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DIFFERENTIAL EFFECT OF NEURAMINIDASE-TREATMENT ON THE SURFACE CHARGE-ASSOCIATED PROPERTIES OF RAT RETICULOCYTES AND ERYTHROCYTES

STUDIES BY PARTITIONING IN TWO-POLYMER AQUEOUS PHASES

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Rat reticulocytes undergo charge-associated surface changes, detectable by cell partitioning in charged dextran-poly(ethylene glycol) aqueous phase systems, as they become mature erythrocytes. Young reticulocytes have a lower partition coefficient, i.e., quantity of cells in the top phase as a percentage of total cells added, than do mature erythrocytes. Sialic acid is the main charge-bearing group on red blood cells and, in the case of the rat, most of the sialic acid can be removed by treatment of the cells with neuraminidase (*Vibrio cholerae*). By combining isotopic ^{59}Fe-labeling of reticulocytes with countercurrent distribution of the entire red blood cell population in charged dextran-poly(ethylene glycol) aqueous phases we have now studied the relative effect of neuraminidase-treatment on rat reticulocytes and mature erythrocytes. It was found that neuraminidase-treatment (a) does not eliminate surface differences, detectable by partitioning, between rat reticulocytes and erythrocytes and (b) reduces the partition coefficient of mature erythrocytes to a greater extent than the partition coefficient of reticulocytes indicating a differential effect of this enzyme on the two cell populations.

Aqueous solutions of dextran and of poly(ethylene glycol) when mixed above certain concentrations give rise to immiscible, liquid two-phase systems suitable for the separation and subfractionation of cell populations by partitioning [1–3]. Some salts (e.g., phosphates) have different affinities for the two phases [4] and an electrostatic potential difference between the phases results with the top phase positive [5]. Cells added to such systems (at appropriate polymer concentrations) will partition according to surface charge-associated properties [6,7].

Using isotopic labeling techniques we have previously found that rat reticulocytes have the lowest partition coefficient, K (i.e., quantity of cells in the top phase as a percentage of total cells added), of any red cells in the peripheral blood [8] and that the value of K rapidly increases as the reticulocytes mature to erythrocytes [8–10]. Since these changes in K of maturing reticulocytes are charge-associated and since sialic acid is the main charge-bearing group on red blood cells [11,12], we have now determined whether sialic acid susceptible to removal from the cell surface by neuraminidase-treatment is responsible for the observed partitioning difference.

A rat blood cell population in which reticulocytes were radioactively labeled (with ^{59}Fe) was subjected, both with and without neuraminidase-treatment, to countercurrent distribution in a dextran-poly(ethylene glycol) aqueous phase system. It was found not only that neuraminidase did not

eliminate partitioning differences between reticulocytes and mature erythrocytes but that the enzyme has a differential effect on these cells. Neuraminidase-treated reticulocytes have a higher mean K than the bulk of neuraminidase-treated mature erythrocytes.

Injection and bleeding of rats. Male, Sprague-Dawley rats (weight range 350–500 g) were injected intravenously via the saphenous vein with 8–12 μCi of [^{59}Fe]ferrous citrate (Mallinckrodt, MO). They were bled by heart puncture 16.5 h after injection using acid-citrate-dextrose as anticoagulant. Red blood cells were then washed three times with cacodylate buffer, pH 6.4 [11], using ten times the cell volume for each washing.

Incubation of labeled rat red cells. Two aliquots of 0.5 ml of washed, packed red cells were taken and suspended in 3.5 ml of cacodylate buffer, pH 6.4. One aliquot (A) was without neuraminidase while the other aliquot (B) received 200 μl (0.2 I.U.) of neuraminidase (*Vibrio cholerae*, Calbiochem-Behring, CA). Both A and B were then incubated with gentle shaking at 37°C for 90 min. After the incubation both aliquots were again washed three times with cacodylate buffer as above. A 'load mix' (see below) was prepared in the cold room for countercurrent distribution.

It has previously been found that most [13], but not necessarily all [14], of the sialic acid on the rat red cell is susceptible to cleavage by neuraminidase and, further, that all of the sialic acid that can be removed by the enzymatic treatment is cleaved within 60 min. Measurement of red cell electrophoretic mobility also showed no further decrease after this time interval (Seaman, G.V.F. and Walter, H., unpublished data).

Preparation of phase system. The phase system used was prepared as previously described [2]. It was composed of 5% (w/w) Dextran T500, lot No. 11648 (Pharmacia, NJ), 4% (w/w) poly(ethylene glycol) 6000 (trade-name Carbowax 6000, recently renamed Carbowax 8000, by Union Carbide, NY), 0.09 M sodium phosphate buffer, pH 6.8, and 0.03 M NaCl. The phase system was allowed to equilibrate at 4–5°C in a separatory funnel and top and bottom phases were then separated.

Countercurrent distribution of labeled rat red blood cells. 0.30 ml of packed rat red blood cell aliquots A and B were each suspended in 3.3 ml of top phase ('load mix'). An automatic thin-layer countercurrent distribution apparatus with circular plates and 120 concentric cavities was used [15]. Cavities 0–2 and 60–62 each received 0.5 ml of bottom phase and 0.7 ml of one of the load mixes. All other cavities received 0.5 ml bottom phase and 0.7 ml top phase. 30 transfers were completed at 4–5°C using a settling time of 6 min and a shaking time of 22 s.

Analysis of cells following countercurrent distribution. After a countercurrent run, cells were collected directly into plastic centrifuge tubes. 0.7 ml of saline was added to each tube to convert the two-phase system into a single phase, homogeneous suspending medium. Adjacent tubes were pooled by threes or fours and centrifuged. The supernatant solutions were discarded and the cells were lysed in a known (3 ml) volume of 20 mosM sodium phosphate buffer, pH 7.2. The lysates were centrifuged at 12000 × g for 10 min to remove the stroma and the absorbance of the supernatant solutions was measured at 540 nm on a Gilford spectrophotometer. Known aliquots of the lysates were counted in a Searle scintillation well-counter.

Presentation of data. Plotting the hemoglobin absorbance (at 540 nm) in different cavities along the extraction train yields the countercurrent distribution curves of the red blood cell populations as a whole. The distribution of reticulocytes (the labeled population) is obtained by plotting the counts per minute in the different cavities. A relative specific activity (RSA) is also shown and is defined as:

$$\mathrm{RSA} = \frac{\text{cpm/hemoglobin absorbance in a given cavity}}{\text{cpm/hemoglobin absorbance in the original cell population}}$$

Results and discussion. When rats are injected with [^{59}Fe]ferrous citrate and bled at different times red blood cell populations are obtained in which cells of ages corresponding to the time elapsed between injection and bleeding are radioactively labeled [8,9]. Between 16 and 18 h after isotope injection it is the youngest reticulocytes that are labeled [8]. When subjected to countercurrent distribution in a charged (e.g., phosphte-containing) dextran-poly(ethylene glycol) aqueous phase system it is found that the labeled reticulo-

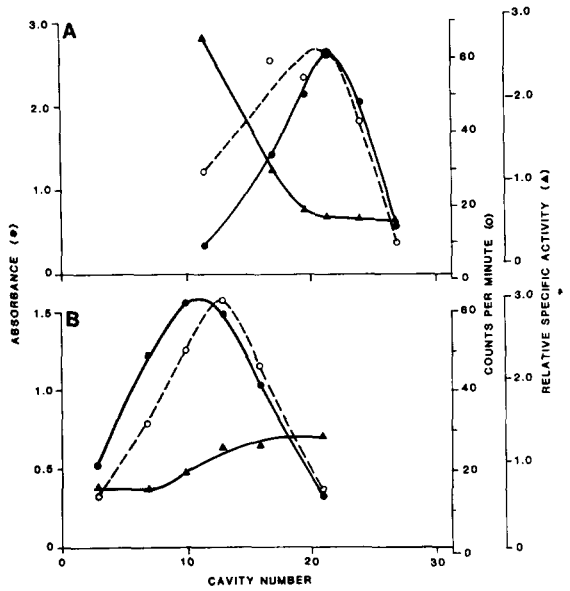

Fig. 1. Countercurrent distribution (CCD) patterns of red blood cells from a rat injected with [^{59}Fe]ferrous citrate and bled 16.5 h later yielding a cell population in which the reticulocytes are labeled. Red cells were subjected to CCD in a phase system containing 5% (w/w) dextran, 4% (w/w) poly(ethylene glycol), 0.09 M sodium phosphate buffer, pH 6.8, and 0.03 M NaCl. Thirty transfers were completed at 4–5°C. Whole red cell distribution is given in terms of hemoglobin absorbance at 540 nm (●); distribution of the labeled reticulocytes in terms of cpm (○); and a relative specific activity is also shown (▲). (A) Original, incubated red blood cell population; (B) cell population as in A but incubated with neuraminidase. The data presented in this figure are representative of those obtained in four separate experiments with four different rats.

cytes have a lower partition coefficient, K, than the bulk of the circulating red cells in the peripheral blood (Fig. 1A). As the reticulocytes mature to young erythrocytes in vivo the value of K increases rapidly [8] so that, in about 48 h, it becomes the highest K of any red cell in the circulation.

Sialic acid is the main charge-bearing group on red blood cells [11,12]. Since partitioning of rat red cells in the phase system used is determined predominantly by the cells' surface charge-associated properties [2,3], we tested whether neuraminidase-treatment of the cells would eliminate the surface differences between reticulocytes and erythrocytes reflected by partitioning (Fig. 1A).

Red blood cells containing labeled reticulocytes were incubated at 37°C for 90 min with or without neuraminidase. Countercurrent distribution of cells incubated with neuraminidase reveals that, while the K values of both erythrocytes and reticulocytes are reduced by this treatment (compare relative positions of distribution curves in Fig. 1B with those in Fig. 1A), the labeled reticulocytes have a markedly higher K than the erythrocytes. Since neuraminidase removes most [13], but not necessarily all [14], of the sialic acid from the surface of the rat red cell population as a whole, the simplest explanation for our results is that more sialic acid per unit area is removed from mature erythrocytes than from reticulocytes. Alternatively, there may be more negative surface charges, other than sialic acid, per unit area on the reticulocyte than on the mature erythrocyte and removal of the sialic acid causes these other charges [14] to become determinant in the cells' relative partitioning behavior.

Whatever the actual chemical basis for our results, we can conclude that (a) the treatment of rat red cell populations with neuraminidase has a differential effect on reticulocytes and erythrocytes and (b) neuraminidase-treatment does not eliminate surface differences between rat reticulocytes and erythrocytes detectable by partitioning in a charged two-polymer aqueous phase system.

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