Role of Sialic Acid in Erythrocyte Survival

By John R. Durocher, Robert C. Payne, and Marcel E. Conrad

The role of membrane sialic acid in erythrocyte survival is unclear, although there is evidence for a reduction in sialic acid and surface charge in older erythrocytes. We reduced the surface charge of human, rat, and rabbit erythrocytes by removing sialic acid with neuraminidase. Reduction in sialic acid correlated with decreases in electrophoretic mobility and loss of PAS staining of membrane glycoproteins on polyacrylamide gels. No changes in ATP levels or deformability were found. $^{51}$Cr erythrocyte survivals in rats and rabbits showed rapid clearance of desialylated erythrocytes with sequestration by the liver. These results suggest that reduction in erythrocyte sialic acid is a mechanism of erythrocyte destruction and may be important in erythrocyte senescence.

The human erythrocyte has a net negative surface charge, and the bulk of this charge is due to ionized sialic acid.1 The role of sialic acid is unclear, but it is located on exterior glycoproteins2 and contains virus receptor sites3 and the M-N antigens.4 Some investigators have found a decreased surface charge5,6 and sialic acid content7 in older erythrocytes, and it is postulated that the decreased electronegativity may be related to senescence.8

Neuraminidase specifically cleaves the glycosidic linkages between sialic acid and mucopolysaccharides.9 This enzyme drastically reduces the surface charge of human erythrocytes and has varying effects on other mammalian erythrocytes.10 We have treated human, rat, and rabbit erythrocytes with neuraminidase and studied its effect on two biophysical parameters postulated to be important in cell survival—namely surface charge and deformability. Erythrocyte survival studies in rats and rabbits suggest the importance of sialic acid.

MATERIALS AND METHODS

Blood was collected in heparin from healthy human volunteers, rats (Walter Reed Carsworth Farm Strain), and rabbits (New Zealand white). The erythrocytes were washed three times in Hank’s balanced salt solution (HBSS), buffered at pH 7.3. After the final wash, 2 ml of cells were incubated in 9 ml of HBSS (pH 7.3) containing either 25 U/ml of Vibrio cholerae neuraminidase (Behring Diagnostics) or heat-inactivated neuraminidase at 37°C for 1 hr in an Eberbach metabolic shaker. The erythrocytes were then washed three times in HBSS before further study.

Deformability

Deformability was measured as filtration time through polycarbonate sieves.11 The erythrocytes were diluted to a 2% suspension in a 12 mM Tris-Ringers buffer (pH 7.4) to which 0.25% human serum albumin was added. When rabbit cells were filtered, rabbit albumin (Sigma) was substituted for human albumin. Our measure of deformability was the time required for 2 ml of the 2% erythrocyte suspension to pass through 3-micropore polycarbonate sieves under 10 cm water negative pressure. Morphology was assessed with a phase microscope. ATP measurements were
done by the method of Kornberg. MCV was determined from the microhematocrit and red blood cell counts on a Coulter Model B.

Electrophoretic Mobility

The surface charge was measured as electrophoretic mobility in a Zeiss cytopherometer fitted with platinum electrodes. Following incubation, the cells were washed with 150 mM NaCl and diluted to approximately 0.25% in a solution containing 4 volumes of 5% sorbitol and 1 volume of 67 mM Sorensen's buffer, pH 7.2. The electrophoretic mobility was measured in the frontal plane of a rectangular cuvette at 23°C with a current of 2 mA and 17.5 V/cm. The mobility of at least ten cells was recorded, and each erythrocyte was measured twice, with the second recording after reversal of polarity of the field in the cytopherometer.

Membrane Proteins and Sialic Acid

Ghosts were prepared from the erythrocytes by the method of Fairbanks et al. Protein determinations were done, and then the ghosts were diluted to 5 mg protein per ml and frozen at −70°C until further study. At the time of electrophoresis they were thawed and solubilized in 50 mM Na2CO3, 2% SDS, and 10% β-mercaptoethanol. Forty-five micrograms of protein were applied to the disc polyacrylamide gel described by Neville using a discontinuous buffer system. The gels were run at 25°C, initially at 0.5 mA per tube until the samples had entered the stacking gel, then at 1.5 mA per tube for 2 hr. The gels were stained with Coomassie blue. For PAS staining, larger amounts of protein were used and the staining procedure of Glossman and Neville used.

For sialic acid and protein determinations, ghosts were prepared by the method of Dodge et al. Sialic acid was hydrolyzed from the glycoproteins by incubating in 0.1 N H2SO4 at 80°C for 1 hr and measured with the thiobarbituric acid method of Warren. Protein was measured as previously described.

Erythrocyte Survival

For erythrocyte survival studies, blood was collected in ACD from rabbits and rats. The samples were incubated with 51Cr (5 μCi/ml) for 30 min at room temperature with frequent mixing. The samples were washed thrice with HBSS, then divided in two and incubated with heat-denatured or active neuraminidase at 37°C for 1 hr. The cells were washed thrice in HBSS, then diluted to approximately a 40% hematocrit. Aliquots were injected into either the dorsal penile vein of the rats or the marginal ear vein of rabbits. Samples (0.1 ml) were collected at 15, 30, 60, 120, 180, 240, and 300 min, then every other day until the CPM of the controls was less than half of the Tg value (extrapolated) after correction for decay of the radioisotope. Gamma counting was done in a Nuclear Chicago Model 4233 automatic gamma counter.

To determine which organs were responsible for removal of the erythrocytes, eight rats were killed 4 hr after receiving either neuraminidase-treated or control 51Cr-labeled erythrocytes. The liver, spleen, kidneys, and lungs were removed, weighed, and homogenized with a tissue grinder. Aliquots were removed for gamma counting and the ratio of CPM of treated to untreated calculated.

Serum-Sialic Acid Incubation

To determine if desialylated erythrocytes could regain membrane sialic acid in the presence of serum and free sialic acid, human, rat, and rabbit erythrocytes were washed six times in HBSS after incubation with neuraminidase. Aliquots were removed for measurement of surface charge and sialic acid content. The remainder of the cells were suspended to approximately a 20% hematocrit in a solution of 50% autologous serum and 50% HBSS, containing 200 U penicillin and 0.2 mg streptomycin per milliliter of suspension. To each flask, sialic acid (Type IV, Sigma) was added to a final concentration of 0.3 mg/ml. The suspensions were incubated for 24 hr at 37°C in a shaking water bath at 80 oscillations per minute. The cells were then washed thrice in 150 mM NaCl and used for measurements of surface charge and sialic acid content.
Acidified Serum Lysis
Erythrocytes were tested for sensitivity to in vitro complement lysis as described in Dacie and Lewis.\textsuperscript{20} Fresh serum was used with control and neuraminidase-treated human, rat, and rabbit erythrocytes. The results are expressed as per cent hemolysis, with complete hemolysis representing 100%.\textsuperscript{\textdegree}

Soybean Agglutinin
To determine if neuraminidase increased exposure of galactosyl groups, erythrocyte agglutination studies were done with purified soybean agglutinin (Miles). After desialylation, erythrocytes were suspended to a 4% concentration in phosphate-buffered saline (pH 7.4).\textsuperscript{21} Soybean agglutinin was serially diluted in the phosphate-buffered saline. For each test, 20\textmu l of the erythrocyte suspension was added to 20\textmu l of the soybean agglutinin in Cooke microtiter plates. The plates were agitated on a rotary table at 1-2 cps for 30 min at room temperature and agglutination measured visually as 0-4 plus.

RESULTS
Neuraminidase had a significant but different effect on the electrophoretic mobility of human, rat, and rabbit erythrocytes (Table I). The degree of reduction was most marked in human erythrocytes and least in rats. A correlation between the extent of sialic acid removal and change in electrophoretic mobility was evident. Neuraminidase did not affect the filtration time of erythrocytes. Furthermore, differences in MCV or ATP levels were not observed. Rabbit erythrocytes had prolonged and erratic filtration times when suspended in human serum albumin, and therefore rabbit serum albumin was substituted. Rabbit cells (which have a smaller MCV than human cells) had longer filtration times than human erythrocytes. No morphologic changes of the enzyme-treated erythrocytes were seen under phase microscopy.

Incubations of enzyme-treated human, rat, and rabbit erythrocytes in serum and sialic acid for 24 hr failed to increase the reduced surface charge or the reduced membrane sialic acid content (Table 2). The concentration of free sialic acid (300 \mu g/ml) represented an excess of free sialic acid to that removed by neuraminidase of greater than 12-, 17-, and 71-fold for human, rat, and rabbit erythrocytes, respectively.

| Table 1. Effect of Neuraminidase on Erythrocyte Surface Charge and Deformability |
|---------------------|--------|--------|--------|--------|--------|
|                     | Electrophoretic Mobility | Sialic Acid | Filtration Time | MCV    | ATP    |
| Human               | (13)   | (6)    | (10)   | (6)    | (5)    |
| Control             | 2.06 ± 0.01 | 140.0 ± 4.1 | 6.1 ± 0.2 | 93.2 ± 0.4 | 1.40 ± 0.27 |
| Neuraminidase       | 0.41 ± 0.02 | 20.5 ± 2.5  | 6.0 ± 0.4  | 93.3 ± 0.4 | 1.33 ± 0.16 |
| Rat                 | (17)   | (7)    | (5)    | (5)    | (5)    |
| Control             | 2.25 ± 0.01 | 116.0 ± 9.4 | 5.0 ± 0.4  | 60.0 ± 0.9 | 1.93 ± 0.17 |
| Neuraminidase       | 1.68 ± 0.02 | 32.7 ± 3.7  | 5.1 ± 0.2  | 59.3 ± 1.0 | 1.84 ± 0.19 |
| Rabbit              | (9)    | (7)    | (5)    | (5)    | (5)    |
| Control             | 0.87 ± 0.01 | 33.6 ± 2.8  | 10.6 ± 0.6 | 71.6 ± 1.4 | 1.52 ± 0.34 |
| Neuraminidase       | 0.44 ± 0.01 | 12.1 ± 2.4  | 9.5 ± 0.6  | 71.0 ± 1.8 | 1.74 ± 0.16 |

Units of measurement: Electrophoretic mobility, \mu sec/V/cm ± 1 SE; sialic acid, \mu g/ml erythrocytes ± 1 SE; filtration time, sec ± 1 SE; MCV, cu \mu ± 1 SE; ATP, \mu M/ml erythrocytes ± 1 SD.
Table 2. Effect of Incubation of Desialylated Erythrocytes With Sialic Acid

<table>
<thead>
<tr>
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<th>Initial</th>
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<tbody>
<tr>
<td></td>
<td>Electrophoretic Mobility (μ)</td>
<td>Sialic Acid (mg/ml)</td>
<td>Electrophoretic Mobility (μ)</td>
</tr>
<tr>
<td>Human</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.08 ± 0.02</td>
<td>145.2 ± 6.2</td>
<td>2.04 ± 0.02</td>
</tr>
<tr>
<td>Neuraminidase</td>
<td>0.45 ± 0.04</td>
<td>18.4 ± 3.1</td>
<td>0.39 ± 0.03</td>
</tr>
<tr>
<td>Rat</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.27 ± 0.02</td>
<td>110.3 ± 8.7</td>
<td>2.25 ± 0.02</td>
</tr>
<tr>
<td>Neuraminidase</td>
<td>1.73 ± 0.02</td>
<td>37.9 ± 4.9</td>
<td>1.68 ± 0.02</td>
</tr>
<tr>
<td>Rabbit</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.91 ± 0.02</td>
<td>36.4 ± 4.1</td>
<td>0.87 ± 0.02</td>
</tr>
<tr>
<td>Neuraminidase</td>
<td>0.43 ± 0.02</td>
<td>10.6 ± 3.1</td>
<td>0.40 ± 0.02</td>
</tr>
</tbody>
</table>

A markedly shortened $^{51}$Cr survival was found after rat and rabbit erythrocytes had been treated with neuraminidase (Fig. 1). In either case, the initial $T_{1/2}$ were under 12 hr, and over 75% of the erythrocytes were cleared from the circulating blood within 24 hr. At this point, the curves became biphasic, and $T_{1/2}$ of 10.25 and 7.25 days for the rats and rabbits, respectively. The control

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Fig. 1. Effect of neuraminidase treatment on erythrocyte $^{51}$Cr survivals; ○, controls; □, neuraminidase treated. Each point represents the mean for six animals.
Table 3. Localization of $^{51}$Cr-labeled Rat Erythrocytes Four Hours After Injection

<table>
<thead>
<tr>
<th>Ratio* (CPM) of neuraminidase to control</th>
<th>Liver</th>
<th>Spleen</th>
<th>Kidneys</th>
<th>Lung</th>
<th>Blood</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3.15 ± 0.74</td>
<td>0.97 ± 0.51</td>
<td>0.47 ± 0.19</td>
<td>0.39 ± 0.06</td>
<td>0.41 ± 0.12</td>
</tr>
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</table>

*Mean of 4 ± 1 SD.

rat erythrocytes survived normally, with a $T_{1/2}$ of 15.3 days. On the other hand, the control rabbit erythrocytes, while surviving longer than the enzyme-treated cells, had a $T_{1/2}$ of approximately 5 days. Neuraminidase did not remove any of the $^{51}$Cr from the erythrocytes because the cpm of injected samples did not vary by more than 4% between control and neuraminidase-treated erythrocytes. Furthermore, no free $^{51}$Cr was found in the enzyme incubation medium.

When rats were examined for localization of $^{51}$Cr-labeled erythrocytes 4 hr after injection (Table 3), the liver had a marked uptake of the labeled enzyme-treated cells. Reduced levels in the kidney, lungs, and blood were noted, and essentially the same amount of radioisotope was found in the spleen as in control animals.

When stained with Coomassie blue, polyacrylamide gels of erythrocyte membrane proteins before and after neuraminidase treatment (Fig. 2) failed to show any difference. Although the high-molecular-weight proteins of human, rat, and rabbit migrated the same distance, a distinct difference was noted between species in the major lower-molecular-weight bands (less than 100,000 daltons).

PAS staining of glycoproteins showed the intensity of bands roughly correlated with amount of sialic acid present (Fig. 3). Human ghosts showed the strongest reaction, and rabbit ghosts stained least. In all cases, neuraminidase led to a marked reduction in staining intensity.

The acidified serum lysis test (Table 4) of control and desialylated erythroy-
cytes showed a moderate increased sensitivity of human desialylated erythrocytes, but not of rabbit and rat red blood cells.

Erythrocyte agglutination studies (Table 5) with soybean agglutinin showed that rabbit red blood cells had increased agglutinability with the lectin compared with human and rat erythrocytes. In all cases, agglutination of desialylated erythrocytes was greater than the control cells.

DISCUSSION

Erythrocyte deformability is important in the removal and subsequent destruction of abnormal or senescent red blood cells from the circulation. Factors affecting deformability and thus impeding the erythrocyte's course through the microvasculature are correlated with shortened erythrocyte survival. These factors include alterations in surface area-to-volume ratio, intracellular inclusions, and intrinsic membrane stiffening. However, the importance in erythrocyte survival of another membrane biophysical parameter, i.e., surface charge, is unclear.

The evidence linking a reduction in net negative surface charge with aging of erythrocytes is indirect: a gradual reduction in surface charge of erythrocytes separated by density ("age") on phthalate esters, an increased surface charge of reticulocytes, and an increased reticulocyte membrane sialic acid content, the primary determinant of the human erythrocyte net negative surface charge. However, there are conflicting reports in the literature, and others have found a decreased or normal surface charge of immature erythrocytes.

### Table 4. Acidified Serum Lysis of Desialylated Erythrocytes

<table>
<thead>
<tr>
<th></th>
<th>Human (5)</th>
<th>Rat (5)</th>
<th>Rabbit (5)</th>
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<tbody>
<tr>
<td>Per cent hemolysis ± 1 SD</td>
<td>0</td>
<td>10.7 ± 1.7</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>Control</td>
<td>Control</td>
</tr>
<tr>
<td>Control</td>
<td>Neuraminidase</td>
<td>Neuraminidase</td>
<td>Neuraminidase</td>
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<td>Neuraminidase</td>
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</table>
Table 5. Agglutination Studies of Desialylated Erythrocytes With Soybean Agglutinin

<table>
<thead>
<tr>
<th>Concentration of Agglutinin</th>
<th>Human Control</th>
<th>Neuraminidase</th>
<th>Rat Control</th>
<th>Neuraminidase</th>
<th>Rabbit Control</th>
<th>Neuraminidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mg/ml</td>
<td>± 0</td>
<td>4+ 3+</td>
<td>± 0</td>
<td>4+ 3+</td>
<td>4+ 3+</td>
<td>4+ 3+</td>
</tr>
<tr>
<td>100 μg/ml</td>
<td>0 0</td>
<td>1+ 0</td>
<td>± 0</td>
<td>± 0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10 μg/ml</td>
<td>0 0</td>
<td>1+ 0</td>
<td>± 0</td>
<td>± 0</td>
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<td>0</td>
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<tr>
<td>1 μg/ml</td>
<td>0</td>
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</tr>
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</table>

and no increase in the sialic acid content of reticulocytes. Furthermore, a rare human blood group, En(a-), has a marked reduction in erythrocyte surface charge and sialic acid but normal erythrocyte survival.

Neuraminidase releases sialic acid from membrane glycoproteins by cleavage of an O-glycosidic linkage and reduces membrane surface charge. Two earlier reports of shortened erythrocyte survivals of rabbit and canine erythrocytes exposed to neuraminidase-producing viruses suggested the importance of sialic acid and surface charge in survival. Recently, intravenous neuraminidase has been found to shorten mouse erythrocyte and rat platelet survival. In the first studies, alterations in surface charge or sialic acid were not described, and in the second, the effect of neuraminidase on cells other than erythrocytes and platelets could not be eliminated.

To clarify the role of membrane sialic acid and surface charge in the destruction of erythrocytes in vivo, we used Vibrio cholerae neuraminidase, an enzyme without proteolytic or cytotoxic properties. Neuraminidase-treated erythrocytes had no changes in deformability, as measured by filtration properties through 3-micropore polycarbonate filters. With tumor cells, neuraminidase treatment actually increases deformability. A marked species difference was noted in erythrocyte electrophoretic mobility and sialic acid content, both before and after neuraminidase treatment. These differences in erythrocyte electrophoretic mobility are similar to those in the literature, although different buffers were used.

Although no change in erythrocyte deformability accompanied the marked reduction in surface charge following neuraminidase treatment, both rat and rabbit erythrocytes were rapidly removed from the circulation. The control rat erythrocytes survived normally, but the rabbit erythrocytes had a shortened survival. The prolonged in vitro incubation was probably injurious to the control rabbit erythrocytes, but the difference in survival between the control and neuraminidase-treated red blood cells was significant. The rapid clearance of neuraminidase-treated erythrocytes was probably not related to metabolism, since the erythrocyte ATP levels were normal, and other studies have shown no alterations in viability or metabolism of neuraminidase-treated cells. Although we found a moderate increase in complement sensitivity of desialylated human erythrocytes (as originally reported by Yachnin and Gardner), there was no increase in acid serum lysis of rabbit or rat erythrocytes. Therefore, an
increased complement sensitivity probably did not account for the rapid destruction of desialylated rat and rabbit erythrocytes.

After the initial rapid clearance of neuraminidase-treated erythrocytes, some remained in the circulation but had shorter survival than cells from the control incubations. None of the injected erythrocytes had normal surface charge, as measured by electrophoretic mobility. We found no change in electrophoretic mobility of erythrocytes incubated in serum and excess sialic acid for 24 hr, but the more normal survival of neuraminidase-treated erythrocytes may represent replacement of sialic acid on the erythrocyte membrane by a sialyltransferase.43,44,48

Most of the erythrocytes were cleared by the liver, and the defect induced by desialylation is probably not subtle. When serum glycoproteins are desialylated, they are rapidly sequestered in the liver.49 However, with removal of the newly exposed galactose moiety, the glycoprotein survival returns toward normal.50 Similarly, removal of the terminal sialic acid on erythrocyte exterior glycoproteins exposes a galactose moiety.51 The soybean agglutinin has a specificity for membrane galactosyl moieties.52 Our agglutination studies, as well as those of Nicolson,21 show an increased exposure of galactose after desialylation. En(a−) erythrocytes have a decreased sialic acid content but may also have a decreased galactose exposure.28 Whether this penultimate galactose is the signal to the reticuloendothelial system for removal of desialylated erythrocytes, both in senescence and following neuraminidase treatment, remains to be determined.

REFERENCES

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transplantable mouse tumor cell after treatment with neuraminidase. Proc Natl Acad Sci USA 69:942, 1972
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