Journal of Chromatography, 375 (1986) 380–385
Biomedical Applications
Elsevier Science Publishers B V , Amsterdam — Printed in The Netherlands

CHROMBIO 2929

Note

Affinity of young and old human erythrocytes for alkyl-Sepharose 6MB gels

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(Received September 18th, 1985)

Differences in the contents of membrane components of young and old human erythrocytes have been studied in many laboratories. It was demonstrated that the contents of cholesterol, phospholipids [1] and bound carbohydrates [1–6] are significantly higher in the membranes of young erythrocytes than in those of old ones.

On the other hand, recently, hydrophobic chromatography using alkyl-Sepharose gels was used as a purification method of human erythrocyte membrane glycoproteins [7, 8]. Furthermore, hydrophobic chromatography was used for the separation of blood cells such as erythrocytes, white blood cells and blood platelets [9] and applied to the discrimination between erythrocytes from different sources [10, 11]. However, the difference in the affinity of young and old erythrocytes for alkyl-Sepharose gels has not yet been investigated In this work, the affinity of young and old human erythrocytes for alkyl-Sepharose 6MB gels was studied.

EXPERIMENTAL

Materials

CNBr-activated Sepharose 6MB was obtained from Pharmacia Fine

Chemicals (Uppsala, Sweden) Alkylamines and bovine serum albumin (pure crystal, BSA) were purchased from Nakarai Chemicals (Kyoto, Japan). Dimethyl phthalate (SG 1 191—1.196) and di-*n*-butyl phthalate (SG 1 047-1.051) were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Neuraminidase (*Streptococcus* sp IID) was obtained from Seikagaku Kogyo (Tokyo, Japan) and trypsin (Type III, two times crystallized trypsin) was obtained from Sigma (St Louis, MO, U S A) Other reagents were of analytical-reagent grade

Preparation of alkyl-Sepharose 6MB

Four alkyl-Sepharose 6MB gels were prepared by coupling CNBr-activated Sepharose 6MB with *n*-alkylamines (*n*-propyl-, *n*-hexyl-, *n*-octyl- and *n*-decylamine) according to the procedure of Shaltiel [12] Alkylamino groups in the gels were determined by the colorimetric method with trinitrobenzenesulphonic acid [13]. Estimation of BSA quantity adsorbed on the four alkyl-Sepharose 6MB gels was carried out by the method of Shaltiel and Halpern [11].

Separation of young and old human erythrocytes

Fresh human bloods of OMN group were collected from normal adults by using 3.8% (w/v) sodium citrate solution as an anticoagulant Age-dependent fractionation of erythrocytes was performed according to Danon and Marikovsky [14] using dimethyl phthalate and di-*n*-butyl phthalate solutions as separating fluids. Erythrocytes were fractionated into three groups, top (< 1.092 density), middle (1 092- 1.100) and bottom (> 1 100) layers, in the ratio 18 65 17 (v/v) The separated erythrocytes were washed five times with buffer I buffer composed of NaCl (8 g), KCl (0 2 g), Na_2HPO_4 (1 15 g), $KH_2PO_4 \cdot H_2O$ (0.2 g), $CaCl_2$ (0 1 g) and $MgCl_2 \cdot 6H_2O$ (0.1 g) in a final volume of 1 l, pH 7.4) [10] The ATP level of erythrocytes was significantly lower in the bottom layer (0.64 ± 0 12 μmol per 10^{10} cells) than in the top layer (0 81 ± 0.09 μmol per 10^{10} cells). This fact shows that the bottom layer contains preponderantly old erythrocytes, while young erythrocytes are concentrated in the top layer

Treatment of young and old human erythrocytes with neuraminidase or trypsin

Packed young or old erythrocytes were incubated with an equal volume of neuraminidase solution (40 U/ml) in phosphate-buffered saline (PBS, pH 7 4) for 1 h at 37°C. Other packed young or old erythrocytes were incubated with an equal volume of trypsin solution (0 5 mg/ml) in PBS for 3 h at 37°C Each of the digestion mixtures was centrifuged at 3000 rpm at 4°C. The separated erythrocytes were washed four times with buffer I and used immediately as desialylated or trypsin-treated erythrocytes

Determination of adsorption rates of young and old human erythrocytes to alkyl-Sepharose 6MB gels

A glass column (10 × 1.6 cm I.D), which was attached with nylon net (80 μm mesh) at its bottom, was used as a container for 1 5 ml of alkyl-Sepharose 6MB gel. A 0.2-ml volume of young or old erythrocyte ($1.5 \cdot 10^{8}$–

$2.5 \cdot 10^8$) suspension in buffer I was gently applied to the column The gel was covered with 0 2 ml of buffer I and allowed to stand at room temperature for 10 min. The column was then gently washed with 10 ml of buffer I to exclude unadsorbed erythrocytes. The excluded erythrocytes were collected and counted with a microscope using a counting chamber (Erma Kogaku, Tokyo, Japan). The adsorption rate of young or old erythrocytes to alkyl-Sepharose 6MB was calculated by the ratio of the number of excluded erythrocytes to that of the applied erythrocytes. The adsorption rates of desialylated or trypsin-treated young and old erythrocytes were also measured by this procedure.

Preparation of the excluded and adsorbed fractions of erythrocytes by octyl-Sepharose 6MB gel column chromatography

A glass column (10 × 1.6 cm I.D) containing 3 0 ml of octyl-Sepharose 6MB gel was used A 0 5-ml volume of buffer I suspension of erythrocytes ($6\ 0 \cdot 10^8$ $-7\ 5 \cdot 10^8$), which was not separated by the density-gradient centrifugation technique, was added to the column. The column was allowed to stand at room temperature for 10 min and gently washed with 20 ml of buffer I to obtain the excluded fraction. The adsorbed fraction was eluted from the column by adding 2.0 ml of BSA solution (20 mg/ml of buffer I) and resuspended in 20 ml of buffer I. Sialic acid content and glucose-6-phosphate dehydrogenase activity of erythrocytes in the excluded and adsorbed fractions were determined by the thiobarbituric acid method [15] and the spectrophotometric method [16], respectively

RESULTS AND DISCUSSION

The concentration of four alkylamines immobilized to CNBr-activated Sepharose 6MB gel was ca. 80—90 μmol/ml of wet gel, as shown in Table I In order to estimate the hydrophobic force of alkyl ligands of the four alkyl-Sepharose 6MB gels, the quantity of BSA adsorbed on the four gels was determined and then the adsorption rates to the gels were calculated. As presented in Table I, the adsorption rates of BSA to the four gels were proportional to the carbon number of their alkyl chains

TABLE I

CONCENTRATION OF ALKYLAMINES COUPLED TO CNBr-ACTIVATED SEPHAROSE 6MB GEL AND ADSORPTION RATES OF BOVINE SERUM ALBUMIN (BSA) TO FOUR ALKYL-SEPHAROSE 6MB GELS

BSA was applied to an alkyl-Sepharose 6MB gel (1 5 ml) column and run with buffer I The first eluate (2 ml) was collected, its adsorbance at 280 nm was measured and the percentage of BSA was calculated Values represent the mean of four separate experiments

Alkylamine	Amount ligand coupled to gel (μmol ligand per ml gel)	Adsorbed BSA (%)
n-Propylamine	88 0	14 3
n-Hexylamine	86 2	35 7
n-Octylamine	82 4	64 3
n-Decylamine	89 6	71 4

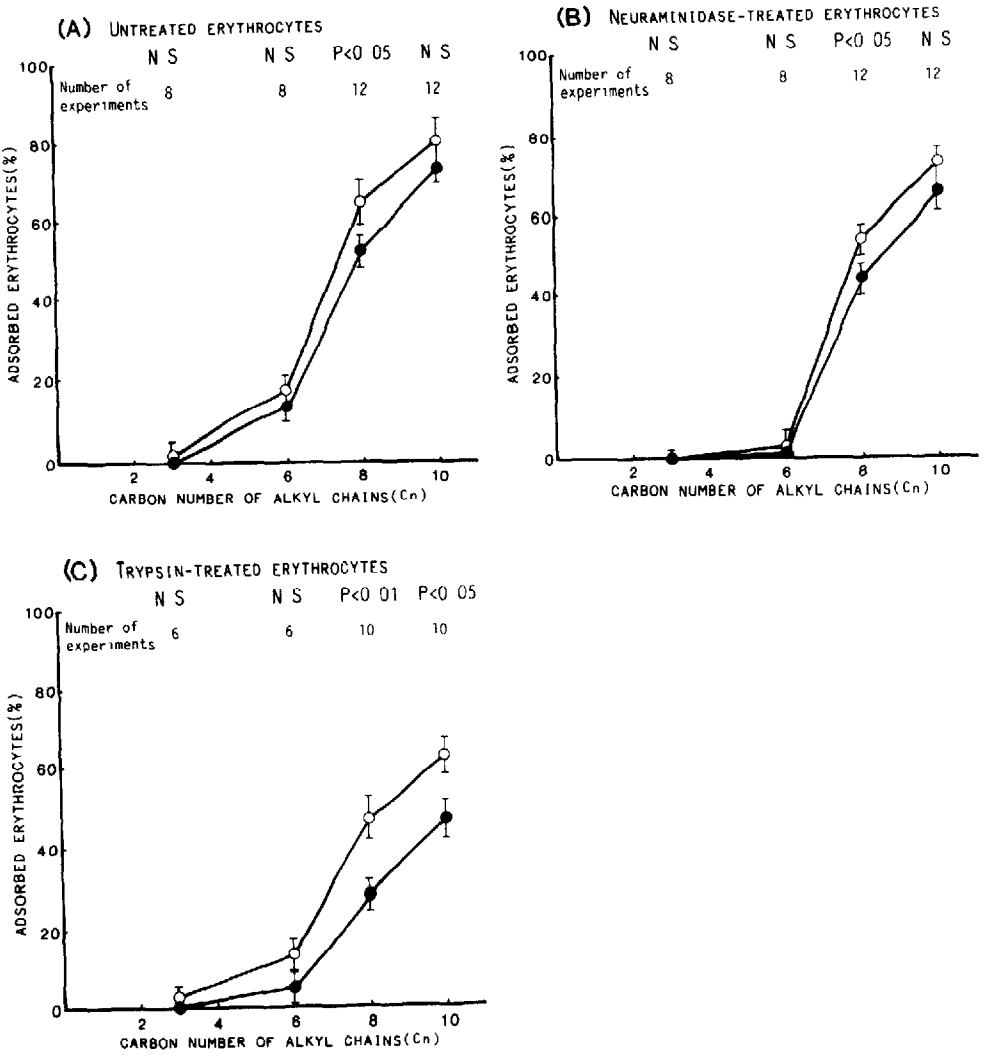

Fig 1 Adsorption rates of young (○) and old (●) human erythrocytes to four alkyl-Sepharose 6MB gels (Cn, n = 3, 6, 8, 10)

The adsorption rates of young and old human erythrocytes to the four alkyl-Sepharose 6MB gels were measured. The results are presented in Fig 1A The adsorption rates of both the erythrocyte groups increased in proportion to the length of the alkyl chains of the four gels The adsorption rate to octyl-Sepharose 6MB gel was distinctly higher ($p < 0.05$) in young erythrocytes than in the old ones. It is known that coupling of alkylamines to CNBr-activated agarose results in the appearance of positive charges in the agarose matrix [17]. Therefore, it is possible that the adsorption rate of erythrocytes to the four gels may be influenced by bound sialic acids on their cell membranes, which are carriers of negative charge [18] So, the adsorption rates of desialylated young and old erythrocytes to the four alkyl-Sepharose 6MB gels were measured. As shown in Fig 1B, the adsorption rates to the four gels of desialylated young and old erythrocytes were ca 10% lower than those of untreated young and old erythrocytes, respectively A decrease in the adsorp-

tion rates of desialylated erythrocytes to the four gels indicates that the carboxyl groups of bound sialic acids on erythrocyte membranes partially participate in binding between erythrocytes and the alkyl-Sepharose 6MB gels. In interaction between octyl-Sepharose 6MB gel and untreated or desialylated erythrocytes, the adsorption rate to the gel was higher ($p < 0.05$) in young erythrocytes than in the old ones (Fig. 1A and B).

The adsorption rates to the four alkyl-Sepharose 6MB gels of young and old erythrocytes treated with trypsin, which releases sialoglycopeptides from their cells, were found to be lower than those of untreated erythrocytes as presented in Fig. 1C and A Therefore, it is clear that trypsin decreases the affinities of both young and old erythrocytes for the four gels Furthermore, trypsin treatment increases the difference in the adsorption rates of young and old erythrocytes to the four gels These facts show that trypsin-sensitive sialoglycoproteins on erythrocyte membranes are responsible for the interaction between erythrocytes and the four alkyl-Sepharose 6MB gels

Erythrocytes that were not treated by the density-gradient centrifugation technique were applied to octyl-Sepharose 6MB gel column, and then excluded and adsorbed fractions were collected. The sialic acid content and glucose-6-phosphate dehydrogenase activity of the excluded fraction were significantly lower ($p < 0.01$) than those of the adsorbed fraction as shown in Table II

It is well known that the sialic acid content [1–6] and glucose-6-phosphate dehydrogenase activity [4] of human young erythrocytes are distinctly higher

TABLE II

SIALIC ACID CONTENT AND GLUCOSE-6-PHOSPHATE DEHYDROGENASE ACTIVITY OF HUMAN ERYTHROCYTES IN EXCLUDED AND ADSORBED FRACTIONS FROM OCTYL-SEPHAROSE 6MB GELS

Values represent mean ± S D of twelve experiments

	Number of erythrocytes ($\times 10^8$)	Sialic acid content (μg per 10^{10} cells)	Glucose-6-phosphate dehydrogenase activity (mU per 10^8 cells)
Applied erythrocytes (control)	6 72 ± 0 70	119 54 ± 13 48	15 83 ± 1 22
Excluded fraction	3 71 ± 0 59 (55 2%)*	118 83 ± 7 94	12 66 ± 1 41
Adsorbed fraction	1 62 ± 0 46 (24 1%)*	140 83 ± 12 46	16 37 ± 0 93
Relative percentage**		84 8	77 6
p of difference observed between excluded and adsorbed fractions		< 0 01	< 0 01

*Value was expressed by the following equation

$$\frac{\text{number of erythrocytes in each fraction}}{\text{number of erythrocytes applied to the column}} \times 100$$

**Relative percentage was expressed by the following equation

$$\frac{\text{value of excluded fraction}}{\text{value of adsorbed fraction}} \times 100$$

than those of old ones Therefore, the results seem to indicate that the excluded fraction contains a more abundant amount of older erythrocytes and the adsorbed fraction is enriched in younger erythrocytes

It may be concluded from the studies described above that the affinity for the four alkyl-Sepharose 6MB gels of young erythrocytes is stronger than that of the old ones Trypsin-sensitive sialoglycoproteins on erythrocyte membranes are responsible for the interaction between erythrocyte surface and the gels, where the sialic acid residues partially participate in the interaction. Column chromatography using octyl-Sepharose 6MB gel may be used as a group-separation technique of young and old erythrocytes

ACKNOWLEDGEMENT

The authors thank Dr A. Ogamo, School of Pharmaceutical Sciences, Kitasato University, for valuable discussions.

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