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THE ROLE OF SIALIC ACID IN THE DETERMINATION OF SURVIVAL OF RABBIT ERYTHROCYTES IN THE CIRCULATION

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ABSTRACT

Evidence is presented to indicate a generalized role for the terminal sialic acid residues of circulating erythrocytes of rabbit. Neuraminidase is shown to remove only sialic acid from these erythrocytes. Neuraminidase-treated and intact rabbit erythrocytes have similar *in vitro* properties, except those of cellular charge and cellular adhesion in their sera. These properties include similar shape, osmotic fragility curve, autohemolysis at 37°, K^+ retention and pyruvate kinase activity. The D-glucose 6-phosphate dehydrogenase and the cholinesterase activities are higher on the neuraminidase-treated erythrocytes than on the intact ones. After injection into rabbits, the sialic acid-less erythrocytes tested, were promptly removed from the circulation; intact erythrocytes, previously incubated under the same conditions but without neuraminidase, were removed from the circulation after a significantly longer period.

INTRODUCTION

About 10% of the total proteins of the human erythrocyte membrane are glycoproteins[1]. Their carbohydrate moieties are antigenic determinants[2,3] and receptors for viruses and plant agglutinins[4]. The sialic acid residues are responsible for most of the negative charge at the cell surface[5] and are involved in the M and N group specificity[6].

Previous reports have established a generalized role for the terminal sialic acid residues of circulating glycoproteins[7,8]. With the exception of transferrin, all the glycoproteins devoid of sialic acid residues, injected into animals, were promptly removed by the liver under conditions where the intact proteins survived normally in the circulation. The rate of clearance has been shown to depend on the resultant exposure of terminal D-galactose residues on the nonreducing ends of the carbohydrate chains of the glycoproteins[8].

The purpose of the present report is to determine whether the terminal sialic acid residues of the erythrocyte membrane are involved in the biochemical phenomena of erythrocyte removal from the circulation. Thus, intact rabbit-erythrocyte pro-

perties are compared *in vitro* with those of sialic acid-less erythrocytes. A comparison is made between the survival rate of intact erythrocytes and sialic acid-less erythrocytes, after intravenous injection into rabbits.

EXPERIMENTAL

Material and methods. — *Vibrio cholerae* neuraminidase (E C 3.2.1.18) (500 units per ml) was obtained from Schwarz/Mann (Orangeburg, N.Y. 10962, U. S. A.). For each experiment, blood was drawn from the ear of a single rabbit, collected into heparinized tubes, and centrifuged immediately. The erythrocytes were washed three times, either in 5 vol. of isotonic sodium chloride before being incubated with neuraminidase, or in isotonic sodium chloride–phosphate buffer solution (pH 7.4) before preparation of the ghost erythrocyte. The washed erythrocytes were used within 25 h.

Removal of sialic acid from erythrocytes. — The packed red-blood cells were suspended in isotonic phosphate buffer (2 vol., pH 7.4, I 0.16). The total volume was accurately determined and an aliquot was removed for red cell count and hematocrit determination. Usually, the hematocrit level was near 26%. The red-cell blood suspension was incubated for 1 h at 37°, after addition of neuraminidase, with gentle shaking, and then immediately cooled to 0°. Some hemolysis always occured. The hemoglobin release did not exceed 1% as measured by absorption at 540 nm.

In the supernatant, sialic acid was determined by the Warren method[9], sugar content by orcinol–sulfuric acid[10], and total amino acid content by ninhydrin. The amino acids were qualitatively analyzed by chromatography, following the method of Stein and Moore[11] modified in our laboratory.

To eliminate the interference of hemoglobin in the determination of sialic acid and total sugars, the supernatant was evaporated to dryness *in vacuo* at 35–40°. Addition of absolute ethanol, followed by evaporation, and then addition of water, followed by centrifugation, removed the denatured hemoglobin[5]. Sialic acid and total sugar determinations were performed on a clear supernatant solution. The red cells were further washed three times with a sodium chloride solution to remove the neuraminidase. Control erythrocytes were treated in the same manner, but without neuraminidase.

Rabbit-erythrocyte ghosts. — The washed erythrocytes were suspended in a 5mM sodium dihydrogen phosphate–15mM disodium hydrogen phosphate hypotonic buffer (pH 7.4) and centrifuged at 10 000*g* for 1 h. The supernatant was decanted and this procedure was repeated five times. The resulting, pink ghosts were suspended in the hypotonic buffer just described (2 vol., pH 7.4) with *Vibrio cholerae* neuraminidase (25 units of neuraminidase, for ghost prepared with 1 ml of erythrocytes), and incubated for 1 h at 37°, with gentle shaking. The ghost suspension was centrifuged again and sialic acid was determined[9] in the clear supernatant. The results were compared with those obtained when these ghosts were hydrolyzed for 1 h at 80° with 0.05M sulfuric acid, and then submitted to a sialic acid determination.

In vitro *properties of intact and sialic acid-less erythrocytes of rabbit.* — Intact

and sialic acid-less erythrocytes of rabbit were examined under a microscope in 0.15M sodium chloride, in a 1mM sodium dihydrogen phosphate–3mM disodium hydrogen phosphate–137mM sodium chloride isotonic buffer (pH 7.4) and in their sera.

The osmotic fragility curve was established according to the method of Dacie[12] and the spontaneous autohemolysis at 37° was determined after 24 and 48 h. The rate of K^+ and Na^+ exchange was measured as described by Phillips and Morisson[13]. The Na^+ and K^+ concentration was determined with a flame photometer (Instrumentation Laboratory Inc., Lexington, Mass. 02173, U. S. A.). The cholinesterase (EC 3.1.1.7) kinetic was determined on hemolysates diluted to 1/10 in water. The enzymic activities were measured on a 50mM phosphate buffer solution (pH 7.2) with 0.25mM 5,5'-dithiobis(2-nitrobenzoic acid) as described by Elleman[14], and various concentrations ($S.10^{-6} = 9.65$; 19.34; 38.67; 77.37; 154.75; 309.5; 619; 1238; 2476; and 4952) of acetylthiocholine iodide (Boehringer, Mannheim, West Germany).

The pyruvate kinase (EC 2.7.1.40) activity was measured as described by Beisenherz[15] with a 0.16M triethanolamine buffer solution (pH 7.5), 0.5 mg per ml LDH, and 0.1M ADP (Boehringer).

The glucose 6-phosphate dehydrogenase (EC 1.1.1.49) activity was measured on a 0.05M tris(2-hydroxyethyl)amine–5mM ethylenediaminetetraacetate buffer solution at pH 7.6 with 10mM $NADP^+$ and 20mM D-glucose 6-phosphate[16] (Biotrol, Paris, France).

Survival time of ^{51}Cr-labeled, intact and sialic acid-less erythrocytes in the rabbit. — Intact and sialic acid-less erythrocytes were incubated for 30 min at 37° with 0.05 mCi/ml in a 0.15M sodium chloride solution of ^{51}Cr (Commissariat à l'Energie Atomique, 91, Gif sur Yvette, France). The ^{51}Cr-labeled erythrocytes were separated from the unreacted isotope by three washes in 0.15M sodium chloride. The resultant erythrocyte preparation had an activity of 12 μCi per ml of packed erythrocytes. Injections were made in the ear vein of male rabbits weighing from 1 to 1.5 kg. Samples of blood were taken at various intervals of time from the other ear, transferred into heparinized tubes, and the radioactivity was determined on a sodium iodide scintillation counter apparatus (Ames, New York, U. S. A.).

RESULTS

Table I shows the amount of sialic acid released from 1 ml of packed rabbit erythrocytes in relation to the quantity of neuraminidase. All membranous sialic acid is liberated when packed erythrocytes are incubated with 25 units of neuraminidase per ml. In these experimental conditions, no sialic acid is found on the corresponding erythrocyte-ghost preparations. Hemolysis always occured, to the same extent in the control erythrocytes incubated without neuraminidase. No significant difference could be found between the amino acid content of the supernatant fluid of the neuraminidase-treated erythrocytes and of the supernatant fluid of the control erythrocytes incubated in a medium without neuraminidase. The orcinol–sulfuric acid

reaction showed that both supernatant fluids did not contain any sugar. Therefore, we can confirm that sialic acid is the only membranous component liberated by the action of *Vibrio cholerae* neuraminidase on rabbit erythrocytes. Rabbit-erythrocytes ghosts, prepared from 1 ml of packed erythrocytes, contain 40–80 nmoles of sialic acid (15–25 μg). Hydrolysis of these ghosts with mineral acids and enzyme gave identical results (Table II). When examined under a microscope, intact and sialic acid-less erythrocytes had normal and comparable shape. The only difference was a much stronger tendency of the sialic acid-less erythrocytes to aggregate in their own sera by forming erythrocytes rouleau. The rate of spontaneous autohemolysis at 37° is lower than 5% at 48 h (Table III). The osmotic fragility curve (Fig. 1), the rate of K^+ and Na^+ exchange (Table IV), and the pyruvate kinase activity are similar

TABLE I

SIALIC ACID LIBERATED BY NEURAMINIDASE TREATMENT OF RABBIT ERYTHROCYTES

Rabbit No.	*Neuraminidase (units)*[a]	*Sialic acid liberated*[a]	
		μg	*μmoles*
718	0	0	0
719	0	0	0
706	0	0	0
706	10	9.2	0.029
706	12.5	12.4	0.040
706	25	14	0.045
709	12	25	0.080
720	10	12	0.038
721	13	24	0.077
719	12.5	10	0.032
718	12.5	13	0.045

[a]Per ml of packed erythrocytes. Each value is an average of two determinations.

TABLE II

SIALIC ACID CONTENT OF RABBIT ERYTHROCYTE

Sialic acid released (μg)[a]	*Rabbit No.*	
	719	721
From stroma		
by acid hydrolysis[b]	12	25
by enzyme hydrolysis[c]	13	24
From cells by enzyme hydrolysis[c]	14	25

[a]Released per ml of packed erythrocytes. [b]Hydrolysis of stroma in 0.05M sulfuric acid. [c]Hydrolysis with 25 units of neuraminidase per ml of packed erythrocytes.

TABLE III

SPONTANEOUS AUTOHEMOLYSIS AT 37° OF INTACT AND NEURAMINIDASE-TREATED RABBIT ERYTHROCYTES IN THEIR OWN SERA[a]

Rabbit erythrocytes	*Autohemolysis (%)*	
	24 h	48 h
Intact	0.33	4
After removal of sialic acid	0.25	2

[a]Each value is an average of two determinations.

TABLE IV

AMOUNT OF EXCHANGE OF K^+ FOR Na^+ OF INTACT AND NEURAMINIDASE-TREATED RABBIT ERYTHROCYTES[a]

Erythrocytes	*Interval of time (min) following erythrocytes incubation*		
	15	90	150
Intact			
K^+ (mEq/l)	75	69	65
Na^+ (mEq/l)	27	33	37
After removal of sialic acid			
K^+ (mEq/l)	72	70	68
Na^+ (mEq/l)	26	28	30

[a]The erythrocytes, prealably incubated in a phosphate isotonic buffer (pH 7.4) containing neuraminidase, or devoid of neuraminidase, were washed three times with the buffer. The amount of Na^+ and K^+ was determined at appropriate time. Each value is an average of two determinations.

TABLE V

GLUCOSE 6-PHOSPHATE DEHYDROGENASE, PYRUVATE KINASE, AND CHOLINESTERASE ACTIVITIES OF INTACT AND NEURAMINIDASE-TREATED RABBIT ERYTHROCYTES[a]

	Erythrocytes	
	Intact	*Neuraminidase-treated*
Pyruvate kinase activity (mu/ml)	30	29
Glucose 6-phosphate dehydrogenase activity (mu/ml)	159	318
Cholinesterase activity (mu/ml)	1363	1598
Cholinesterase kinetic (Hill constant)	1.7	1.7

[a]Each value is an average of two determinations.

TABLE VI

HALF-LIFE OF ^{51}Cr-LABELED INTACT ERYTHROCYTES AND SIALIC ACID-LESS ERYTHROCYTES

Rabbit No.	*Neuraminidase (units)*[a]	*Blood volume (liter)*	*Half-life of ^{51}Cr-labeled erythrocytes (days)*
706	0	0.14	9
709	0	0.13	9.5
718	0	0.09	11.5
719	0	0.085	10.5
720	0	0.100	10
721	0	0.09	11
706	10	0.14	2
709	12	0.13	1.9
718	12.5	0.09	0.5
719	12.5	0.085	0.28
720	10	0.100	0.31
721	13	0.09	0.5

[a]Per ml of packed erythrocytes.

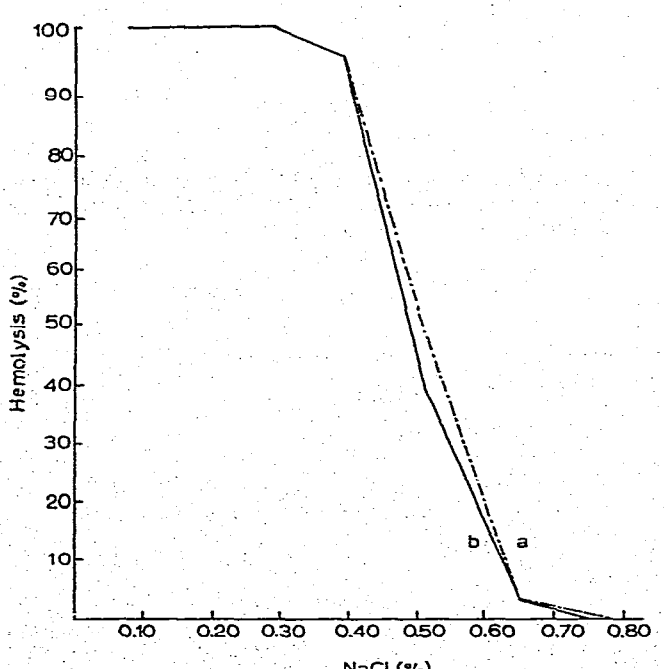

Fig. 1. Osmotic fragility curve of (a) intact rabbit erythrocytes, and (b) sialic acid-less rabbit erythrocytes.

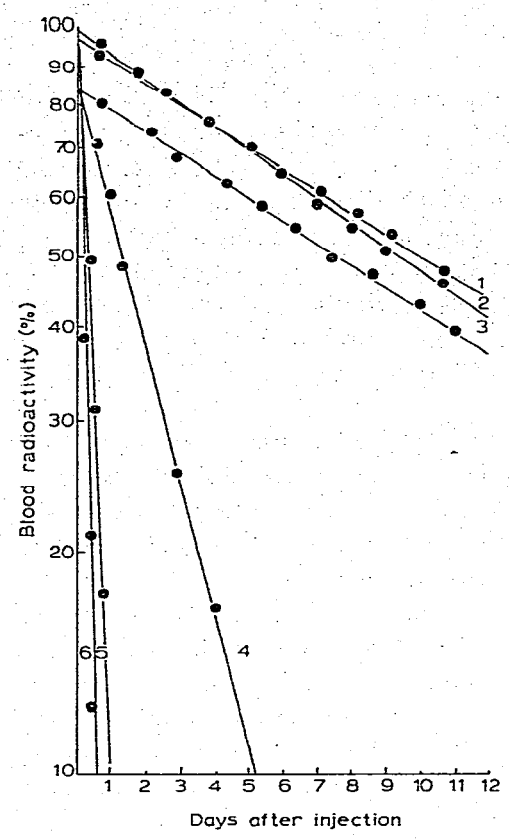

Fig. 2. Disappearance from the circulation of ^{51}Cr-labeled intact (1, 2, and 3) and sialic acid-less rabbit erythrocytes (4, 5, and 6). Each point is the average of values obtained from two animals.

(Table V). The glucose 6-phosphate dehydrogenase activity is twice as high on the sialic acid-less erythrocytes than on the intact erythrocytes. Cholinesterase activity is slightly higher on the sialic acid-less erythrocytes than on the intact ones. Cholinesterase kinetics of both erythrocytes were characterized by a Hill[17] constant of 1.7 (Table V); therefore, no conformational modification of the membrane could be detected on the sialic acid-less erythrocytes.

Table VI shows a significant difference between the half-life of ^{51}Cr-labeled intact erythrocytes and that of ^{51}Cr-labeled, sialic acid-less erythrocytes. The half-life of intact erythrocytes of rabbit is 9–11 days. The half-life of the sialic acid-less erythrocytes is longer in rabbits having a blood volume of 0.13–0.14 liter (half-life: 2 days) than in rabbits having a blood volume lower than 0.1 liter (half-life: 7–12 h). The half-life does not seem to correlate with the amount of sialic acid liberated (Table I and VI). It is important to note that the hematocrits remained the same during all the experiments for a given rabbit.

DISCUSSION

Sialic acid is the only component of the membrane liberated by action of *Vibrio cholerae* neuraminidase on rabbit erythrocytes. The total sialic acid content that can be liberated from rabbit erythrocytes is located at the outer surface of the membrane, since the amount liberated from the stroma by weak acid is equal to the amount released by enzyme from intact cells. Intact and sialic acid-less erythrocytes have the same life expectancy *in vitro*. No enzymic deficiency could be detected on the sialic acid-less erythrocytes. On the contrary, an activation of one membranous and of one cytoplasmic enzyme was noted; this activation was more important for the glucose 6-phosphate dehydrogenase than for the cholinesterase. The activation mechanism remains unclear.

No conformational modification of the erythrocyte membrane could be detected by studies of the cholinesterase kinetics: intact and sialic acid-less erythrocytes being characterized by comparable cholinesterase kinetics (Hill constant: 1.7).

No abnormal fragility could be detected *in vitro* on the sialic acid-less erythrocytes: the osmotic fragility curve, spontaneous autohemolysis at 37°, and K^+ retention were similar to those observed on the intact erythrocytes. Thus, we can affirm that sialic acid removal does not disturb the properties of rabbit erythrocytes, *in vitro*, except those of cellular charge[5] and cellular adhesion in their sera.

The most striking result is that sialic acid-less erythrocytes, when injected into their original animal, are characterized by a significantly reduced half-life, under conditions where control erythrocytes survived during a much longer period. The half-life of sialic acid-less erythrocyte does not seem to correlate with the amount of sialic acid liberated, but with the rabbit blood-volume.

Our observation should be compared with that of Ashwell *et al.*[7,8] who described a reduced half-life for most of the circulating glycoproteins devoid of sialic acid. Recently, a significant difference between the sialic acid content of young and old human erythrocytes has been shown; the old erythrocytes have a lower content of sialic acid[18].

In our observation, sialic acid removal by neuraminidase could amplify one of the processes of physiological aging of erythrocytes, and expose an underlying pattern of molecular recognition by the catabolic cells. Or, the removal of sialic acid reported here could be followed by an *in vivo* nonrecognition phenomena of the neuraminidase-treated erythrocytes. Baxley *et al.*[19] have shown that human, peripheral blood lymphocytes and tonsillar lymphocytes bind to neuraminidase-treated, human red-blood cells, but not to intact, human red-blood cells. The two hypotheses remain to be tested.

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