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

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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$^{2+}$ 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 and inflammation, was exclusively bound to transferrin in the blood. It is well known that transferrin is the major serum iron-transport protein. Transferrin provides the developing erythroid cells, which require a large amount of iron for heme synthesis, with iron. Reticulocytes have a larger number of transferrin receptors than erythrocytes. Reticulocytes have been used for the study of receptor-mediated endocytosis, cell proliferation, and cell differentiation. 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 radio-labeling of transferrin, the method of iodination with $^{125}$I or reductive methylation 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.

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, 4,14C] Putrescine dihydrochloride (14C-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 intraperitoneally 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/ml. 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. Each rat was intravenously injected with $^{125}$I-Tf in a dose of 5 kBq (200 µl).

Administration of cold-FeCl₃: Each rat was intravenously injected with 100 µl of cold-FeCl₃ (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:

\[ \text{Uptake ratio} = \frac{A}{B} \]

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 x 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. with minor modifications. The final assay reaction mixture contained 50 mM Tris-HCl (pH 7.4), 10 mM dithiothreitol, 10 mM (CaCl₂, 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. 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 FeCl₃ 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 given phenylhydrazine gradually increased and reached the maximum at 3 days after the last administration of phenylhydrazine solution.
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.\(^\text{17}\) 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.\(^\text{10}\) 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.\(^\text{11}\) 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.\(^\text{18,19}\) 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 phenyl-

**REFERENCES**


