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SIMPLE AND RAPID PURIFICATION OF INSIDE-OUT VESICLES FROM HUMAN ERYTHROCYTES

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Summary

The preparation of inside-out vesicles from human erythrocytes requires their separation from contaminating right-side-out vesicles. We have taken advantage of the fact that there are no glycoproteins on the internal side of the erythrocyte membrane; therefore, inside-out vesicles do not interact with the lectin, concanavalin A, while right-side-out vesicles do interact. A concanavalin A-cellulose affinity matrix has been utilized to separate easily inside-out vesicles with a purity comparable to those prepared by prolonged centrifugation.

Introduction

Sealed inside-out vesicles have been very useful in the characterization of transport systems in erythrocyte membranes [1—3]. Steck et al. [4,5] reported a method for preparation of inside-out vesicles which used ultracentrifugation in a dextran density gradient to purify the vesicles. This purification procedure requires 3 h of centrifugation [3,5] followed by extensive washing. We have developed an alternative purification method which relies on the observation that glycoproteins are located only on the external surface of erythrocyte membranes [6] and that they have an affinity for concanavalin A [7]. This characteristic of erythrocyte membranes provides a basis for simplified purification of inside-out vesicles.

Materials and Methods

Concanavalin A and α-cellulose were purchased from Sigma Chemical Co. (Saint Louis, MO) and dextran T-70 from Pharmacia (Upsala, Sweden).

All reagents used were of analytical grade.

Preparation of concanavalin A-cellulose. Concanavalin A was coupled to α-cellulose by a modification of the method described by Sanderson and Wilson [8]. 2 g of α-cellulose (approx. 10 ml of hydrated bed volume), were washed with distilled water and excess water decanted. The cellulose was then oxidized with 10 ml of 100 mM sodium periodate in water for 1 h at room temperature. The oxidized α-cellulose was collected on a coarse sintered-glass funnel and washed with 1 l of distilled water. 60 mg of concanavalin A in 30 ml of phosphate-buffered saline prepared from 9 parts of 0.154 M NaCl to 1 part of 0.1 M Na_2HPO_4/NaH_2PO_4, pH 8.0, were added to the moist α-cellulose and the mixture gently agitated for 24 h at 4°C. The particles were then washed with 1 l of phosphate-buffered saline and treated with 20 ml of 100 mM $NaBH_4$ in water for 15 min. The particles were washed again with phosphate buffered saline and suspended in 0.5 mM Tris-HCl, pH 8.0, containing 1 mM each of $CaCl_2$ and $MnCl_2$. Under the conditions employed, the coupling yield was approx. 18 mg of concanavalin A per g cellulose. This yield was determined by the estimation of the protein concentration in the supernatant of the coupling suspension. A control cellulose suspension was carried through the same procedure except for the periodate oxidation. The control preparation did not bind intact erythrocytes.

Preparation of erythrocyte membrane. Fresh blood from normal donors, collected in 1 mg EDTA per ml of blood, was freed of leucocytes and most platelets by passing through a small column of α-cellulose and microcrystalline cellulose [9]. The erythrocytes were then washed three times with 0.12 M KCl containing 0.02 M K_2HPO_4/KH_2PO_4, pH 7.3 [10]. Ghosts were prepared by lysing the erythrocytes in 25 vols. of 10 mM Tris-HCl, pH 7.4, and washing in the same buffer until their color was white.

Preparation of sealed vesicles. Vesicles were prepared from the red cell membranes at 4°C as described by Steck and Kant [5]. The pelleted ghosts were suspended in 40 vols. of ice-cold 0.5 mM Tris-HCl, pH 8.0, for 2 h, washed twice with the same buffer and centrifuged at 28 000 × *g* for 30 min. The pellet was then vesiculated by passing through a 27 gauge needle five times; this produces a mixture of right-side-out and inside-out vesicles.

Analytical methods. Protein concentrations were estimated by using the method of Lowry et al. [11] with bovine serum albumin or concanavalin A as a standard. Glyceraldehyde phosphate dehydrogenase and acetylcholinesterase were assayed as described previously [12]. The assay conditions used for glyceraldehyde-phosphate dehydrogenase and acetylcholinesterase are sufficiently gentle that the vesicles are not lysed and that, therefore, only enzyme exposed on the outer surface of the vesicle is measured.

Experimental Procedure and Results

10 ml of homogenized vesicles with a hematocrit of 15% were added to 10 ml of a 20% (v/v) suspension of concanavalin A-cellulose. After 10 min at room temperature, the concanavalin A-cellulose was poured onto a coarse sintered-glass funnel and washed with 50 ml of 0.5 mM Tris-HCl, pH 8.0. The vesicles not bound to the concanavalin A-cellulose were concentrated

TABLE I

PREPARATION OF INSIDE-OUT VESICLES

Accessibility was calculated as (activity without detergent/activity with detergent) × 100.

Preparation	Volume (ml)	Protein (mg/ml)	(%)	Glyceraldehyde-phosphate dehydrogenase (I.U./mg protein)	Acetyl-cholinesterase (I.U./mg protein)
Unsealed membranes	10	1.34	(100)	2.90	1.82
Homogenized with needle	10	1.32	(100)	3.77	0.76
Concanavalin A-cellulose treated	5.5	1.75	(71.8)		
Without detergent				5.55	0.38
With detergent *				6.01	2.10
Accessibility (%)				92.3	18.1

* Triton X-100 in a final concentration of 0.02% was added as a detergent.

by centrifugation at 26 000 × *g* for 20 min. The sideness of these vesicles was determined by measurement of glyceraldehyde-phosphate dehydrogenase as a marker of the erythrocyte inner surface, and acetylcholinesterase as a marker of the erythrocyte outer surface [5]. As a control, the vesicles were dissolved in Triton X-100 detergent to allow assay of total glyceraldehyde-phosphate dehydrogenase and acetylcholinesterase. The characteristics of the vesicles are summarized in the tables. It is apparent that the vesicles which did not bind to the concanavalin A-cellulose had almost all (92.3%) of their glyceraldehyde-phosphate dehydrogenase exposed on the outer surface of the vesicle while 82% of the acetylcholinesterase was on the internal surface of the vesicle.

In order to compare the proposed method with that originally used by Steck and Kant [5], sealed vesicles were prepared and then divided into two portions, one was separated into inside-out and right-side-out vesicles by centrifugation for 3 h at 65 000 × *g*, while the other portion was treated with concanavalin A-cellulose as described above. As shown in Table II, the inside-out vesicles purified by the two techniques are virtually the same with regard to the accessibility of glyceraldehyde-phosphate dehydrogenase and acetylcholinesterase.

TABLE II

COMPARISON OF ACCESSIBILITY OF INSIDE-OUT VESICLES PREPARED BY THE CONCANAVALIN A-CELLULOSE METHOD AND THE METHOD OF STECK AND KANT

Accessibility as defined in Table I. The means and the ranges of two experiments are presented.

	Glyceraldehyde-phosphate dehydrogenase	Acetyl-cholinesterase
Concanavalin A-cellulose method (this paper)	89.8% (88.6%—91.0%)	19.8% (18.8%—20.8%)
Method of Steck and Kant [5]	88.8% (87.9%—89.6%)	20.1% (20.0%—20.2%)

In another experiment, inside-out vesicles prepared by the concanavalin A-cellulose method were analyzed by the ultracentrifugation procedure of Steck and Kant [5]. Almost all of the vesicles banded at a density of 1.030–1.035 g/cm^3, which corresponds to the reported density of inside-out vesicles. No vesicles were present at the density range at which right-side-out vesicles are found.

Discussion

The glycoproteins of the red cell membrane extend their chains of sugars outwards into the plasma. Thus, right-side-out vesicles prepared from erythrocytes expose chains of sugars on their exterior surface, and are, therefore, able to bind concanavalin A. Inside-out vesicles do not have any exposed carbohydrate and do not interact with concanavalin A. In preliminary studies, we found that commercially available concanavalin A-Sepharose was ineffective in binding right-side-out vesicles, presumably because much of the concanavalin A was bound to internal portions of the gel matrix and sterically restricted from binding the vesicles. This problem was overcome by using α-cellulose as the solid phase for attachment of concanavalin A. Using this system, it was possible to purify inside-out vesicles with a very high yield from a mixture of inside-out and right-side-out vesicles. A small amount of acetylcholinesterase activity was exposed in the final preparation of inside-out vesicles. This might suggest that some of the right-side-out vesicles were not removed by the concanavalin A-cellulose but, more likely, may indicate that some of the vesicles were leaking or fragmented, and that it was therefore possible for the substrate for acetylcholinesterase to enter. A similar amount of acetylcholinesterase was accessible in inside-out vesicles prepared by other investigators [3,5].

The preparation of concanavalin A-cellulose is simple, and it may be used repeatedly merely by washing with 0.1 M α-methylmannoside. The method we describe provides, with a minimum of effort, inside-out vesicles of high purity identical to that obtained by the method of Steck and Kant [5].

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