brain tissue is probably dependent on the modification of protease expression and, in particular, those exerting esterase activity. This suggests that brain SPECT imaging with ECD might more accurately reflect rCBF than HMPAO because modulating the expression of esterase activity in brain cells could be less important than modification of redox tissue in cerebral disease.

Our data provide in vitro evidence supporting the hypothesis that esterase activity is the major determinant of the overall retention of ECD, but these data show further that the membranar esterase enzymes play an inverse role by inhibiting the transmembranar passage of ECD.

ACKNOWLEDGMENTS

We are indebted to Professor Pierre Magistretti and Dr. Franz Buchegger for helpful comments and stimulating discussions. We thank Nicole Pittet and Laurence Porret for skillful technical assistance. This research was supported in part by FNSRS grant 31-33781.92 (DOS) and by INSERM (MRJS and BSP).

REFERENCES

1. Moretti JL, Caglar M, Weinmann P. Cerebral perfusion imaging tracers for SPECT: which one to choose? *J Nucl Med* 1995;36:359–363.
2. Podreka I, Suess E, Goldenberg G, et al. Initial experience with technetium-99m-HMPAO brain SPECT. *J Nucl Med* 1987;28:1657–1666.
3. Lassen NA, Andersen AR, Friberg L, Paulson OB. The retention of ^{99m}Tc-D,L-HMPAO in the human brain after intracarotid bolus injection: a kinetic analysis. *J Cereb Blood Flow Metab* 1988;8(suppl):S13–S22.
4. Andersen AR. ^{99m}Tc-D,L-hexamethylene-propyleneamine oxime (^{99m}Tc-HMPAO): basic kinetic studies of a tracer of cerebral blood flow. *Cerebrovasc Brain Metab Rev* 1989;1:288–318.
5. Neirinckx RD, Canning LR, Piper IM, et al. Technetium-99m-D,L-HMPAO: a new radiopharmaceutical for SPECT imaging of regional cerebral blood perfusion. *J Nucl Med* 1987;28:191–202.
6. Neirinckx R, Burke JF, Harrison RC, et al. The retention mechanism of technetium-99m-HMPAO: intracellular reaction with glutathione. *J Cereb Blood Flow* 1988;8:S4–S12.
7. Gemmell HG, Evans NTS, Besson JAO, et al. Regional cerebral blood flow imaging: a quantitative comparison of technetium-99m-HMPAO SPECT with C-15 O2 PET. *J Nucl Med* 1990;31:1595–1600.
8. Walovitch RC, Hill TC, Garrity ST, et al. Characterization of technetium-99m-L,L-ECD for brain perfusion imaging, part 1: pharmacology of technetium-99m-ECD in nonhuman primates. *J Nucl Med* 1989;30:1892–1901.
9. Léveillé J, Demonceau G, De Roo M, et al. Characterization of technetium-99m-L,L-ECD for brain perfusion imaging, part 2: biodistribution and brain imaging in humans. *J Nucl Med* 1989;30:1902–1910.
10. Walovitch RC, Franceschi M, Picard M, et al. Metabolism of ^{99m}Tc-L,L-ethyl cysteinate dimer in healthy volunteers. *Neuropharmacology* 1991;30:283–292.
11. Tsuchida T, Nishizawa S, Yonekura Y, et al. SPECT images of technetium-99m-ethyl cysteinate dimer in cerebrovascular diseases: comparison with other cerebral perfusion tracers and PET. *J Nucl Med* 1993;35:27–31.
12. Devous MD, Payne JK, Lowe JL, Leroy RF. Comparison of technetium-99m-ECD to xenon-133 SPECT in normal controls and in patients with mild to moderate regional cerebral blood flow abnormalities. *J Nucl Med* 1993;34:754–761.
13. Tamgac F, Moretti J-L, Defer G, et al. Nonmatched images with ^{123}I-IMP and ^{99m}Tc-bicisate single-photon emission tomography in the demonstration of focal hyperemia during the subacute phase of an ischemic stroke. *Eur J Nucl Med* 1994;21:254–257.
14. Lassen NA, Sperling B. ^{99m}Tc-bicisate reliably images CBF in chronic brain diseases but fails to show reflow hyperemia in subacute stroke: report of a multicenter trial of 105 cases comparing ^{133}Xe and ^{99m}Tc-bicisate (ECD, Neurolite) measured by SPECT on the same day. *J Cereb Blood Flow Metab* 1994;14(suppl):S44–S48.
15. Jacquier-Sarlin MR, Polla BS, Slosman DO. Oxido-reductive state: the major determinant for cellular retention of ^{99m}Tc-HMPAO. *J Nucl Med* 1996;37:1413–1416.
16. Slosman DO, Pittet N, Donath A, Polla BS. Fluorodeoxyglucose cell incorporation as an index of cell proliferation: evaluation of accuracy in cell culture. *Eur J Nucl Med* 1993;20:1084–1088.
17. Slosman DO, Pugin J. Lack of correlations between 3-H deoxyglucose, ^{201}Tl and ^{99m}Tc-MIBI cell incorporation under various cell stresses. *J Nucl Med* 1994;35:120–126.
18. Darte C, Beaufay H. Analytical subcellular fractionation of cultivated mouse resident peritoneal macrophages. *J Exp Med* 1983;157:1208–1228.
19. Orlandi C, Crane PD, Platts SH, Walovitch RC. Regional cerebral blood flow and distribution of ^{99m}Tc-ethyl cysteinate dimer in nonhuman primates. *Stroke* 1990;21:1059–1063.
20. Di Rocco RJ, Silva DA, Kuczynski BL, et al. The single-pass cerebral extraction and capillary permeability-surface area product of several putative cerebral blood flow imaging agents. *J Nucl Med* 1993;34:641–648.
21. Pupi A, Castagnoli A, De Cristofaro MTR, et al. Quantitative comparison between ^{99m}Tc-HMPAO and ^{99m}Tc-ECD: measurement of arterial input and brain retention. *Eur J Nucl Med* 1994;21:124–130.
22. Greenberg JH, Araki N, Karp A. Correlation between ^{99m}Tc-bicisate and regional CBF measured with iodo-[C-14] antipyrine in a primate focal ischemia model. *J Cereb Blood Flow Metab* 1994;14:S36–S43.