The Effect of Cyclosporin Concentration on the Efficiency of in Vitro Technetium-99m Radiolabeling of Red Blood Cells

June A. Reisdorff, Lori D. Trevino, David Velasquez IV, and Michael Jackson

School of Allied Health Professions, University of Nebraska Medical Center, Omaha, Nebraska

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Previous work suggested that the immunosuppressive drug cyclosporin (CsA) was associated with significant decreases in red blood cell (RBC) radiolabeling efficiencies. To test this relationship, we studied five normal, adult male subjects who had received a single oral dose of CsA. Blood samples were drawn at 0, 1, 2, and 4 hours postdosage. In vitro labeling of RBCs by a commercial stannous-pyrophosphate preparation method was done on all samples and the labeling efficiencies calculated. Mean labeling efficiency for all subjects was 95.7% ± 1.7%. CsA concentration was determined on these samples by fluorescent polarization immunoassay (with a polyclonal antibody which detects the parent compound and some metabolites), using an Abbott TDx. The concentrations ranged from 0 ng/ml at predose to peak concentrations up to 1,897 ng/ml postdose. We found no significant effect (r = 0.17) of CsA concentration in whole blood up to 1,897 ng/ml upon labeling efficiency of RBCs. This is contrary to the original work of Allen et al. We postulate that the stannous-pyrophosphate used in our labeling procedure may be responsible for overcoming binding difficulties encountered with CsA in previous work.

Medical advancements in the field of transplantation have incorporated the diagnostic capabilities of nuclear medicine procedures to aid in diagnosis, follow-up, and maintenance of the growing range of transplant patients. Some nuclear medicine procedures such as gastrointestinal bleeding and gated heart studies require labeling red blood cells (RBCs) with a radioactive isotope. In transplant patients, immunosuppressive drugs are critical for life-long suppression of graft-versus-host disease and to prevent acute and chronic rejection episodes. For example, CsA has been proven to substantially improve the survival rates of liver transplant patients (*1*). In addition, the use of CsA seems to have resulted in fewer episodes of infections when compared to azathioprine regimens (*2*).

CsA is known to be extremely hydrophobic and 60%–70% binds to erythrocytes in whole blood (*3*). CsA binds to cytosolic proteins within the erythrocyte and saturates the erythrocytic membrane bilayer. This suggests that CsA binding to erythrocytes may limit the binding of other chemicals, which, when introduced into the blood, may normally bind to the RBCs. Such drugs as digoxin and prozasin are known to decrease the labeling efficiency of RBCs (*4*). It is possible that CsA might also decrease the labeling efficiency of RBCs. In addition, CsA has been linked with the interference of ion transportation and inhibition of the sodium-potassium (Na-K) pump in human RBCs (*5*). Temperature, hematocrit, and metabolite concentrations of CsA affect the balance between the plasma and cellular component of whole blood of CsA (*6*).

A study by Allen et al., has indicated a negative relationship between CsA concentration and RBC labeling efficiency. Our follow-up study involved the same issue of whether or not CsA concentration affects RBC labeling efficiency.

MATERIALS AND METHODS

Our study was conducted with five normal, male volunteers. Their ages ranged from 20 to 29 yr. All subjects were given 10 mg/kg of oral CsA according to their total body weight. Four 2-ml samples of whole blood were drawn into separate heparinized tubes from each individual. The first samples were baseline, drawn before CsA administration. Next, samples were drawn at 1, 2, and 4 hours post-CsA administration.

A comparison study was also conducted on the addition of CsA to whole blood taken from a normal control volunteer. The study was set up using five test tubes; each contained heparinized whole blood. Tube 1 was the control tube (containing no CsA), while varying amounts of CsA were added to the remaining tubes (500, 1,000, 1,500, and 2,000 ng/ml of CsA, respectively). The samples were incubated for 3 hr and then labeled in vitro with the following method.

The RBCs from all subjects were labeled with technetium-99m (^{99m}Tc) pertechnetate using a modified in vitro labeling technique (*7*). Modifications included the use of two 1-ml

For reprints contact: June A. Reisdorff, University of Nebraska Medical Center, Department of Nuclear Medicine, 600 South 42nd Street, Omaha, NE 68198-1045.

whole blood aliquots for duplication, 0.1 ml of stannous pyrophosphate (maximum: 36-μg tin ion), 0.1 ml of 4.4% ethylenediaminetetraacetic acid (EDTA), and 1.3 ± 0.3 ml of ^{99m}Tc pertechnetate.

CsA concentrations in each of the samples were measured by both high pressure liquid chromatography (HPLC) and fluorescent polarization immunoassay (FPIA). FPIA measures parent compound and some metabolite components, while the HPLC method measures only parent compound (see Table 1).

RESULTS

RBC labeling efficiency was plotted against in vivo CsA concentration in whole blood. Individual curves for each of the five patients were recorded for baseline, 1, 2, and 4 hr post-CsA dosage. Mean labeling efficiencies were 95.1%, 94.7%, 95.4%, 96.8%, and 96.9%, respectively, corresponding to an overall mean for all patients of 95.7% (s.d. = 1.7), for the range of CsA concentration 0–1,897 ng/ml (see Fig. 1 and Table 2). Linear regression analysis upon all points revealed a correlation of r = 0.17 (no statistical significance) for the relationship of CsA concentration upon RBC labeling efficiency.

An addition of CsA to normal whole blood also showed no decrease in labeling with increased CsA concentration. The mean labeling efficiency was 94.6% (s.d. = 2.5) and a nonsignificant correlation (r = 0.3) was found for CsA concentration affecting the labeling efficiency of RBCs (see Fig. 2 and Table 3).

TABLE 1. In Vivo CsA Concentrations of Parent and Metabolite Compounds

Subject	Time (hr)	CsA with Metabolites (ng/ml)	CsA Parent Drug (ng/ml)
1	0	<100	3.08
	1	431.18	499.52
	2	1892.11	1847.24
	4	1666.52	1218.88
2	0	<100	6.41
	1	510.64	636.94
	2	1821.91	1632.14
	4	1897.89	1654.36
3	0	<100	8.49
	1	658.01	724.66
	2	1660.10	1521.20
	4	1656.82	1370.97
4	0	<100	8.98
	1	1442.68	1402.52
	2	1683.47	1463.28
	4	1850.80	1617.80
5	0	<100	7.60
	1	1201.53	1245.58
	2	1458.81	1174.65
	4	864.95	612.18

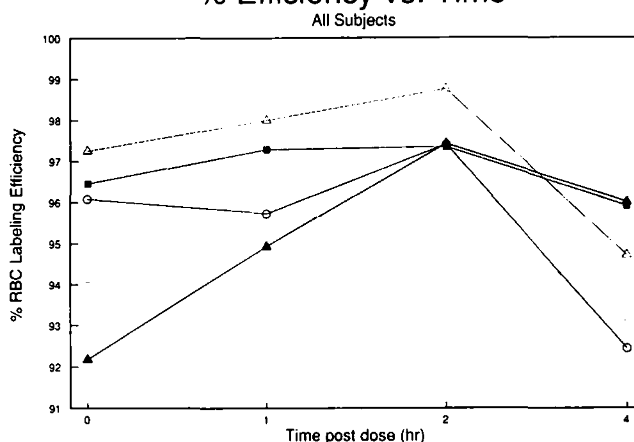

FIG. 1. Comparison of RBC labeling efficiency in vivo from 0–4 hr post-CsA administration for all five subjects.

DISCUSSION

Parent Compound and Metabolite Affinity for RBCs

CsA's known affinity for RBCs in whole blood (*8–10*) was suggested by Allen et al. as a possible cause for decreased RBC labeling efficiency in cardiac transplant patients. The authors found that trough blood levels had a tagging efficiency of 96% compared with a 2-hr postdose efficiency of 44% (*11*). In contrast, our study of single-dose subjects showed no significant changes in labeling efficiency with CsA concentrations up to 2,000 ng/ml, as determined by the HPLC method.

A review of previous research studies reveals that parent CsA is metabolized by a liver enzyme into as many as 25 different metabolites present in whole blood (*12*). Of these metabolites, M17 > M1 > CsA, in the affinity for RBCs, and together they predominate in both spiked normal blood and in transplant patients (*6,10*). In addition, metabolites have been found to exceed parent CsA in total concentration in whole blood, in liver, heart, and kidney transplant patients (*13,14*). Therefore, further research into possible CsA metabolite interference with the ^{99m}Tc labeling process, rather than

TABLE 2. Percent RBC Labeling Efficiency for Various CsA Concentrations

	CsA Concentration (ng/ml)			
Baseline	0–500	500–1,000	1,000–1,500	1,500–2,000
92.2	97.3	95.7	95.4	97.4
94.1		93.1	96.2	96.0
96.1		94.9		97.4
96.5				92.4
97.3				97.4
				95.9
				98.8
				94.7
X̄:95.2	97.3	94.6	95.8	96.5

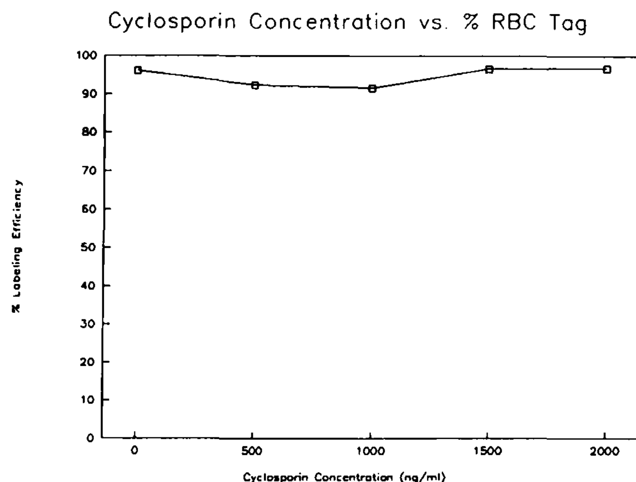

FIG. 2. CsA diluted in normal whole blood shows no significant decrease in RBC labeling efficiency, in vitro, for CsA concentrations up to 2,000 ng/ml.

parent CsA per se, may shed additional light on the mechanisms involved.

Possible Contribution of Labeling Methods to Efficiency Differences

A sharp contrast was found between our study and that performed by Allen et al. (*11*). Although in vitro methods of labeling were utilized in both studies, the in vitro methods were not the same. Allen et al. utilized an in vitro kit similar to that developed by Brookhaven National Laboratory. This in vitro kit is cited predominantly in literature where the labeling efficiency of RBCs exceeded 98%, providing kit precautions were followed (*15*). However, our study did not use a kit designed especially for in vitro RBC labeling; we used commercially available pyrophosphate kits (Mallinckrodt Medical, St. Louis, MO) and ETDA. This procedure is currently in use in our radiopharmacy and labeling efficiencies have been greater than 95% (*7*).

The possibility that differences in labeling methods are responsible for the discrepancy between the results of the two studies is puzzling. Maysheva et al. (*16*) and Srivastava et al. (*17*) reported that the labeling efficiency is dependent upon the quantity and the chemical form of the stannous ion. Since both methods of labeling generally yield at least 95% efficiency, it is intriguing to ask why a labeling method that uses a pyrophosphate kit as the source of stannous ion results in such high labeling efficiencies in the presence of large concentrations of CsA.

At the current time, the labeling mechanism is believed to involve the binding of reduced ^{99m}Tc pertechnetate to the intracellular proteins, specifically the beta chain of the hemoglobin, within the RBCs (*15,18,19*). RBC-associated CsA is bound greater than 90% to a cytosolic protein (*20*). However, when based upon weight, hemoglobin is present at a concentration ~500 times more than that of the CsA-binding protein (*21*). Since CsA binds to a cytosolic protein rather than hemoglobin, our results lead us to question the importance of hemoglobin as the primary binding site in the labeling process, when a pyrophosphate kit is used as a source of stannous ions.

The exact role that the stannous pyrophosphate complex plays in the labeling procedure is not known. However, it is known that pyrophosphate has an affinity for RBCs (*22*) and that it absorbs onto the surface of RBCs (*15*). Surface labeling of the RBCs in vitro, due to an unexplained mechanism of the stannous pyrophosphate complex, would explain the lack of a labeling efficiency response to increased concentrations of CsA.

Previous research has questioned the possibility of surface labeling. When investigating the effect of RBC surface charge on labeling efficiency, Seldin et al. (*23*) initially suggested that the majority of the label is not associated with hemoglobin. As much as 80% of the label could possibly be associated with intracellular components or the membrane itself. However, this hypothesis could not be substantiated; eventually they hypothesized that increased RBC membrane permeability, due to loss of RBC surface charge, was responsible for their results.

Mackey et al. (*24*) studied the effect of the anticoagulants, heparin and acid-citrate-dextrose (ACD), on hemoglobin and membrane-binding of mongrel RBCs. The distribution of membrane activity was reported to be as high as 58% ± 9% when ACD was used instead of heparin, during in vitro studies (*24*). Therefore, different combinations of reactants and methods of labeling may affect binding efficiency, as well as the distribution and site of the labeling itself.

TABLE 3. In Vitro CsA Concentrations with Corresponding Labeling Efficiencies in Whole Blood

CsA (ng/ml)	Total Assay (mCi)	Plasma Assay (mCi)	Volume Correction Factor	Labeling Efficiency (%)
2,000	4.20	0.139	2.711	96.7
1,500	4.34	0.151	2.719	96.5
1,000	3.90	0.328	2.736	91.5
500	3.73	0.289	2.735	92.3
0	4.10	0.161	2.711	96.0

CONCLUSION

We found no significant correlation between the concentration of CsA and the in vitro labeling efficiency of RBCs with ^{99m}Tc pertechnetate. Although our results can not be definitively explained, we feel there is a possible link with the in vitro labeling method that utilizes a stannous pyrophosphate preparation and the "unresponsiveness" of RBC labeling efficiencies to high CsA concentrations. Regardless of whether the CsA concentration was due to subjects taking the drug, or an addition of the drug to normal blood samples, the labeling yield was above 90%. Since our subject samples contained mainly CsA parent compound, the effects of CsA metabolites on labeling efficiency remains unknown. Further studies on

the involvement of the RBC membrane, the binding of CsA and its metabolites to erythrocytes, and the stannous pyrophosphate complex in the labeling process are suggested.

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REFERENCES

1. Starzl TE. New approaches in the use of cyclosporine: with particular reference to the liver. *Transplant Proc* 1988;20(suppl):356–360.
2. Mereish KA. Alteration of cyclosporin: a bioavailability through complexation. (PhD thesis.) Omaha, NE: University of Nebraska Medical Center, Pharmaceutical Sciences Graduate Program; 1984:216–224.
3. Gilman A, Rall TW, Nies AS, Taylor P. *The pharmacological basis of therapeutics.* 8th ed. New York, NY: Pergamon Press; 1990:1267–1270.
4. Lee HB, Wexler JP, Scharf SC, Blaufox MD. Pharmacologic alterations in ^{99m}Tc binding by red blood cells: concise communication. *J Nucl Med* 1983;24:397–401.
5. Ihara H, Hosokawa S, Ogino T, Arima M, Ikoma F. Activation of K+ channel and inhibition of Na+-K+ ATPase of human erythrocytes by cyclosporin: possible role in hyperpotassemia in kidney transplant recipients. *Transplant Proc* 1990;22:1736–1739.
6. Lensmeyer GL, Wiebe PA, Carlson IH. Distribution of cyclosporin A metabolites among plasma and cells in whole blood: effect of temperature, hematocrit, and metabolite concentration. *Clin Chem* 1989;35:56–63.
7. Augustine SC, Hankins JH, Poster EC, et al. A practical method for in vitro labeling of red blood cells with stannous pyrophosphate and EDTA: preparation, quality control and clinical application. (Presented at SNM Missouri Chapter Annual Meeting, September 18, 1988.)
8. Lemaire M, Tillement JP. Role of lipoproteins and erythrocytes in the in vitro binding and distribution of cyclosporine A in the blood. *J Pharm Pharmacol* 1982;34:715–718.
9. Keown PA, Stiller CR, Sinclair NR, et al. The clinical relevance of cyclosporine blood levels as measured by radioimmunoassay. *Transplant Proc* 1983;15(suppl):2438–2441.
10. Maurer G, Lemaire M. Biotransformation and distribution in blood of cyclosporine and its metabolites. *Transplant Proc* 1986;18(suppl):25–34.
11. Allen ML, McPherson AL, Mertes BJ, Preston DF, Robinson RG. Effects of cyclosporine in lowering red blood cell technetium-99m labeling efficiencies. *J Nucl Med Technol* 1990;18:191–193.
12. Lee GC, Rosano T, Ptachcinski R. Pharmacology: profiles, parameters, interpretations, and drug interactions. *Transplant Proc* 1988;20(suppl). 715–721.
13. Lensmeyer GL, Weibe PA, Carlson JH. Disposition of nine metabolites of cyclosporin in human tissues, bile, urine and whole blood. *Transplant Proc* 1988;20:614–622.
14. Rabini RA, Testa I, Corvetta A, et al. Cyclosporin effect on sodium and potassium transport across erythrocytes in rheumatoid arthritis. *Scand J Rheumatol* 1990;19:356–362.
15. Sharp PF, Gemmell HG, Smith FW, eds. Radiopharmaceuticals in nuclear medicine practice. *Practical nuclear medicine.* New York: Oxford University Press; 1989:212–213.
16. Srivastava SC, Straub RF, Richards P. Mechanistic aspects of the technetium-99m-tin-RBC labeling reactions. *J Label Comp Radiopharm* 1984; 21:1055–1056.
17. Srivastava SC, Straub RF. Blood cell labeling with 99m-Tc: progress and preparation. *Semin Nucl Med* 1990;20:41–51.
18. Dewanjee MK. The chemistry of 99m-Tc-labeled radiopharmaceuticals. *Semin Nucl Med* 1990;20:5–27.
19. Rehani MM, Sharma SK. Site of Tc-99m binding to the red blood cell: concise communication. *J Nucl Med* 1980;21:676–678.
20. Foxwell BF, Frazier G, Winters M, et al. Identification of cyclophilin as the erythrocyte cyclosporin-binding protein. *Biochem-Biophys-Acta* 1988; 938:447–455.
21. Agarwal P, Threatte GA, McPherson RA. Temperature-dependent binding of cyclosporin to an erythrocyte protein. *Clin Chem* 1987;33:481–485.
22. Mallinckrodt Medical, Inc. Technical product data: TechneScan PYP kit for the preparation of technetium Tc-99m pyrophosphate. 1989.
23. Seldin DW, Simchon S, Jan KM, Chein S, Alderson PO. Dependence of technetium-99m red blood cell labeling efficiency on red cell surface charge. *J Nucl Med* 1988;29:1710–1713.
24. Mackey ST, Dewanjee MK, Chowdhury S, Wahner HW. Intracellular distribution of radionuclides and turnover in red cells labeled with Cr-51, In-111, and Tc-99m in dogs. *J Label Comp Radiopharm* 1984;21:1058.