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FRACTIONATION OF ARGINASE-LOADED ERYTHROCYTE GHOSTS WITH PERCOLL DENSITY GRADIENTS

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Summary. Arginase-loaded human erythrocyte ghosts prepared by a slow dialysis technique were fractionated into enzyme-enriched populations by density-gradient centrifugation in Percoll. The enzyme-enriched populations contain up to 12-times more arginase than normal intact erythrocytes and up to 7-times more arginase than the average of the arginase-loaded erythrocyte ghost pool. The ability to isolate, rapidly and preparatively, ghosts which are highly enriched with enzyme can be useful for enzyme replacement studies.

INTRODUCTION

The suitability of erythrocytes or their ghosts as carriers of an enzyme in enzyme replacement therapy was suggested by Ihler *et al.* (1). Since then it has been demonstrated that resealed erythrocyte ghosts have transport characteristics (2), permeability coefficients (3), and volumes and shapes (4) similar to those of normal erythrocytes.

Loaded erythrocyte ghosts offer several advantages over direct injection of enzyme *in vivo*. First, encapsulation of the enzyme can protect it from reaction with other proteins in the circulation and possible inactivation. Second, with isologous or autologous blood, immunologic reactions to the carrier can be avoided. Hudson *et al.* (5) have demonstrated the immunologic advantages of erythrocyte-ghost carriers to liposome carriers and direct injection of enzyme *in vivo*. Third, erythrocytes are filtered out by the

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spleen and liver; this fact may be of special advantage in treating diseases manifested in those organs. In our recent study (6), we suggested a way to deliver enzyme to the cytoplasm of mammalian cells. Since human erythrocytes do not have a cell nucleus or typical cytoplasm, contamination of tissue cells by interfering components is less likely to occur.

The slow dialysis method of loading erythrocytes is easy and conserves precious isolated enzyme. In addition, significant levels of active enzyme can be entrapped inside the ghosts (6,7,8), an important consideration if this form of therapy is to be successful. One factor affecting loading is molecular size. Although there are exceptions, a progressive exclusion occurs with increasing molecular size of the macromolecule (1,7). The amount of protein taken up by the ghosts appears to be proportional to the protein concentration in the surrounding medium during lysis (7,9,10). A factor that has a major effect on the average amount of macromolecule loaded is the percentage of the total ghost pool that reseals with enzyme (4). Beutler's group has injected ghosts loaded with β-glucosidase into patients with Gaucher's disease (11). Although no untoward effects to the patient were noticed with this method of therapy, they questioned whether a sufficient quantity of active enzyme was being administered, since they had been unable to increase the amount of enzyme loaded into the ghosts. During loading of the erythrocytes with an enzyme the amount loaded into the cells is variable and results in a heterogeneous population of loaded ghosts in which a portion of the ghost population consists of lysed cells and another portion of cells unloaded with enzyme (4,9).

Percoll^{R}, a commercially available polyvinyl-pyrrolidone-coated colloidal silica, has been used for whole cell separation and subcellular fractionation (12). Percoll is nontoxic to cells, can be made isotonic, and allows rapid separation of cells at low centrifugal forces. In addition, sterility can be easily maintained as is necessary for enzyme replacement trials.

We demonstrate that enzyme-loaded ghosts can be preparatively and rapidly separated by Percoll density gradient centrifugation and that a population of ghosts highly enriched with an enzyme can be obtained which could be used for enzyme replacement, either by direct injection *in vivo* or by microinjection *in vitro*.

MATERIALS AND METHODS

Intact human erythrocytes were isolated from freshly drawn, heparinized blood. Whole blood was centrifuged at 1,000 x g for 10 min at 4° C. The supernatant fraction and buffy coat were removed by aspiration. The packed cells were washed three times in 10 volumes of phosphate-buffered saline (PBS, 5 mM sodium phosphate buffer, pH 7.4, 0.15 M NaCl) and then suspended 1:10 (v/v) in PBS for density gradient centrifugation. Cell counts of erythrocytes and ghosts in suspension were taken with a Spencer hemocytometer (no. 1492 Brightline Counting Chamber, American Optical Co., Buffalo, NY) or an electronic cell counter (Royco model 920A, San Francisco, CA) set at a discriminator value of three.

Hemoglobin was determined as methemoglobin (13) against a hemoglobin standard (Hemotrol; Clinton Laboratories, Santa Monica, CA). Erythrocytes were loaded with bovine liver arginase (Sigma) as described previously (6). The resealed ghosts were pelleted and washed three times in PBS, and after the last pelleting, were resuspended in 10 volumes of the same buffer. Arginase was assayed by the cleavage of [guanido-^{14}C]arginine (sp. act. 43.5 Ci/mol; RPI Corp., Elk Grove Village, IL) to urea and ornithine and the conversion of the [^{14}C]urea to $^{14}CO_2$ by urease as described (6).

Percoll (Pharmacia, Fine Chemicals) was rendered isotonic by addition of one-tenth volume of 8.5% NaCl. This solution was then diluted with PBS to the appropriate concentrations. Three types of gradients (step, continuous, and preformed) were made with 7 ml of Percoll in 15 ml Corex tubes siliconized with Prosil-28^{R} (PCR Research Chemicals, Gainsville, FL). The preformed gradients were made with 50, 60 or 70% concentrations of Percoll and were centrifuged in a fixed angle rotor (Sorvall SS-34 head, RC-2B centrifuge) at 12,000 x g for 30 min. The step gradients were made with 1 ml each of 75, 70, 65, 60, 50, 30, and 15% concentrations of Percoll. Continuous gradients were made with 3.5 ml each of 15 and 75% Percoll with the aid of a gradient maker and peristaltic pump.

To each type of gradient, 1 or 2 ml of the 1:10 (v/v) suspension of erythrocytes or ghosts was layered on top of the gradient and brought to isopycnic equilibrium by centrifuging at 10° C in a IEC (Model PR-2) centrifuge at 1000 rpm for 10 min. One ml fractions were collected. Since erythrocytes or ghosts cannot be maintained in Percoll for long due to aggregation effects, they were washed free of Percoll with two 5 ml washes in PBS. The fractions were analyzed for arginase, hemoglobin content and cell number.

RESULTS

All three types of gradients (e.g. step, continuous, and preformed) satisfactorily separated arginase-enriched populations of erythrocyte ghosts from others in the heterogeneously-loaded pool. Preformed and continuous

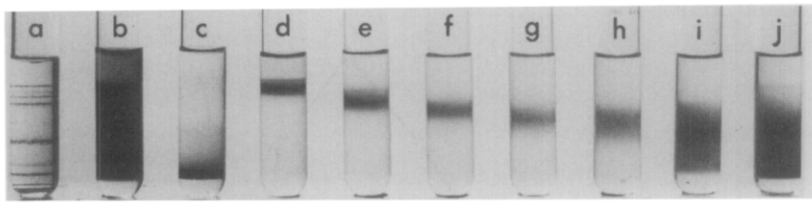

Figure 1. Sedimentation of density marker beads (a), arginase-loaded erythrocyte ghosts (b) and intact erythrocytes (c) on a 7 ml 60% preformed Percoll gradient. One ml fractions, collected from top to bottom, were removed from tube b and washed with PBS. The fractions were resuspended in 1 ml of PBS and reapplied to individual 60% preformed gradients, then recentrifuged under the previous conditions. The banding characteristics of each fraction are shown (d-j).

gradients achieved similar separations: a major band at the top, followed by a diffuse continuum of cells, and then a major band at the bottom. The step gradient had several visible bands throughout. In all three gradients the intact, unloaded erythrocytes banded near the bottom of the gradients (density ca. 1.11).

Of the three types of gradients, the 60% preformed gradients had a density profile most suitable for separation of the arginase-loaded erythrocyte ghosts. Fig. 1 demonstrates the separation of nine density marker beads (a), the spreading of the erythrocyte ghosts throughout the gradient (b), and the sedimentation of normal erythrocytes to near the bottom of the gradient (c). One ml fractions were collected from tube (b), washed and resuspended in 1 ml of PBS, and reapplied to identical but separate preformed gradients and recentrifuged under the previous conditions. The cells maintained their previous banding characteristic except for the two bottom fractions (Fig. 1, d-j). Data obtained for each one ml fraction taken from another experiment are given in Fig. 2, which shows the arginase and hemoglobin contents per 10^6 ghosts, the cell number per fraction, and the density pattern for the gradient. Fractions 3-6, sedimenting between densities of 1.062 and 1.08 had the highest arginase content per 10^6 ghosts. The cell numbers in those fractions were, however, relatively low. Fraction 1 must contain many completely lysed cells since this fraction had the lowest

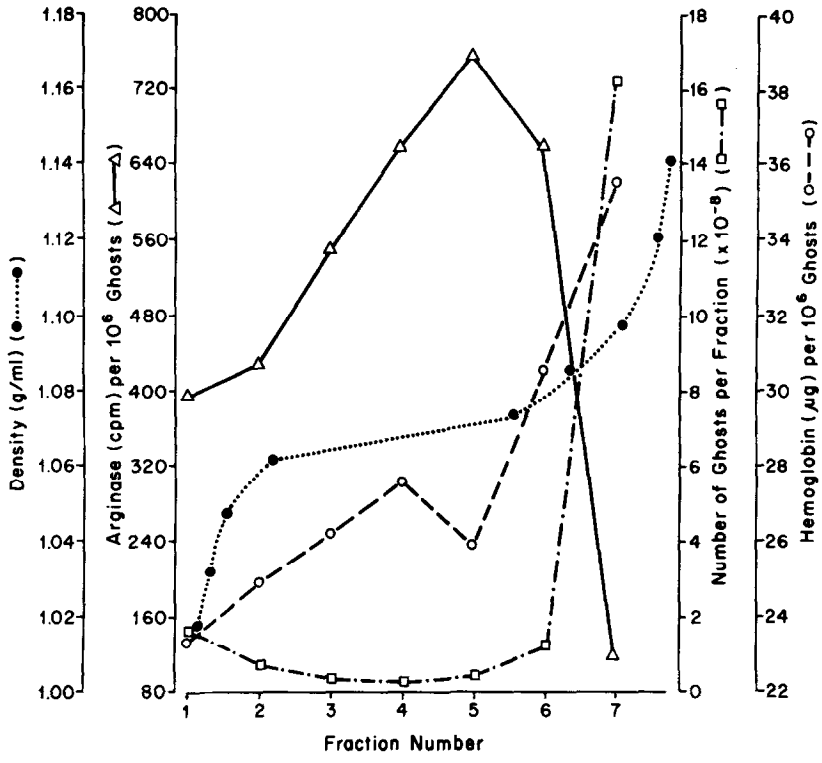

Figure 2. Data obtained for one ml fractions (1-7) from arginase-loaded erythrocyte ghosts centrifuged in a 60% preformed Percoll gradient. The arginase and hemoglobin contents per 10^6 ghosts and the total ghost number for each fraction are given along with the density pattern of the gradient. Normal erythrocytes contained an arginase activity equal to 20 cpm/10^6 ghosts and a hemoglobin content of 39 μg/10^6 ghosts. The arginase-loaded erythrocyte ghost pool contained an arginase activity equal to 120 cpm/10^6 ghosts (e.g. a six-fold increase) and a hemoglobin content of 32 μg/10^6 ghosts.

hemoglobin content per cell. Fraction 7 must contain many unloaded cells since they banded at the density of the normal erythrocytes and had the highest hemoglobin and lowest arginase contents. In other similar experiments we found maximum concentrations of arginase in fractions 3-5. The spreading of the two bottom fractions on recentrifuging in fresh gradients (Fig. 1, tubes i,j) was consistently observed. It is possible that some of the ghosts aggregate in the Percoll and these clumps, together with normal erythrocytes, would sediment to near the bottom of the gradient. However, the washing of these bottom fractions with PBS may disaggregate the clumps and on

sedimentation in fresh gradients the ghosts band isopycnically over a wider range of the gradient.

DISCUSSION

The experiments demonstrated not only the heterogeneity of the resealed erythrocyte ghosts in respect of their densities, but also in respect of their loading with an enzyme. Thus, after centrifugation in a 7 ml preformed gradient, collection of the fractions in the density range of 1.062 to 1.08 can provide quickly a cell population with the highest arginase content. All three types of gradients tried are satisfactory for the fractionation of resealed erythrocyte ghosts, but the preformed gradients are the most convenient as they are easily prepared under sterile conditions. Although Percoll is not toxic to cells (11) and had no effect on arginase activity, it should be washed away from the ghosts quickly to prevent their aggregation. Our observations provide additional evidence for the enzyme being entrapped within the erythrocyte ghosts and not merely adsorbed to the surface of the cells. Percoll gradients have been shown to be suitable for separation of erythrocytes of different ages (14), but this is the first demonstration of the fractionation of resealed erythrocyte ghosts on similar gradients.

REFERENCES

1. Ihler, G., Glew, R.H. and Schnure, F.W. (1973) Proc. Natl. Acad. Sci. USA 70, 2663-2666.
2. Hubbard, A.R., Sprandel, U. and Chalmers, R.A. (1980) Biochem. J. 190, 653-658.
3. Levin, S.W., Levin, R.L. and Solomon, A.K. (1980) J. Biochem. Biophys. Mtds. 3, 255-272.
4. Schwoch, G. and Passow, H. (1973) Mol. Cell. Biochem. 2, 197-218.
5. Hudson, L.D.S., Fiddler, M.B. and Desnick, R.J. (1980) In Birth Defects: Original Article Series, Vol. XVI, Desnick, R.J. (ed.), pp. 163-178, Alan Liss, New York.
6. Kruse, C.A., Spector, E.B., Cederbaum, S.D., Wisnieski, B.J. and Popják, G. (1981) Biochim. Biophys. Acta 645, 339-345.
7. Rechsteiner, M. (1978) Natl. Cancer Inst. Monogr. 48, 57-64.
8. Adriaenssens, K., Karcher, D., Lowenthal, A. and Terheggen, H.G. (1976) Clin. Chem. 22, 323-326.
9. Rechsteiner, M. (1975) Exp. Cell Res. 93, 487-492.
10. Wasserman, M., Zakai, N. and Loyter, A. (1976) Cell 7, 551-556.

11. Beutler, E., Dale, G.L. and Kuhl, W. (1980) Birth Defects: Original Article Series, Vol. XVI, Desnick, R.J. (ed.), pp. 369-381, Alan Liss, New York.
12. Pertoft, H. and Laurent, T.C. (1977) In Methods of Cell Separation, Vol. 1, Catsimpoolas, N. (ed.), pp. 25-65, Plenum Press, New York.
13. Wintrobe, M.M. (1976) In Clinical Hematology, 7th Edition, pp. 114-115, Lea and Febiger Pub., Philadelphia, PA.
14. Rennie, C.M., Thompson, S., Parker, A.C. and Maddy, A. (1979) Clin. Chim. Acta 98, 119-125.