Annals of Nuclear Medicine Vol. 6, No. 1, 59–62, 1992

Application of ^{67}Ga for the estimation of reticulocyte production

Yasuhito OHKUBO,* Shigeto TAKASU,* Midori ITAKURA,* Shinsuke KATOH,*
Akiko SASAYAMA,* Hiroyuki KOHNO* and Akiko KUBODERA**

*Department of Radiopharmacy, Tohoku College of Pharmacy
**Department of Radiopharmacy, School of Pharmaceutical Science, Science University of Tokyo

In order to estimate the production of reticulocytes, which have a larger number of transferrin receptors than erythrocytes, we used ^{67}Ga which is exclusively bound to transferrin in the blood. The pattern of uptake of ^{67}Ga by reticulocytes was quite similar to the time course of transglutaminase activity which might be involved in receptor-mediated endocytosis. The preinjection of Fe^{3+} decreased the uptake of ^{67}Ga by reticulocytes. These results suggested that ^{67}Ga in a transferrin-bound form was taken up by reticulocytes via receptor-mediated endocytosis. It was showed that the application of ^{67}Ga is very easy and useful for the estimation of reticulocyte production.

Key words: ^{67}Ga uptake, transferrin, reticulocyte production

INTRODUCTION

IT HAS BEEN PRODUCED that ^{67}Ga, a diagnostic agent of tumor[1] and inflammation,[2,3] was exclusively bound to transferrin in the blood.[4,5] It is well known that transferrin is the major serum iron-transport protein.[6] Transferrin provides the developing erythroid cells, which require a large amount of iron for heme synthesis, with iron.[7–9] Reticulocytes have a larger number of transferrin receptors than erythrocytes.[10] Reticulocytes have been used for the study of receptor-mediated endocytosis,[11] cell proliferation, and cell differentiation.[10] In order to extend these studies further, it is necessary to estimate reticulocyte production, that is to evaluate the number of transferrin receptors produced. Ordinarily radio-labeled transferrin is employed for these studies. For radiolabeling of transferrin, the method of iodination[12] with ^{125}I or reductive methylation[13] with ^{3}H has been employed. In the present study, we used ^{67}Ga by which transferrin was labeled *in vivo* instead of ^{125}I- or ^{3}H-labeled transferrin. Consequently we attempted to more readily estimate reticulocyte production with ^{67}Ga.

Received June 10, 1991, revision accepted October 25, 1991.

For reprints contact: Yasuhito Ohkubo, Department of Radiopharmacy Tohoku College of Pharmacy, 4–4–1 Komatsushima, Aoba-ku Sendai, 981, JAPAN.

MATERIALS AND METHODS

Animals: Male Wistar rats weighing 150–200 g were purchased from Shizuoka Laboratory Animal Center (Hamamatsu, Japan), and were housed in wire mesh cages at a room temperature of 23 ± 1°C and a relative humidity of 55 ± 5%.

Chemicals: Phenylhydrazine chloride and ferric chloride were purchased from Nakarai Tesque (Japan). [1, 4-^{14}C] Putrescine dihydrochloride (^{14}C-PUT, specific activity=3.87 GBq/mmol) was obtained from New England Nuclear (USA). N, N,-Dimethylcasein was purchased from Sigma (USA). All other reagents were of analytical grade.

Production of reticulocytes: Rats were intraperitonealy administered with phenylhydrazine hydrochloride (40 mg/kg body weight/day) for 3 days. One to five days after the last (the third) administration, blood was collected from the abdominal vein, with heparin as an anticoagulant.

Administration of ^{67}Ga: Gallium-67 citrate (kindly supplied by Daiichi Radioisotope Laboratory Ltd, Tokyo, Japan) was diluted with saline to 185 kBq/m*l*. Each rat was intravenously injected with ^{67}Ga in a dose of 37 kBq (200 μl).

Preparation of ^{125}I-transferrin: ^{125}I-transferrin (^{125}I-Tf) was prepared by the method described previously.[14] Each rat was intravenously injected with ^{125}I-Tf in a dose of 5 kBq (200 μl).

Administration of cold-$FeCl_3$: Each rat was intraveneously injected with 100 μl of cold-$FeCl_3$ (2.50 μmole/ml saline) 5 min before the administration of ^{67}Ga.

Determination of radioactivity: Four hours after the injection of ^{67}Ga or ^{125}I-Tf, the reticulocytes were collected from the abdominal vein of rats given phenylhydrazine, were washed twice with ice-cold saline and the buffy coat was removed during the washing. The radioactivity of the cells was determined with a well-type NaI-scintillation counter (Aloka, ARC-300). The ratio of uptake of ^{67}Ga in reticulocytes was expressed in the following formula:
Uptake ratio=A/B

A=sample activity (cpm)/sample weight (g)

B=total activity administered (cpm)/rat body weight (g).

Fractionation of reticulocyte cytosol: After hemolyzing cells with hypo-osmotic buffer (3 mM Tris, 1 mM EDTA, 0.5 mM dithiothreitol), the lysate was centrifuged at 20,000$\times$g for 40 min. This process was repeated twice. Supernatants were collected and then used as the cell cytosol fraction.

Determination of transglutaminase activity: The transglutaminase activity of cell cytosol fraction from red blood cells was assayed by the incorporation of ^{14}C-PUT into N, N′-dimethylcasein by the filter paper technique described by Lorand et al.[15] with minor modifications. The final assay reaction mixture contained 50 mM Tris-HCl (pH 7.4), 10 mM dithiothreitol, 10 mM ($CaCl_2$, 0.5 mg of N, N′-dimethylcasein, 1 mM PUT (including 1.85 kBq of ^{14}C-PUT), and 10 μl of the sample solution. The reaction was initiated by the addition of a sample solution of cell cytosol fraction, and was carried out at 37°C in a total volume of 100 μl. The enzyme reaction was terminated by spotting a 20 μl volume of the mixtures onto a Whatman 3MM filter paper which was immersed in 10% trichloroacetic acid (TCA) solution and fixed on a multi vacuum-filter unit, and the filter paper was washed twice with 1 ml volume of 10% TCA solution. The filters were transferred to scintillation counting vials and 5 ml of scintillation counting fluid (ASC II, Amersham) was added. Radioactivity was determined with a liquid scintillation counter (Beckman, LS-7800). Determination of protein content of red blood cell cytosol fraction was carried out by the method of Bradford et al.[16] with bovine serum albumin used as a standard. The enzyme activity was expressed as nmol PUT incorporation into dimethylcasein min^{-1} mg $protein^{-1}$.

RESULTS

Figure 1 shows the uptake of ^{67}Ga by reticulocytes at various days after the last administration of phenylhydrazine solution. The uptake of ^{67}Ga by the cells reached the maximum at 3 days after the last administration of phenylhydrazine solution and the uptake ratio was about 6 times that for normal rat erythrocytes. The preinjection of $FeCl_3$ remarkably decreased the uptake of ^{67}Ga by the cells. This result shows that ^{67}Ga had bound to transferrin.

Figure 2 shows the uptake of ^{125}I-transferrin by reticulocytes at various days after the last administration of phenylhydrazine solution. The uptake of ^{125}I-transferrin by the cells of rats given phenylhydrazine gradually increased and reached the maximum at 3 days after the last administration of phenylhydrazine solution.

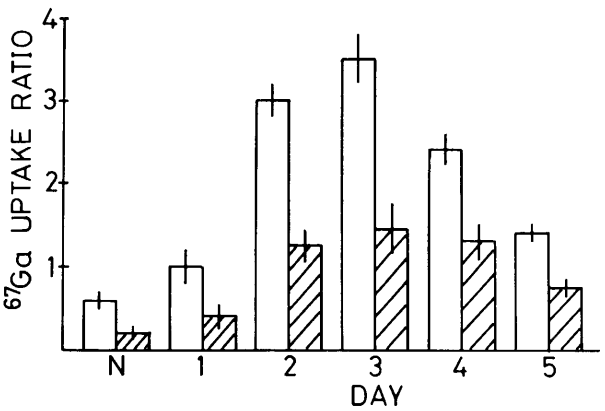

Fig. 1 The uptake of ^{67}Ga by reticulocytes at various days after the last administration of phenylhydrazine. N: normal rats. (□): saline was preinjected before ^{67}Ga injection. (▨): $FeCl_3$ was preinjected before ^{67}Ga injection. Each point represents the mean±SEM for five rats.

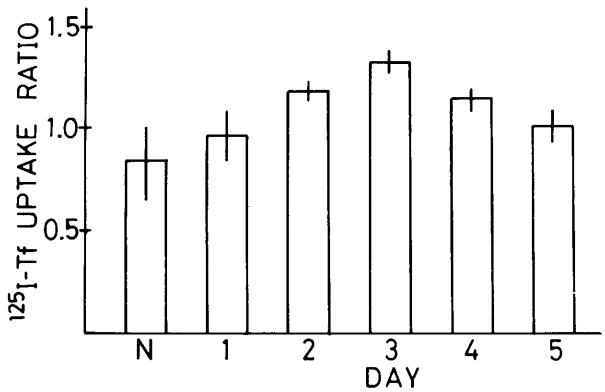

Fig. 2 The uptake of ^{125}I-transferrin uptake by reticulocytes at various days after the last administration of phenylhydrazine. N: normal rats. Each point represents the mean±SEM for five rats.

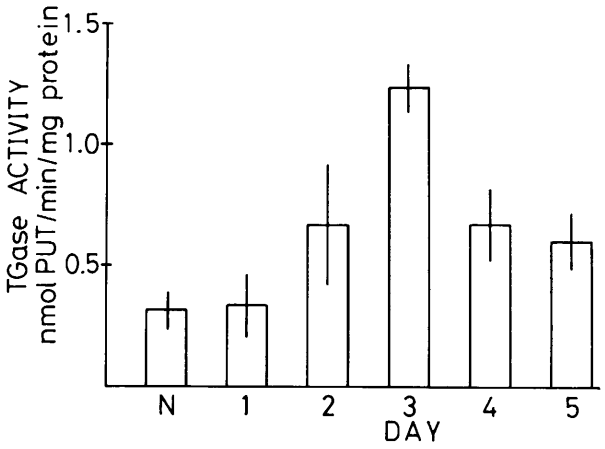

Fig. 3 Transglutaminase activity of the cytosol fraction from reticulocytes at various days after the last administration of phenylhydrazine. Each point represents the mean±SEM for five rats.

Figure 3 shows that transglutaminase (TGase) activity of the cytosol fraction of reticulocytes at various days after the last administration of phenylhydrazine solution. The activity reached the maximum at 3 days after the last administration of phenylhydrazine.

DISCUSSION

Tavassoli et al. reported that phenylhydrazine affected cell proliferation in the regenerating marrow stroma implanted.[17] It has also been reported that reticulocytes induced by the injection of phenylhydrazine had transferrin receptors and as the reticulocytes matured, the density of the receptors on the surface decreased.[10] There must be a close relation between iron uptake and both cell proliferation and differentiation. Therefore, reticulocytes can be used as a good tool for the investigation of cell proliferation and differentiation. It was reported that transferrin might be taken up by rat reticulocytes via receptor-mediated endocytosis.[11] It has been reported that transglutaminase, which is a calcium-dependent enzyme and catalyzes the covalent cross-linking of proteins, might be involved in receptor-mediated endocytosis.[18,19] These reports show that transglutaminase must be related to transferrin internalization into reticulocytes. In the present study, transglutaminase activity reached the maximum at 3 days after the last administration of phenylhydrazine solution. This suggests that the receptor-mediated endocytosis on reticulocytes is maximum at 3 days after the last administration of phenylhydrazine solution. The uptakes of ^{125}I-transferrin and ^{67}Ga by reticulocytes also reached the maximum at 3 days after the last administration of phenylhydrazine solution, and the pattern of uptake of ^{67}Ga by reticulocytes was very similar to the time course of transglutaminase activity of the cells. Moreover, ^{67}Ga in a transferrin-bound form might be taken up by reticulocytes since Fe^{3+} decreased the uptake of ^{67}Ga by reticulocytes. These results suggested that ^{67}Ga in a transferrin-bound form was taken up by the cells via receptor-mediated encocytosis. Consequently, it was suggested that reticulocyte production reached a maximum at 3 days after the last administration of the phenylhydrazine. As ^{67}Ga is injected into the blood, ^{67}Ga is immediately bound to transferrin. Therefore, it is not necessary to label transferrin with a radioisotope, e.g., ^{125}I or ^{3}H, in advance in vitro since transferrin is readily labeled with ^{67}Ga *in vivo*. Moreover, the pattern of ^{67}Ga uptake by reticulocytes is sharper than that of ^{125}I-transferrin uptake. This may be because the ^{67}Ga taken up into reticulocytes in a transferrin-bound form is removed from transferrin at an intracellular site and is accumulated in reticulocytes. The concentration of ^{67}Ga in the blood at 4 h after subcutanous injection is nearly identical to that at 4 h after intravenous injection.[20] Therefore ^{67}Ga has the advantage of being easily injected, whereas radiolabeled transferrin must be intravenously injected. Moreover, the determination of ^{67}Ga activity is easier than that of ^{3}H activity, for which preparation of the sample, e.g., oxidation or solubilization, is needed. From the results of this study we think that the application of ^{67}Ga is very easy and useful for the estimation of reticulocyte production. Furthermore, ^{67}Ga will be available for other studies of physiological events in which transferrin is involved.

REFERENCES

1. Johnston GS: Clinical applications of gallium in oncology. *Int J Nucl Med Biol* 8: 249–255, 1981
2. Hoffer PB: Gallium and infection. *J Nucl Med* 21: 484–488, 1980
3. Hoffer PB: Use of gallium-67 for detection of inflammatory diseases: A brief review of mechanisms and clinical applications. *Int J Nucl Med Biol* 8: 243–247, 1981
4. Tsan MF, Scheffel U, Tzen KY, et al: Factors affecting the binding of gallium-67 in serum. *Int J Nucl Med Biol* 7: 270–273, 1980
5. Ohkubo Y, Shibuya A, Kohno H, et al: Involvement of transferrin in the uptake of ^{67}Ga in inflammatory and normal tissues. *Nucl Med Biol* 16: 337–341, 1989
6. Aisen P, Litstowsky I: Iron transport and storage proteins. *Annu Rev Biochem* 49: 357–393, 1980
7. Huebers H, Huebers E, Csiba E, et al: Iron uptake from rat plasma transferrin by rat reticulocytes. *J Clin Invest* 62: 944–951, 1978
8. Glass J, Nunez MT, Fischer S, et al: Transferrin receptors, iron transport and ferritin metabolism in

friend erythroleukemia cells. *Biochim Biophys Acta* 542: 154–162, 1978
9. Takahashi K, Tavassoli M: Biphasic uptake of iron-transferrin complex by L1210 murine leukemia cells and rat reticulocytes. *Biochim Biophys Acta* 685: 6–12, 1982
10. Van Bockxmeer FM, Morgan EH: Transferrin receptors during rabbit reticulocyte maturation. *Biochim Biophys Acta* 584: 76–83, 1979
11. Kohno H, Tokunaga R: Transferrin and iron uptake by rat reticulocytes. *J Biochem* 97: 1181–1188, 1985
12. Markwell MAK: A new solid-state reagent to iodinate protein. I. Conditions for the efficient labeling of antiserum. *Anal Biochem* 125: 427–432, 1982
13. Means GE, Feeney RE: Reductive alkylation of amino groups in protein. *Biochemistry* 7: 2192–2196, 1968
14. Ohkubo Y, Sawamura H, Katoh S, et al: Studies on ^{67}Ga uptake by mouse granuloma tissues. *Nucl Med Biol* 18: 205–208, 1991
15. Lorand L, Campbell-Wilkes LK, Cooperstein L: A filter paper assay for transglutaminase enzymes using radioactive amine substrates. *Anal Biochem* 50: 623–631, 1972
16. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248–254, 1976
17. Tavassoli M, Maniatis A, Grosby WH: The effects of phenylhydrazine-induced haemolysis on the behaviour of regenerating marrow stroma. *Br J Haematol* 23: 707–711, 1972
18. Maxfield FR, Willingham MC, Davies PJA, et al: Amines inhibit the clustering of α-macroglobulin and EGF on the fibroblast cell surface. *Nature* 277: 661–663, 1979
19. Davies PJA, Davies DR, Levitzki A, et al: Transglutaminase is essential in receptor-mediated endocytosis of α-macroglobulin and polypeptide hormones. *Nature* 283: 162–167, 1980
20. Kohno H, Suzuki T, Ohkubo Y, et al: Comparative pharmacokinetics of ^{67}Ga among the different routes of injection. *Radioisotopes* 33: 269–272, 1984