RETENTION OF PURIFIED PROTEINS IN RESEALED HUMAN ERYTHROCYTE GHOSTS AND TRANSFER BY FUSION INTO CULTURED MURINE RECIPIENT CELLS

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1. Introduction

In order to study the biological action of exogenous proteins in cultured mammalian cells a technique is necessary by which these macromolecules may be transferred into recipient cells without being exposed to degradative enzymes in lysosomes. As one possible solution to this experimental problem, Furusawa et al. [1] demonstrated that fluorescein isothiocyanate could be trapped by hemolyzed and resealed erythrocyte ghosts. Subsequently these filled ghosts were fused with human amniotic cells under conditions where the contents of the ghosts were introduced into the cytoplasm of the recipient cells. It had been shown earlier, that macromolecules such as rat β-galactosidase (EC 3.2.1.23) and β-glucosidase (EC 3.2.1.21) [2], ferritin [3,4], dextran [6] and colloidal gold [3] could be trapped by hemolyzed resealed erythrocytes. While our studies were in progress reports from two other laboratories described the fusion of resealed erythrocyte ghosts with mammalian recipient cells. Loyter et al. demonstrated qualitatively the introduction of ferritin and latex beads into mouse cells [5] while Schlegel and Rechsteiner reported quantitative results on the microinjection of bovine serum albumin and murine thymidine kinase (EC 2.7.1.21) into murine recipient cells [7].

This communication describes quantitative data on the transfer of human ^{125}I-serum albumin into murine LM (TK$^-$) cells which confirm in a different experimental system the results of Schlegel and Rechsteiner [7,8]. In addition we demonstrate that human serum albumin (mol. wt. 64 000) can be trapped in resealed erythrocytes at about the same concentration as present in the hemolysis medium while a larger protein, *Escherichia coli* β-galactosidase (EC 3.2.1.23) (mol. wt. 520 000), is more than 100-fold less efficiently retained in red cell ghosts.

2. Materials and methods

The murine recipient cells, (LM (TK$^-$)) [9], were grown on glass cover slips or on plastic tissue culture flasks (Falcon) in Dulbecco's modified Eagle's Medium (Gibco) containing 10% fetal bovine serum (Flow), penicillin (50 units/ml) and streptomycin (50 μg/ml). β-Propiolactone-inactivated Sendai virus was obtained from Connaught Laboratories (Toronto, Canada). Fluorescein isothyocyanate and Dowex (mesh 200–400) were purchased from Serva (Heidelberg), β-mercaptoethanol was bought from Fluka AG (Neu Ulm). Rabbit antihuman red blood cell serum was obtained from Cappel Laboratories (Downingtown, USA), human serum albumin (HSA) and rabbit anti-HSA from Behringwerke AG (Marburg). ^{125}I (carrier free, 300 mCi/ml) was bought from Amersham-Buchler (Braunschweig) and *E. coli* β-galactosidase (30 U/mg) from Boehringer (Mannheim). All other chemicals were purchased from Merck (Darmstadt). Red ghosts were prepared as described by Schwoch and Passow [10]. Briefly human erythrocytes, type O, not older than 3 days, were centrifuged at room temperature for 10 min at 1600 *g* and washed three times with 0.9% NaCl solution (saline). A suspension (50% v/v) of packed cells in ice-cold saline was added to the 10-fold excess of hypotonic hemolysis medium, 1.2 mM acetic acid pH 3.2, 4.0 mM $MgSO_4$. Due to the immediate hemolysis the pH increased to 6.0. After 5 min

incubation at 0°C the medium was adjusted to isotonicity with 20% NaCl solution and the pH was adjusted to 7.2 by addition of 1 N Tris-OH. Then the cells were kept for 90 min at 37°C. Subsequently the resealed ghosts were washed 3–5 times with saline and resuspended in BSS^- [1]. HSA or β-galactosidase (dialyzed against Z-buffer [12]) was added to the hemolysis medium after hemolysis. The ^{125}I-labeling procedure of HSA was carried out according to Greenwood et al. [11]. Radioactivity was measured in a Packard Gamma Scintillation Spectrophotometer, model 3002 (counting efficiency: 50%). For analysis of the trapped β-galactosidase the resealed ghosts were counted in a hemocytometer, centrifuged (10 min, 1600 *g*) lysed, in distilled H_2O (10 volumes of packed cells), and incubated for 30 min at 37°C. Then the suspension was centrifuged for 30 min at 34 700 *g* in order to remove cell membranes. The supernatant was dialyzed overnight at 4°C against 5 liters of Z-buffer [12]. To the dialyzed supernatant a solution of *o*-nitrophenyl galactoside (1/5 vol., 4 mg/ml Z-buffer) was added. The incubation was carried out for 4 h at 37°C and then stopped by chilling and addition of Na_2CO_3 (0.42 M final concentration). Ice-cold trichloroacetic acid (8.2% final concentration) was added in order to precipiate hemoglobin which has an absorption maximum very close to the absorption of *o*-nitrophenol. The solution was kept for 60 min at room temperature, centrifuged (3 min, 34 700 *g*), and the supernatant was filtered through a Millipore filter (25 mm, 0.45 μm pore size). The filtrate was readjusted to alkaline pH with 0.1 volume of 1 M Na_2CO_3. The absorption was measured at 420 nm in a Perkin-Elmer double beam spectrophotometer, model 124. The amount of trapped β-galactosidase was calculated according to a reference curve which was obtained by analysing different amounts of enzyme mixed with erythrocyte ghosts which had been prepared in protein-free hemolysis medium. In order to determine the amount of HSA in resealed erythrocytes the cells were washed, counted and lyzed by three cycles of rapid freezing and thawing. The lysate was centrifuged (4°C, 50 min, 48 000 *g*) and aliquots (1 μl) were applied on cellogel strips (5.7 × 14 cm, Chemetron, Milan, Italy) which had been uniformly coated with anti-HSA serum. Quantitative immunoelectrophoreses were carried out according to Laurell [14] at 120 V for 75 min in 0.05 M sodium diethyl barbiturate buffer.

The fusion was carried out as described by Furusawa et al. [1]. 2000 hemagglutinating units of β-propiolactone-inactivated Sendai virus and 2 ml of suspended red ghosts (1.5% volume fo packed cells/volume) were added to about 5×10^5 recipient cells. The fusion index was determined by staining the recipient cells with fluorescein isothiocyanate-labelled antiserum against human red blood cells and counting the fluorescent cells in the fluorescence microscope. Labeling of the antiserum by fluorescein isothiocyanate was carried out according to the procedure of Clark and Shepard as described by Raff et al. [3].

3. Results and discussion

When human erythrocytes were hypotonically treated and resealed according to Schwoch and Passow [10] the resulting red ghosts retained the biconcave, disk-like shape and the size of normal erythrocytes. This was confirmed by quantitative measurements on microscopic photos. Spherical ghosts were present as less than 0.1%. Thus we assumed the mean volume of resealed red ghosts to be identical to the mean volume (90 μm^3) of normal erythrocytes [10]. In order to measure the extent with which large protein molecules can be trapped by red ghosts we had chosen to work with *E. coli* β-galactosidase (mol. wt. 520 000). Previously Ihler et al. [2] had qualitatively shown that rat β-galactosidase (mol. wt. 180 000 as quoted in [2]) can be taken up by red cells during the hemolysis process. Our data in table 1 demonstrate that only about 0.6% of the β-galactosidase concentration present in the hemolysis medium was detected in the resealed erythrocytes. Presumably the average pore size of erythrocytes during hemolysis is too small for efficient uptake of large molecules like *E. coli* β-galactosidase. Human serum albumin (mol. wt. 64 000), however, can be trapped to more than 83% of the concentration in the hemolysis medium. The uptake of HSA is probably slightly higher than represented by this figure as not all counted red ghosts may have been filled with the exogenous protein. Our preparation may have contained up to 5% leaky ghosts and intact erythrocytes [10]. No HSA could be detected in red ghosts which had been incubated with HSA (25 mg/ml) *after resealing* and

Table 1
Retention of purified proteins by resealed erythrocyte ghosts

	Concentration in hemolysis medium (mg/ml)	Concentration in red ghosts (mg/ml)	Efficiency of retention (%)
β-galactosidase (*E. coli*)	0.6	3.4×10^{-3}	0.56
serum albumin (human)	1.8	1.5	83.3

which had been washed 5 times. These results suggest that HSA may be trapped by hemolysed erythrocytes under our experimental conditions at about the same concentration as present in the hemolysis medium.

Table 2 lists results of a fusion experiment in which ^{125}I-labelled HSA was transferred into murine LM (TK$^-$) cells by fusion with resealed red ghosts. Using fluorescent antibodies against membranes of human red blood cells we determined a fusion index of 10%. This is lower than values reported by other authors [1,7]. Possibly the preparation of inactivated Sendai virus which we used was not optimally active. In addition some fused recipient cells of the relatively loosely attached LM (TK$^-$) cell line might have been removed together with the excess of unfused red ghosts during the repeating washings. The radioactivity detected after fusion in the recipient cells is slightly higher than the fusion index determined by counting the fluorescent cells. This result could in part be explained by fusion of a second resealed ghost with the same recipient cell (statistically about 0.6%). The contamination of the recipient cells with unfused red ghosts was less than 1% based on counts in the phase contrast and fluorescence microscope. The amount of transferred ^{125}I-labelled HSA listed in Table 2 has been corrected by the amount of radioactive HSA determined in recipient cells during a control experiment without inactived Sendai virus.

In confirmation of independent studies by Schlegel and Rechsteiner [7] our results demonstrate that human serum albumin can be efficiently transferred into murine recipient cells. Probably the total amount of HSA trapped in the red ghosts is injected during fusion into the corresponding recipient cells. In addition our results suggest that the less efficient trapping of larger proteins such as *E. coli* β-galactosidase by red ghosts may point to a limitation of this new technique of cell biology. In order to transfer efficiently by this method very large protein molecules they would have be used at relatively high concentrations to compensate for their less efficient trapping by red cell ghosts.

Table 2
Transfer of human ^{125}I-labelled serum albumin into murine LM (TK$^-$) cells by fusion with resealed ghosts

Concentration of human ^{125}I-labelled serum albumin in hemolysis medium:	109 700 cpm/μl 4.5 mg/ml
Total radioactivity in 1.18×10^7 recipient cells	14 700 cpm
Total amount of HSA in 1.18×10^7 recipient cells	0.64 mg
Calculated number of fused recipient cells	1.5×10^6
Calculated fusion index	11.7%
Observed fusion index	approx. 10%

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