

Role of sialic acid in survival of erythrocytes in the circulation: Interaction of neuraminidase-treated and untreated erythrocytes with spleen and liver at the cellular level

(agglutination/erythrocyte aging/Kupffer cells/neuraminic acids/reticuloendothelial system)

DAVID AMINOFF, WILLIAM F. VORDER BRUEGGE, WILLIAM C. BELL, KEITH SARPOLIS, AND REVIUS WILLIAMS

Departments of Internal Medicine (Simpson Memorial Institute) and Biological Chemistry, University of Michigan, Ann Arbor, Michigan 48109

Communicated by J. L. Oncley, February 7, 1977

ABSTRACT Sialidase (neuraminidase; acylneuraminyl hydrolase; EC 3.2.1.18)-treated erythrocytes obtained from different species are susceptible to rapid elimination from the circulation and are sequestered in the liver and spleen. The present studies were concerned with the mechanism of this clearance and how it may relate to the normal physiological process of removing senescent erythrocytes from the circulation. The results obtained indicate a preferential recognition of sialidase-treated as compared to normal erythrocytes by mononuclear spleen cells and Kupffer cells of the liver. This recognition manifests itself in both autologous and homologous systems by adhesion of the complementary cells in the form of rosettes, and as such could explain the removal of enzyme-treated erythrocytes from the circulation with their accumulation in liver and spleen. This phenomenon may represent a normal physiological mechanism for removal of senescent erythrocytes containing decreased sialic acid.

Sialic acid is an indigenous component of the erythrocytes of many different animal species (1-3). Treatment of these erythrocytes with sialidase (neuraminidase; acylneuraminyl hydrolase; EC 3.2.1.18) releases >95% of the sialic acid (4). Other investigators have shown (5-8) that the sialic acid content of circulating erythrocytes varies with the age of the erythrocyte. In older erythrocytes sialic acid content is 10-15% less than in younger erythrocytes (6).

Autologous transfusion of sialidase-treated erythrocytes results in their rapid disappearance from the circulation. This is true in several different species, such as rats (9, 10), rabbits (4, 10-13), dogs (4), goats (4), and human beings (14). Removal of only 10% of the total sialic acid present on the surface of erythrocytes is sufficient to eliminate these sialidase-treated erythrocytes from the circulation (4). Moreover, the ⁵¹Cr label used to tag the sialidase-treated erythrocytes accumulates in the liver (9, 10) and spleen (9).

The present studies were concerned with the process by which the sialidase-treated erythrocytes are sequestered in the liver and spleen. These studies were initiated with the concept that the physiological removal of senescent erythrocytes from the circulation may be analogous to the removal of sialidase-treated erythrocytes. The results of this study demonstrate that sialidase-treated erythrocytes preferentially adhere to Kupffer cells in the liver or to mononuclear cells in the spleen. These investigations correlate well with the histological findings of Schauer (personal communication).

MATERIALS AND METHODS

Materials. Sialidase was prepared from *Vibrio cholerae* as described (3). *Clostridium perfringens* sialidase was obtained commercially from Sigma Biochemicals (Type VI, lot 30C-8010) and *Cl. histolyticum* collagenase was purchased from

either Sigma Biochemicals (No. C-0130, lot 15C-0037) or Worthington Biochemicals (Class III, lot 46D084). Elemental iron particles 3-4 μ m in size were obtained from the G.A.F. Corp. ("carbonyl iron, S-F special"). Bovine serum albumin in crystalline form was obtained from Pentex Biochemicals (lot 18). Ficoll-Paque lymphocyte isolation medium was obtained from Pharmacia Fine Chemicals (lot C5P001).

Enzymatic Treatment of Erythrocytes. Blood was collected in EDTA (1.4 mg/ml) from 200- to 250-g male rats (Sprague-Dawley strain) maintained on standard laboratory chow and tap water ad lib. After anesthetic induction with ethyl ether, the blood was withdrawn by a sterile cardiac puncture. The erythrocytes were treated with sialidase as described (4). The erythrocytes were washed twice in 0.9% saline (pH 7.0) and the buffy coat was removed. After a final wash with 0.83% saline in 10 mM CaCl₂, the erythrocytes were incubated at 37° for 30 min with 0.2 unit* of sialidase per ml of packed cells or incubated for 30 min at 37° in CaCl₂/saline only. The erythrocytes were then washed three times in saline before further study, and the supernatants were assayed for free sialic acid by the quantitative thiobarbituric acid method (15). The erythrocytes were prepared before the liver or spleen cells because they were stable at 4° for at least 3 hr.

Isolation of Rat Liver Hepatocytes and Kupffer Cells. The method is based on that of Wincek *et al.* (16). A 200- to 250-g male rat (Sprague-Dawley strain) maintained on standard laboratory chow and tap water ad lib, was anesthetized with ethyl ether, and 200 USP units of sodium heparinate was injected into the portal vein. The abdominal vena cava was ligated above the right renal vein, and one cannula was inserted into the portal vein and another through the right atrium into the thoracic vena cava. The animals were sacrificed by cutting the diaphragmatic muscles just prior to cannulation of the thoracic vena cava. To remove residual blood, the liver was perfused via the portal vein with 400 ml of calcium-free Hanks' balanced salt solution at a flow rate of 20 ml/min. A suspension of colloidal iron (0.3 g/ml) in 2.2 M sucrose was slowly (over approximately 20 sec) injected into the portal vein cannula. Ten minutes were allowed for the injected iron to be phagocytized, followed by a 100-ml perfusion with calcium-free Hanks' solution to remove nonphagocytized iron. The collected perfusate was discarded. The liver was then perfused with 0.05% (wt/vol) collagenase (alone and with no hyaluronidase) in calcium-free Hanks' solution at a flow rate of 20 ml/min. This solution was perfused throughout the liver and recirculated for 8-10 min. During this interval the liver became amorphous, preventing

* In these studies, 1 unit is defined as the amount of enzyme that liberates 1 μ mol of *N*-acetylneuraminic acid from ovine submaxillary mucin in 1 min at 37° and pH 5.5.

Table 1. Rosette formation of sialidase-treated and untreated rat erythrocytes with rat Kupffer, hepatocytes, and spleen cells

Cell type	Erythrocytes	
	Treated	Untreated
Kupffer	20–25	2–5
Hepatocytes	1–2	1
Spleen	10–15	1

The values are given as percentages of viable liver or spleen cells that form rosettes, and represent the mean of at least three animals tested.

further perfusion. All perfusion solutions were oxygenated with 100% O₂ and maintained at 37°. The liver was excised, placed in 20 ml of calcium-free Hanks' solution containing 1% (wt/vol) bovine serum albumin at 4°, and gently teased until the liver cells were dissociated. The cell suspension was filtered through Dacron gauze and Kupffer cells were separated from the hepatocytes as described (16). The cells were used immediately. It is important to note that the liver cells are easily damaged by mechanical abrasion and so must be handled gently throughout the preparative procedure. The Kupffer cells represent 5–10% of the total liver cells in the suspension. The viability of Kupffer cells and hepatocytes was 80–90% on the basis of the trypan blue dye exclusion test. The appearance and viability of the cells was ascertained at the beginning of each experiment.

Isolation of Spleen Cells. A rat was injected with 200 USP units of sodium heparinate into the abdominal vena cava. After 3 min the spleen was excised, placed in 3 ml of calcium-free Hanks' solution containing bovine serum albumin at 4°, and minced with scissors. The minced spleen suspension was swirled for 3 min and then filtered through Dacron gauze. The filtrate was layered onto 4 ml of Ficoll-Paque and centrifuged at 2000 × *g* at room temperature for 15 min on a swing-out head. The spleen mononuclear cell layer was aspirated from the Ficoll-Hanks' solution interface, washed twice in calcium-free Hanks' solution containing bovine serum albumin at 4° to remove the Ficoll, and then used immediately as a 1% suspension (vol/vol) in the same solution. The viability of spleen cells was 80–90% on the basis of the trypan blue dye exclusion test.

Adhesion Assay. The method used was based on the formation of "rosettes" (17). To perform the test, one drop of a liver or spleen cell suspension (1% vol/vol) was added to one drop of an erythrocyte suspension (1% vol/vol) in a small plastic tube (75 × 7 mm) which was centrifuged at room temperature at 2000 × *g* using a swing-out head. The button of cells was then gently broken up by drawing it into a pasteur pipette. A wet slide was prepared for light microscope examination (×430) and a slide was stained with Wright's stain. A Spencer "Brightline" hemacytometer was used to quantitate observations. A liver or spleen cell with three or more erythrocytes on its surface was arbitrarily considered a "rosette."

RESULTS

Sialidase treatment removed >95% of the erythrocyte sialic acid. As shown in Table 1, these asialo-erythrocytes (both autologous and homologous) formed rosettes with a significant portion of the Kupffer and spleen cell populations, while very few rosettes formed around hepatocytes. Untreated erythrocytes, on the other hand, did not form significant numbers of rosettes with either hepatocytes or spleen cells; however, a small number of rosettes were observed around Kupffer cells.

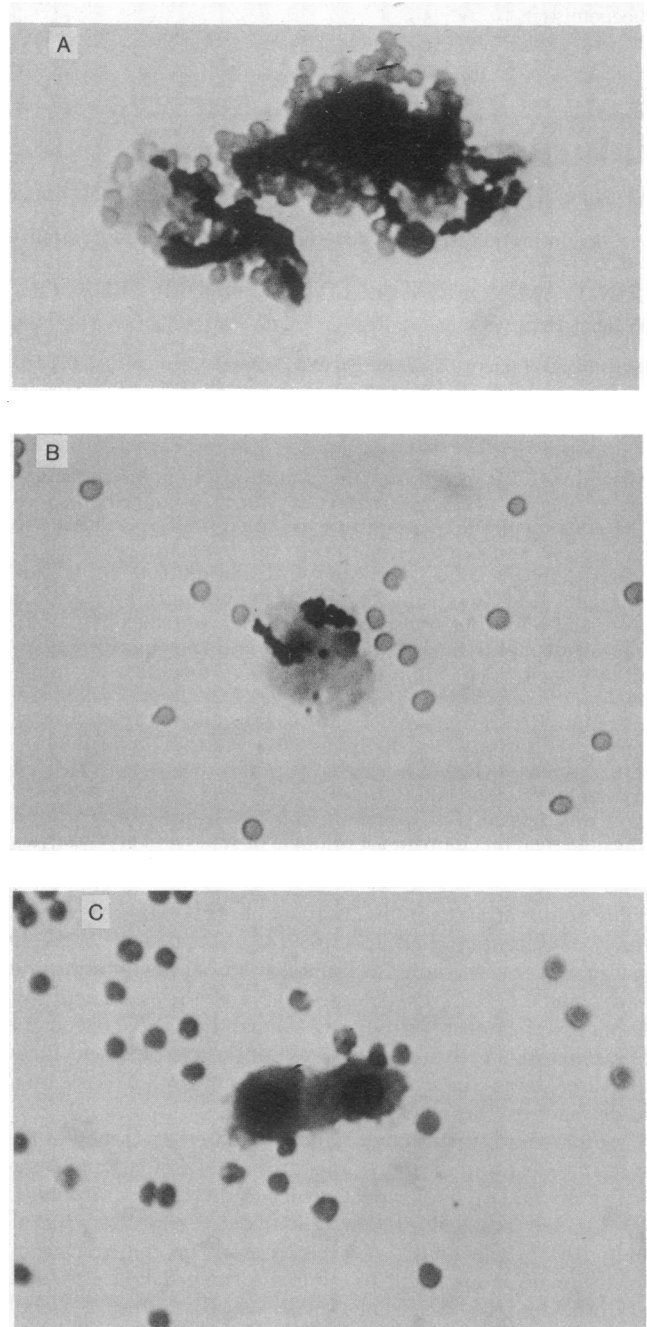


FIG. 1. Rat Kupffer cells, identified by partial phagocytosis of colloidal iron filings, form rosettes with sialidase-treated rat erythrocytes (A), but not with untreated erythrocytes (B). Rat hepatocytes with sialidase-treated rat erythrocytes did not form rosettes (C). (Wright's stain.)

Rosettes obtained from asialo-erythrocytes with Kupffer cells (Fig. 1A) and spleen cells (Fig. 2A) are characteristic microscopic clusters, in which a single layer of erythrocytes closely surround the central cell. In Fig. 1C, hepatocytes are shown among asialo-erythrocytes with no evidence of rosette formation. Likewise, rosette formation is not evident among the spleen cells and untreated erythrocytes (Fig. 2B). The Kupffer cells are shown to contain elemental iron in various stages of phagocytosis (Fig. 1A and B), while hepatocytes are iron-free (Fig. 1C). The erythrocytes maintain normal discocyte morphology throughout the experimental procedure.

Rosettes could be destroyed by the shearing forces of pasteur

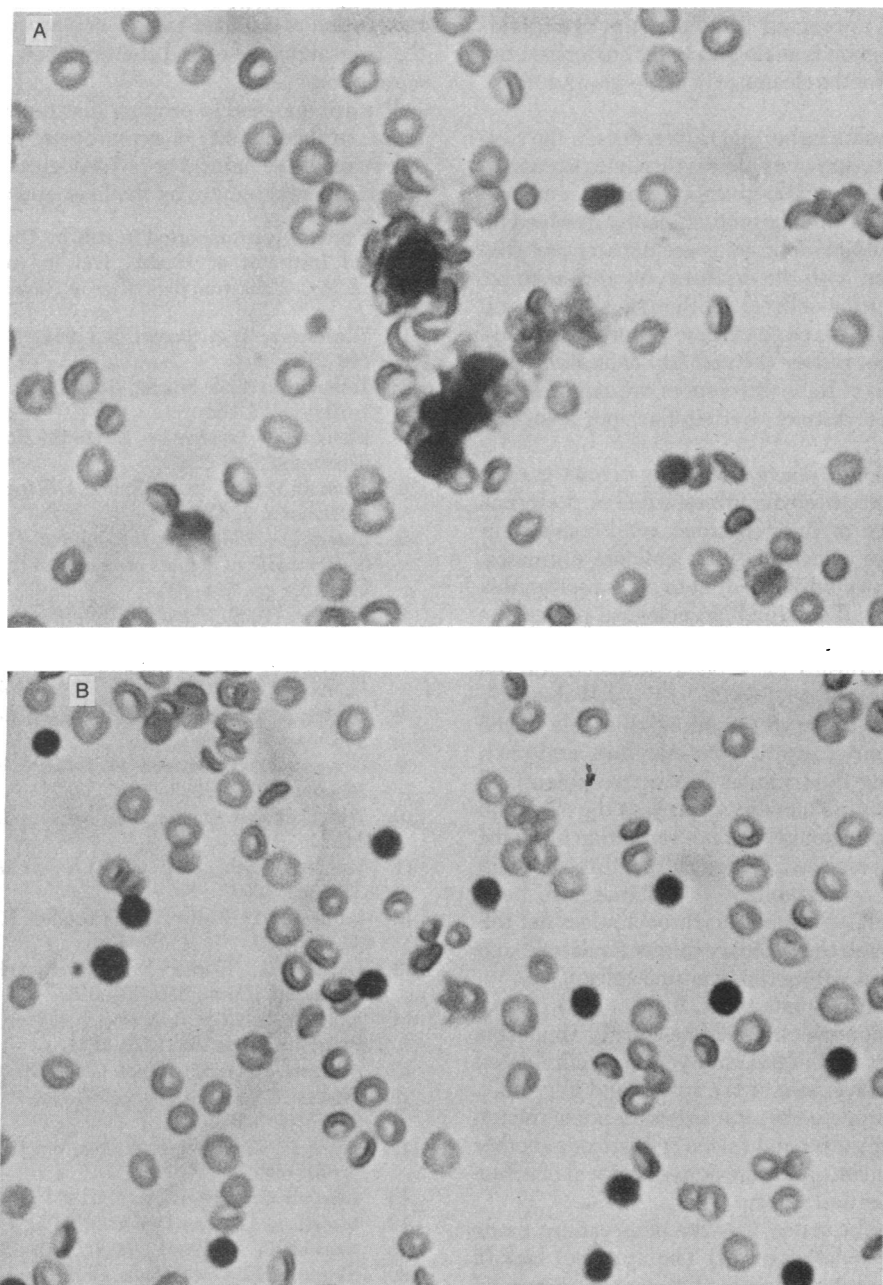


FIG. 2. Rat spleen mononuclear cells form rosettes with sialidase-treated rat erythrocytes (A), but not with untreated erythrocytes (B). (Wright's stain; $\times 140$.)

pipette trituration, with the number of rosettes decreasing significantly after two triturations. "Polyagglutination" of rat erythrocytes (that is, adhesion of one erythrocyte to another in the absence of liver or spleen cells or iron filings) occurred to a greater extent among the sialidase-treated erythrocytes. This erythrocyte "clumping" was almost entirely eliminated by gentle agitation of the rosette pellet.

Preliminary attempts at visual quantitation of the rosette formation indicated that sialidase-treated rat erythrocytes formed rosettes at least 10 times as often with either rat Kupffer or spleen cells than with rat hepatocytes (Table 1). Untreated erythrocytes almost entirely lacked the capacity to form rosettes, although a small number were present among the Kupffer cells. Erythrocytes treated with either *V. cholerae* or *Cl. perfringens* sialidase were indistinguishable with respect to rosette formation.

DISCUSSION

Liver cells to which iron filings are attached can be positively identified as Kupffer cells (16). The Kupffer cell fraction contains less than 1% hepatocytes (16), and the hepatocyte fraction contains less than 1% Kupffer cells (16). We suggest that the occasional rosettes observed with the hepatocyte cell population are attributable to these few contaminating Kupffer cells and that, in essence, the ability to form rosettes is specific for Kupffer cells. The spleen cell preparation consisted of a heterogeneous cell population. These data indicate that there is at least one cell type in the spleen that can form rosettes with asialo-erythrocytes. These results were obtained with autologous as well as homologous systems.

It is not apparent from these observations whether the absence of sialic acid or the exposure of the penultimate sugar is

responsible for the effect observed. We favor the latter, and believe that the phenomenon is analogous to that described by Ashwell and Morell (18) for the clearance of asialo-glycoproteins from the circulation.

There are, however, some important differences in the two systems. (a) The sequestering of asialo-erythrocytes occurs in the liver and spleen while the asialo-glycoproteins are removed by the liver only. (b) The liver cells predominantly involved in the sequestration of asialo-glycoproteins are hepatocytes (19, 20), while those involved with the erythrocytes appear to be predominantly the Kupffer cells. (c) Although oxidation of asialo-glycoproteins with galactose oxidase restores their "viability" (21), this does not restore the viability of asialo-erythrocytes (22). On the basis of these differences, we can presume that they represent two distinct, but similar, physiological systems.

The nature of the adhesion reaction resulting in rosettes may explain why the asialo-erythrocytes are retarded or preferentially held in the spleen or liver sinusoids until completely phagocytized. Likewise, since normal sialidase-untreated erythrocytes also react to a limited extent to give rosettes, this mechanism could explain the normal physiological process for removal of senescent erythrocytes in mammals.

Two principal mechanisms have been proposed for the "natural senescence" of erythrocytes (23): (a) a slow decrease in metabolic activity of the erythrocytes attributable to the progressive denaturation of erythrocyte enzymes, and (b) a progressively decreasing intracellular "reductive potential," resulting in the erythrocyte's inability to prevent the oxidation and denaturation of hemoglobin with the formation of Heinz bodies. These changes result in a "sluggish" erythrocyte with decreased membrane deformability. It has therefore been proposed that the normal senescent erythrocyte does not tolerate well passage through the capillary microcirculation, and is ultimately sequestered within the liver and spleen.

This schema seems inadequate to us. It involves a passive mechanism of elimination which should be equally applicable within the capillaries of any organ, and yet the accumulated data suggest direct involvement of the spleen and liver only. We would therefore propose that the adhesion phenomenon described here for the Kupffer and spleen cells affords another and more specific mechanism for the active removal of effete erythrocytes in the liver and spleen.

In summary, it may be stated that the observations made support the following conclusions. (a) The apparent lack of reactivity of the hepatocytes is an illustration of the nature of specificity of the reaction. (b) The Kupffer cells of liver and mononuclear cells of spleen preferentially react with sialidase-treated as compared to normal erythrocytes, be they of autologous or homologous origin. (c) The adhesion phenomenon observed in the formation of rosettes could explain the selective

retardation of sialidase-treated erythrocytes in the sinusoids of the liver and spleen, and thereby their elimination from the circulation.

We are tempted to propose that the consequence of desialation on the viability of erythrocytes in circulation is but an extension of the natural or physiological process of removal of senescent erythrocytes by the liver and spleen.

This project was supported in part by Grant HL 17881 from the National Institutes of Health, and in part by Contract ONR N00014-76-C-2069 from the Office of Naval Research.

1. Yamakawa, T. & Suzuki, S. (1951) *J. Biochem. (Tokyo)* **38**, 199-212.
2. Yamakawa, T. & Suzuki, S. (1953) *J. Biochem. (Tokyo)* **40**, 7-10.
3. Klenk, E. & Lauenstein, K. (1952) *Hoppe Seylers Z. Physiol. Chem.* **291**, 249-258.
4. Aminoff, D., Bell, W. C., Fulton, I. & Ingebrigtsen, N. (1976) *Am. J. Hematol.* **1**, 419-432.
5. Danon, D. (1966) *Eleventh Congress of the International Society of Hematology*. Papers presented at the plenary sessions of the Congress, pp. 394-405.
6. Yaari, A. (1969) *Blood* **33**, 159-163.
7. Greenwalt, T. J., Steane, E. A. & Pine, N. E. (1971) in *Glycoproteins of Blood Cells and Plasma*, eds. Jamieson, G. A. & Greenwalt, T. J. (Lippincott, Philadelphia) pp. 235-244.
8. Baxter, A. & Beeley, J. G. (1975) *Biochem. Soc. Trans.* **3**, 134-136.
9. Gregoriadis, G., Putman, D., Louis, L. & Neerunjun, D. (1974) *Biochem. J.* **140**, 323-330.
10. Durocher, J. R., Payne, R. C. & Conrad, M. E. (1974) *Blood* **45**, 11-20.
11. Jancik, J. & Schauer, R. (1974) *Hoppe Seylers Z. Physiol. Chem.* **355**, 395-400.
12. Gattegno, L., Bladier, D. & Cornillot, P. (1974) *Carbohydr. Res.* **34**, 361-369.
13. Gattegno, L., Bladier, D. & Cornillot, P. (1975) *Hoppe Seylers Z. Physiol. Chem.* **356**, 391-397.
14. Jancik, J., Schauer, R. & Streicher, H. J. (1975) *Hoppe Seylers Z. Physiol. Chem.* **356**, 1329-1331.
15. Aminoff, D. (1961) *J. Biol. Chem.* **81**, 384-391.
16. Wincek, T. J., Hupka, A. L. & Sweat, F. W. (1975) *J. Biol. Chem.* **250**, 8863-8873.
17. Brain, P., Gordon, J. & Willets, W. (1970) *Clin. Exp. Immunol.* **6**, 681-688.
18. Ashwell, G. & Morell, A. G. (1974) *Adv. Enzymol.* **41**, 99-128.
19. Morell, A. G., Van Den Hamer, C. J. A., Scheinberg, I. H. & Ashwell, G. A. (1966) *J. Biol. Chem.* **241**, 3745-3749.
20. Morell, A. G., Gregoriadis, D., Scheinberg, I. H., Hickman, J. & Ashwell, G. A. (1971) *J. Biol. Chem.* **246**, 1461-1467.
21. Morell, A. G., Irvine, R. A., Sternlieb, I., Scheinberg, I. H. & Ashwell, G. A. (1968) *J. Biol. Chem.* **243**, 155-159.
22. Bell, W. C., Williams, R. & Aminoff, D. (1976) *Fed. Proc.* **35**, 1432.
23. Pranker, J. A. J. (1969) *Ser. Hematol.* **2**, 52.