Biochimica et Biophysica Acta, 734 (1983) 368–372
Elsevier

BBA Report

BBA 70121

THE EFFECT OF NEURAMINIDASE ON THE RELATIVE SURFACE CHARGE-ASSOCIATED PROPERTIES OF RAT RED BLOOD CELLS OF DIFFERENT AGES

HARRY WALTER [a,b], CHERRY H. TAMBLYN [c], EUGENE J. KROB [a] AND GEOFFREY V.F. SEAMAN [c]

[a] *Laboratory of Chemical Biology-151, Veterans Administration Medical Center, Long Beach, CA 90822,* [b] *Department of Physiology and Biophysics, University of California, Irvine, CA 92717, and* [c] *Department of Neurology, Oregon Health Sciences University, Portland, OR 97201 (U.S.A.)*

(Received November 23rd, 1982)
(Revised manuscript received July 11th, 1983)

Key words: Neuraminidase; Surface charge; Erythrocyte; Sialic acid; Aging; (Rat)

Approx. 70% of the sialic acid on the rat erythrocyte surface is susceptible to cleavage by neuraminidase (*Vibrio cholerae*). Neuraminidase treatment results in a reduction in the partition coefficient (K) of the red cells in a charged dextran-poly(ethylene glycol) aqueous phase system and in the electrophoretic mobility of the cells. Countercurrent distribution of rat neuraminidase-treated erythrocytes, containing ^{59}Fe-labeled mature red cells of distinct age, indicates that (a) the electrophoretic mobilities of red cells in different cavities along the extraction train increase with increasing K, as is the case with untreated erythrocytes, and (b) the cell age-related differences in surface charge-associated properties are neither eliminated nor altered by the enzyme action.

When aqueous solutions of dextran and of poly(ethylene glycol) are mixed above certain concentrations, immiscible, liquid two-phase systems are obtained with one of the phases rich in one of the polymers and the other phase rich in the second polymer [1]. Such systems, when rendered isotonic, are suitable for the separation and subfractionation of cell populations by partitioning [1,2]. Some salts (e.g., phosphates) have unequal affinities for the two phases [3] and an electrostatic potential difference between the phases results, with the top phase positive with respect to the bottom phase [4]. In such a system, cells will partition according to surface charge-associated properties [2,5]. Combining isotopic labeling techniques, in which rat red blood cells of different but distinct ages were labeled, with countercurrent distribution (a multiple extraction procedure) in a charged dextran-poly(ethylene glycol) aqueous phase system we have found previously that erythrocytes of different ages have characteristic partition coefficients, K (i.e., quantities of cells in the top phase as a percentage of total cells added). The K of rat mature erythrocytes decreases with cell age [5]. Furthermore, a concomitant decrease in rat erythrocyte electrophoretic mobility with decreasing K through the countercurrent distribution extraction curve was observed [6], although the charge reflected by partitioning and that measured by cell electrophoresis is not necessarily the same [7]. It appears that rat red cells with increasing in vivo age have decreasing surface charge as measured both by partitioning and electrophoresis. Recent experiments [8,9] have shown that in the case of human red cells no subfractionation based on cell age is obtained by countercurrent distribution, as evidenced by the overlap of the distribution curves of cell fractions enriched in young or old red cells obtained by the Murphy centrifugation technique [10], and by the constancy of the electrophoretic mobility of cells from different parts of the extraction train [8].

Our interest has turned to the chemical basis for the age-dependent difference in membrane properties of rat mature red blood cells. In the present work we examined the effect of neuraminidase on the relative surface charge-associated properties of rat red blood cells of different ages.

Blood was obtained by heart puncture from adult, male Sprague-Dawley rats weighing between 300 and 500 g, using acid-citrate-dextrose as anticoagulant. Erythrocytes were routinely washed three times (using at least 10-times the packed cell volume) with either phosphate-buffered saline (pH 7.0) or, in the case of experiments in which cells were treated with neuraminidase, cacodylate buffer (pH 6.4) [11]. In some experiments, rats were injected with about 12 μCi of [^{59}Fe]ferrous citrate (Mallinckrodt) via the saphenous vein, and each was bled at a different time thereafter.

Cell counts on aliquots of erythrocytes used in sialic acid assays were obtained by use of an Electrozone Celloscope (Particle Data, Chicago), fitted with a 76-μm orifice tube.

Mobilities of cells were measured in a cylindrical chamber apparatus at 25°C using Ag|AgCl electrodes [12]. Cell samples were washed three times and examined in 'standard saline' [12].

For neuraminidase treatment, 0.5 ml of washed, packed red blood cells were suspended in 3.5 ml of cacodylate buffer (pH 6.4) and an aliquot was taken for an electronic cell count. 200 μl (0.2 IU) of neuraminidase (*Vibrio cholerae*, Calbiochem-Behring) was added and the suspension incubated for 90 min at 37°C with constant gentle swirling. 'Untreated' cells (depicted in Fig. 1, top) were suspended and incubated in a similar manner except that no neuraminidase was added. Cells were washed three times as above immediately after completion of the incubation. Aliquots were taken for measurement of electrophoretic mobility; others for countercurrent distribution or sialic acid assays (see below).

To determine total sialic acid, an electronic cell count was obtained on an aliquot of a suspension of washed red cells. Another aliquot (representing about 0.5 ml of packed cells) was used to prepare erythrocyte ghosts by the method of Dodge et al. [13]. Aliquots of the ghost suspension were made 0.05 N with sulfuric acid and heated at 80°C for 1 h [11]. Sialic acid was assayed on the neutralized supernatant solutions using the thiobarbituric acid method of Warren [14].

To determine that sialic acid released by neuraminidase, cell suspensions incubated with neuraminidase as described above were sampled at 60 and at 90 min, immediately chilled in ice and centrifuged to remove the cells. Sialic acid was assayed on a known aliquot of the supernatant solutions by Warren's method [14].

The phase system used was prepared as described by Walter [2] and had the following composition: 5% (w/w) Dextran T-500, lot No. 11648 (Pharmacia Fine Chemicals), 4% (w/w) poly(ethylene glycol) 6000 (recently renamed '8000' by Union Carbide) and 0.11 M sodium-phosphate buffer (pH 6.8). The phases were equilibrated at 3–5°C in separatory funnels and top and bottom phases were then separated.

Countercurrent distribution studies on normal or neuraminidase-treated rat red cells were carried out as previously described [15]. Since our countercurrent plates have 120 cavities [16], 40 or 50 transfers were carried out on normal and neuraminidase-treated erythrocytes in a given experiment simultaneously and without overlap by loading the two preparations on opposite sides of the plate. Conditions for the countercurrent distributions are given in the captions to Figs. 1 and 2.

At the end of a countercurrent distribution experiment, cell suspensions in different cavities along the extraction train were collected directly in centrifuge tubes. Saline (about 0.7 ml) was added to each tube to convert the two-phase system into homogeneous suspending medium. Analysis was then carried out as previously described: cells in certain cavities were washed and their electrophoretic mobilities were examined [8]; cells in other selected cavities were lysed in a known aliquot of 20 mosM sodium phosphate buffer (pH 7.2), the stroma were removed by centrifugation and the cell concentration was obtained in terms of the hemoglobin absorbance at 540 nm [15]. In ^{59}Fe isotope experiments, a known aliquot of the lysate was also counted in a scintillation well-counter [15].

The quantity of sialic acid released (either by neuraminidase or by sulfuric acid hydrolysis) is given in terms of fg/cell [12]. Electrophoretic mo-

bilities are in $\mu m/s$ per V per cm. Countercurrent distribution curves of erythrocytes are in terms of hemoglobin absorbance at 540 nm, and that of the isotopically labeled subpopulation of red cells of distinct age in counts per minute (cpm).

Sialic acid is the main charge-bearing group on mammalian red blood cells [11,17] and we investigated whether the treatment of rat erythrocytes with neuraminidase would (a) eliminate the surface charge-associated fractionation by partitioning feasible with untreated rat red cells [2,5] and, if not, (b) eliminate the age-related differences in the partition coefficient (K) of the cells.

In initial experiments, it was determined (Table I) that neuraminidase released approx. 5.9 fg/cell of sialic acid (corresponding to about 0.19 μmol/10^{10} cells), while the total sialic acid on the rat red cell (as determined after sulfuric acid hydrolysis of red cell stroma) is 8.2 fg/cell. The former value corresponds reasonably well with that of 6.8 fg/cell reported for neuraminidase-treated rat red cells by Tenforde [18]. The electrophoretic mobility of fresh rat red cells was found to be -1.25 $\mu m/s$ per V per cm, while that of the enzyme-treated cells was -0.80 $\mu m/s$ per V per cm. Neuraminidase thus releases approx. 70% of the total sialic acid * on the rat erythrocyte while reducing the electrophoretic mobility of the cells by 35%.

Normal (Fig. 1, top) and neuraminidase-treated (Fig. 1, bottom) rat erythrocytes were separately but simultaneously subjected to countercurrent distribution study in a dextran-poly(ethylene glycol) aqueous phase system, having an electrostatic potential difference between the phases and reflecting charge-associated surface properties

TABLE I

SIALIC ACID (NANA) RELEASED BY NEURAMINIDASE (*VIBRIO CHOLERAE*) FROM THE SURFACE OF RAT RED BLOOD CELLS AND THE TOTAL QUANTITY OF NANA ON RAT RED-CELL MEMBRANES (ASSAYED AFTER SULFURIC ACID HYDROLYSIS)

Electrophoretic mobilities ($\mu m/s$ per V per cm) were determined on rat red cells before neuraminidase treatment and at 60 and 90 min. They were, respectively, -1.25 ± 0.02; -0.82 ± 0.04; and -0.80 ± 0.02. Hence, the mobility reached a constant reduced level by 60 min while the NANA level showed a very small additional decrease between 60 and 90 min.

Rat No.	NANA released by neuraminidase (fg/cell)		Total NANA (fg/cell)
	at 60 min	at 90 min	
1070H	5.4	5.9	8.1
1071H	5.2	5.8	8.1
1072H	6.0	6.1	8.3

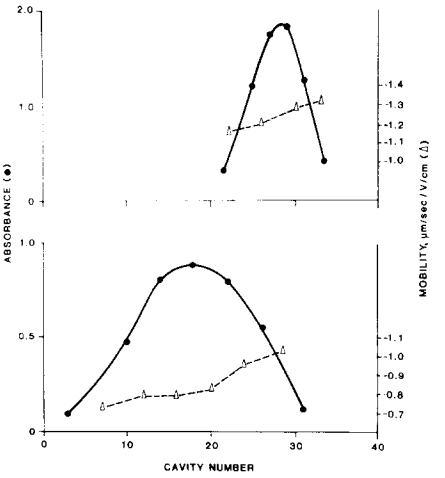

Fig. 1. Countercurrent distribution patterns for rat normal (top) and neuraminidase-treated (bottom) red blood cells. The treated cells were incubated with neuraminidase for 90 min at 37°C as described in the text. Countercurrent distribution was carried out at 3–5°C in a charged phase system composed of 5% (w/w) Dextran T-500, 4% (w/w) poly(ethylene glycol) 6000 (recently renamed '8000'), and 0.11 M sodium phosphate buffer (pH 6.8). 40 transfers were completed using a settling time of 6 min and a shaking time of 27.5 s. ●, gives the distribution in terms of hemoglobin absorbance at 540 nm in different cavities along the extraction train. Cells from selected cavities were washed three times with buffered saline and their electrophoretic mobilities were then determined. △ shows the mobilities of cells in different cavities in $\mu m/s$ per V per cm.

* To establish that no significant loss of membrane occurred during ghost preparation (an event which could give an erroneous value for the fraction of NANA/cell released by neuraminidase) the following experimental control was run: Ghosts were prepared and treated with neuraminidase. The supernatant solution was assayed for NANA (as described above). The enzyme-treated membranes were then subjected to sulfuric acid hydrolysis (again as above) and this supernatant solution assayed for NANA as well. The quantity of NANA released by neuraminidase under these conditions was 67% of the total NANA released. 33% of total NANA was released by sulfuric acid hydrolysis of neuraminidase-treated membranes (see also Table I).

[2,5–7]. The untreated red cells have a higher K (i.e., are further to the right) than the neuraminidase-treated erythrocytes, indicating that the removal of sialic acid (and the accompanying reduction in surface charge) reflects itself in the partitioning behavior of the cells. As found earlier [6], the increase in K of cells through the extraction train of rat normal erythrocytes (Fig. 1, top) is concomitant with an increase in cell electrophoretic mobility. Interesting is the fact (Fig. 1, bottom) that neuraminidase-treatment does not eliminate surface charge-associated differences between red cells, since an increase in K is still accompanied by an increase in cell mobility.

When rats are injected with [^{59}Fe]ferrous citrate and bled at different times thereafter, the isotopically labeled cells in the red cell population are those which correspond in age to the time elapsed between injection and bleeding. When erythrocytes from a series of rats containing labeled subpopulations of different but distinct age are subjected to countercurrent distribution one finds that the K of the labeled cell population decreases with increasing cell age [2,5]. In Fig. 2, we show the results of a typical experiment of this type undertaken, not with a labeled normal erythrocyte population as we have previously done [19], but rather with a labeled rat cell population (obtained 3 days after isotope injection) which was treated with neuraminidase prior to countercurrent distribution. Note that the labeled cell subpopulation (i.e., the 3-day-old erythrocytes) has a markedly higher mean K (i.e., the curve is to the right) than does the whole cell population. These results are analogous to those previously obtained with similarly labeled, untreated red cells [19]. As in Fig. 1, bottom, it is again apparent that the electrophoretic mobilities increase with increasing K.

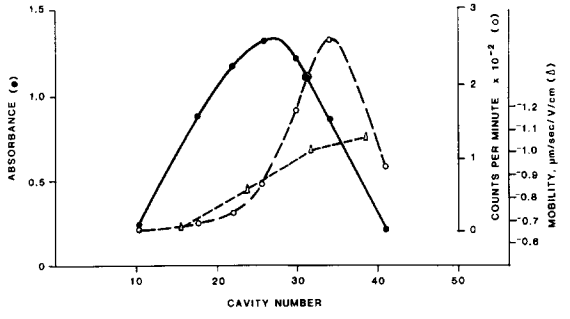

Fig. 2. Countercurrent distribution pattern of neuraminidase-treated red blood cells obtained from a rat injected with [^{59}Fe]ferrous citrate 3 days prior to bleeding. Conditions as in Fig. 1 except that 50 transfers were carried out. In addition to the cell distribution (●) and the cell electrophoretic mobilities (△), we also show the distribution of the isotopically labeled (3-day-old) red cells (○). For additional details see text.

We conclude that sialic acid susceptible to release by neuraminidase is not responsible for the difference in charge-associated properties of rat mature red cells of different age, since sialic acid removal by this enzyme has no effect on their relative partition coefficients. Since cell electrophoresis measures charge at the shear plane while partitioning appears also to reflect charge deeper in the membrane [7], we can further conclude, from the very fact that electrophoretic mobility increases with K (Fig. 2), that the charge difference at the outer surface of rat red cells of different ages is maintained after neuraminidase treatment.

The different K values and mobilities of rat erythrocytes of different ages may be due to ganglioside-bound sialic acid (which is not fully released by neuraminidase, [18]). Rats have a fairly large ganglioside content (about 6.3% of total lipid [18]). Human red cells, on the other hand, have a negligible ganglioside content and virtually all of their sialic acid residues are susceptible to cleavage by neuraminidase [11]. It is of interest that, as mentioned above, human erythrocytes do not change surface charge, as reflected by cell electrophoresis and partitioning, with age [8,9].

Experiments are in progress, using rat aldehyde-fixed erythrocytes from which lipids (including gangliosides) can be extracted, to determine whether ganglioside-linked sialic acid, or other charged groups, are responsible for the surface charge differences associated with rat erythrocytes of different ages.

This work was supported by the Medical Research Service of the Veterans Administration and by grant HL 24374 from the U.S. Public Health Service.

References

1 Albertsson, P.Å. and Baird, G.D. (1962) Exp. Cell Res. 28, 296–322

2 Walter, H. (1977) in Methods of Cell Separation (Catsimpoolas. N., ed.), Vol. 1, pp. 307–354, Plenum Press, New York
3 Johansson, G. (1970) Biochim. Biophys. Acta 221, 387–390
4 Reitherman, R., Flanagan, S.D. and Barondes, S.H. (1973) Biochim. Biophys. Acta 297, 193–202
5 Walter, H. (1982) in Cell Separation: Methods and Selected Applications (Pretlow, T.G. and Pretlow, T., eds.), Vol. 1, pp. 261–299, Academic Press, New York
6 Brooks, D.E., Seaman, G.V.F. and Walter, H. (1971) Nature New Biol. 234, 61–62
7 Walter, H., Tung, R., Jackson, L.J. and Seaman, G.V.F. (1972) Biochem. Biophys. Res. Commun. 48, 565–571
8 Walter, H., Krob, E.J., Tamblyn, C.H. and Seaman, G.V.F. (1980) Biochem. Biophys. Res. Commun. 97, 107–113
9 Walter, H., Krob, E.J. and Ascher, G.S. (1981) Biochim. Biophys. Acta 641, 202–215
10 Murphy, J.R. (1973) J. Lab. Clin. Med. 82, 334–341
11 Eylar, E.H., Madoff, M.A., Brody, O.V. and Oncley, J.L. (1962) J. Biol. Chem. 237, 1992–2000
12 Seaman, G.V.F., Knox, R.J., Nordt, F.J. and Regan, D.H. (1977) Blood 50, 1001–1011
13 Dodge, J.T., Mitchell, C. and Hanahan, D.J. (1963) Arch. Biochem. Biophys. 100, 119–130
14 Warren, L. (1959) J. Biol. Chem. 234, 1971–1975
15 Walter, H., Miller, A., Krob, E.J. and Ascher, G.S. (1971) Exp. Cell Res. 69, 416–424
16 Albertsson, P.Å. (1970) Sci. Tools 17, 53–57
17 Cook, G.M.W., Heard, D.H. and Seaman, G.V.F. (1961) Nature (London) 191, 44–47
18 Tenforde, T. (1970) Adv. Biol. Med. Sci. 13, 43–105
19 Walter, H. and Krob, E.J. (1978) Br. J. Haematol. 38, 43–55