

Adhesion and erythrophagocytosis of human senescent erythrocytes by autologous monocytes and their inhibition by β -galactosyl derivatives

(erythrocyte aging/erythrocyte senescence factor/sialoglycoconjugates).

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ABSTRACT Senescent human erythrocytes (RBC) are able to adhere to and be phagocytized by autologous monocytes *in vitro* to a greater extent than are young RBC. This adhesion and erythrophagocytosis of senescent RBC is inhibited by D-galactose, N-acetyl-D-galactosamine, their corresponding derivatives of bovine serum albumin, and lactose. On the other hand, D-glucose, D-mannose, L-fucose, N-acetyl-D-glucosamine, and their corresponding derivatives of bovine serum albumin are noninhibiting. The glycopeptides released by tryptic digestion of senescent RBC and purified on immobilized peanut agglutinin are the most effective inhibitors of both RBC adhesion and phagocytosis by autologous monocytes obtained from peripheral blood.

There is a difference in sialic acid content between young and senescent erythrocytes (RBC).[§] This has suggested the possibility that *in vivo* senescence involves desialation of RBC with their subsequent sequestration from circulation (1, 33). The hypothesis has been supported by the observations that asialo-RBC (i) were rapidly sequestered from circulation (2-8) and (ii) readily gave rosettes with Kupffer cells and spleen monocytes in the absence of any additional serum factors (9, 10). This postulated a lectin-like interaction between asialo-RBC and, indirectly, between senescent RBC and macrophages. Many investigators demonstrated that this interaction could be inhibited by simple sugars and oligosaccharides containing galactosyl residues (11-13). Further credence was given to this hypothesis with the isolation of a sialic acid-free glycopeptide from senescent RBC that was able to bind to spleen monocytes (14). Treatment of this glycopeptide with β -galactosidase destroyed this property (14).

An independent line of investigation demonstrated that senescent RBC have more bound IgG molecules compared to young RBC (15-17). The specificity of this IgG was characterized as anti-galactosyl (18). That raised the possibility that galactosyl residues are involved in the detection and sequestration of senescent RBC by autologous macrophages and that this might be mediated by both IgG and lectin-like receptors on the macrophages (19).

In this study we demonstrate the ability to specifically inhibit the adhesion to and erythrophagocytosis of human senescent RBC by autologous blood monocytes. The most active inhibitor detected to date is a glycopeptide obtained from a tryptic digest of human senescent RBC (14). A preliminary report of these studies already has appeared.[¶]

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MATERIALS AND METHODS

Materials. Human blood, obtained from healthy donors of the Seine Saint Denis Blood Transfusion Center, was collected by venipuncture into heparinized tubes and processed within 2 hr.

$\text{Na}_2^{51}\text{CrO}_4$ was purchased from New England Nuclear; D-galactose, from Carlo-Erba (Milan, Italy); D-glucose, L-fucose, D-mannose, and melibiose, from Merck; GlcNAc, GalNAc, bovine serum albumin (BSA), *p*-nitro-phenyl derivatives of β -D-galactose, β -D-GalNAc, α -D-mannose, α -L-fucose and β -D-GlcNAc, from Sigma; and peanut agglutinin (PNA) immobilized on beaded agarose (1.5 mg of lectin per ml), from Sigma.

Fractionation of RBC According to Their Biological Age. Senescent human RBC were separated from young RBC on the basis of differences in their density (20). Briefly, whole blood was centrifuged at $2000 \times g$ for 120 min at 15°C; plasma and buffy coat were removed and discarded. The top 5% and the bottom 5% layers of RBC were collected by aspiration, and the effectiveness of this age-dependent fractionation was checked by measurement of the pyruvate kinase activity in the two fractions. The specific activity was 2.1 ± 0.5 times greater in the RBC from the top (young RBC) than in the bottom fraction (senescent RBC).

RBC Labeling. Young and senescent RBC were washed three times with isotonic phosphate-buffered saline [NaCl (8.7 g/liter)/ $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ (2.76 g/liter)/ $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ (2.76 g/liter), pH 7.4] and then incubated with an equal volume of $\text{Na}_2^{51}\text{CrO}_4$ in saline (1 mCi/ml; 1 Ci = 37 GBq) for 30 min at 37°C. After three washings with phosphate-buffered saline, the cells were suspended in Hanks' balanced salt solution and adjusted to 1.5×10^8 cells per ml.

Isolation of Tryptic Glycopeptides (TP). TP specifically adsorbed and eluted from immobilized PNA (14) (TP-PNA⁺) were prepared from human RBC separated according to their age (20). The young and senescent RBC were washed three times with phosphate-buffered saline (pH 7.4). TP were prepared by incubating young and senescent RBC in a solution of trypsin (200 units/ml) in phosphate-buffered saline at pH 8 for 1 hr at 37°C. After trypsin treatment, the supernatant was extracted with 90% phenol. The aqueous phase was treated with diethyl ether to remove the remaining

Abbreviations: PNA, peanut agglutinin; RBC, erythrocyte(s); TP, tryptic peptides; TP-PNA⁺, TP specifically adsorbed and eluted from peanut agglutinin; BSA, bovine serum albumin.

[§]We have chosen to use the term senescent RBC in lieu of densest. This choice was made because by most criteria the densest population of RBC do show the greatest number of time-dependent changes.

[¶]Gattegno, L., Vaysse, J., Bladier, D. & Aminoff, D., International Lectin Meeting, Sept. 3-8, 1984, Poznan, Poland.

phenol and then dried under vacuum at 37°C. The dry residue was dissolved in a minimal volume of distilled water, and the solution was desalted by gel filtration. In some experiments the TP were labeled at this stage with galactose oxidase and tritiated borohydride (21). The TP fraction then was passed through a PNA-agarose column (0.7 × 19 cm) preequilibrated with phosphate-buffered saline (pH 7.4). After the unbound material was washed with phosphate-buffered saline (pH 7.4), elution of TP-PNA⁺ was achieved with 20 ml of a 0.2 M lactose solution in phosphate-buffered saline (pH 7.4) at a 10 ml/hr flow rate. After removal of salts and lactose by gel filtration, the TP-PNA⁺ solution was dried under vacuum, and the dry residue was dissolved in 1 ml of distilled water. When labeled TP were processed, the radioactivity of the applied TP and of the collected TP-PNA⁺ was determined. The correlation between the tritium incorporated and the number of galactosyl + galactosaminyl residues oxidized with galactose oxidase was determined with standard solutions of D-galactose after correction for the acid-stable tritium (22). An average of 1.32 ± 0.11 dpm was found to be incorporated per nmol of galactose (*n* = 5).

Monocyte Isolation. Mononuclear cells were isolated from peripheral blood by centrifugation over Ficoll/Hypaque as described by Boyum (23). Of the collected cells, 3 × 10⁷ were suspended in 2 ml of RPMI 1640 medium supplemented with 20% human AB serum and were incubated in Petri dishes for 90 min at 37°C in humidified air containing 5% CO₂. Nonadherent cells were removed by washing four times with 2 ml of RPMI medium, and the adherent cells were collected with a rubber policeman and suspended in RPMI medium. Of these nucleated cells, 95% were identified as monocytes, either by staining with acridin or by phagocytosis of latex particles.

Phagocytosis Assay Using ⁵¹Cr-labeled RBC. Monocytes (10⁵) suspended in 50 μl of serum-free RPMI medium were allowed to adhere to Falcon culture tubes (12 × 75 mm) for 30 min at 37°C in humidified air containing 5% CO₂. To these were added 2 × 10⁶ ⁵¹Cr-labeled autologous young or senescent RBC. Incubation was carried out for 75 min at 37°C in humidified air containing 5% CO₂. Nonadherent RBC were removed by washing twice with 2 ml of Hanks' balanced salt solution. Finally, attached but not internalized RBC were lysed by addition of 2 ml of 140 mM NH₄Cl/Tris buffer, pH 7.4, for 3 min at 20°C.

The radioactivity of the lysate (RL) and that of the washed monocyte layer (RM), which contains the ingested cells, was counted. Total radioactivity of the ⁵¹Cr-labeled RBC added for each assay also was determined (RT). The phagocytic activity was expressed as the percentage of the total added radioactivity that remained associated with the monocytes after the lytic step—namely,

$$\% \text{ phagocytosis} = \frac{\text{RM} \times 100}{\text{RT}}$$

The percentage of adhering but not internalized RBC also was determined:

$$\% \text{ adhesion} = \frac{\text{RL} \times 100}{\text{RT}}$$

All experiments were performed in quadruplicate or quintuplicate. Results were expressed as the mean value ± SEM of several experiments performed with RBC and autologous monocytes from different donors.

In order to investigate the effect of glycosyl residues on the phagocytosis of RBC, the monocytes were preincubated for 3 min with monosaccharides, disaccharides, neoglycoproteins, or TP-PNA⁺ before the addition of ⁵¹Cr-labeled RBC. Different concentrations (ranging from 4 to 25 mM) of the following inhibitors were tested: D-galactose, D-glucose, D-mannose, L-fucose, GlcNAc, GalNAc, lactose, and melibiose. Synthetic glycoproteins prepared by coupling

monosaccharides with BSA—1 mol of BSA containing 25 mol of D-galactose, 27 mol of D-GalNAc, 15 mol of L-fucose, or 20 mol of D-mannose (24)—were used at concentrations ranging from 3 to 25 μM. The inhibitory activity was expressed as the percentage of inhibition calculated from the following equations:

$$\% \text{ inhibition of phagocytosis} = \frac{P - P_I}{P} \times 100,$$

in which P is the percentage of phagocytosis without added inhibitor and P_I is the percentage of phagocytosis with inhibitor; and

$$\% \text{ inhibition of adhesion} = \frac{A - A_I}{A} \times 100,$$

in which A is the percentage of adhesion without inhibitor and A_I is the percentage of adhesion with inhibitor.

In some experiments to test whether a direct interaction between the sugar and the RBC may occur in the second step of the assay, senescent RBC first were incubated for 60 min at 37°C with lactose or galactose at a 25 mM final concentration, then washed, labeled, and finally added to the monocytes. Controls were performed by incubating senescent RBC in buffer alone.

Visual Evaluation of Phagocytosis. In some experiments, mononuclear cells were adjusted to a concentration of 2 × 10⁶ cells per ml of RPMI 1640 medium supplemented with 20% AB serum. Aliquots (1 ml) were layered onto 16-mm flat-bottomed wells (24 wells per plate, Costar, Cambridge, MA) containing 12 × 12 mm sterile glass coverslips and were incubated for 2 hr at 37°C in humidified air with 5% CO₂. Nonadherent cells were removed by four washings with RPMI 1640 medium. The adherent monocytes were cultured in the medium containing 2 μmol of glutamine, 100 units of penicillin, and 100 μg of streptomycin per ml and 20% AB serum. After a 24-hr incubation, the medium was gently aspirated, and 0.5 ml of RPMI 1640 medium with or without inhibitor was added to each well. After 3 min, a 0.1-ml suspension of young or senescent RBC suspension (10⁷ cells) was added in RPMI 1640 medium. Incubation was continued for 2 hr at 37°C under humidified air containing 5% CO₂. The glass coverslips were washed twice with Hanks' medium to remove the unbound RBC, and then the bound cells were fixed with methanol, dried, and stained with May-Grunwald-Giemsa reagent. Random fields of these coverslips were examined microscopically at ×1000 magnification under oil, and at least 500 monocytes were scored for binding and ingestion of RBC. All of the experiments were run in duplicate for each blood donor, and the results were expressed as the average of several different donors. The percentage of monocytes that ingested one or more RBC, and the percentage of monocytes that bound one or more RBC were determined; these two indices provide information with regard to the extent of adhesion and phagocytosis.

RESULTS

Phagocytosis of senescent RBC by autologous monocytes was significantly higher than that of young RBC in both the ⁵¹Cr and visual assays (Table 1). Moreover, human senescent RBC adhered to the monocytes in significantly greater numbers than did young RBC (Table 1).

The sugars most commonly found as glycoconjugates on the RBC surface then were examined for their ability to inhibit both the adhesion and phagocytosis of the senescent RBC. Under comparable experimental conditions, no statistically significant inhibition of adhesion or phagocytosis was observed with D-glucose, D-mannose, L-fucose and GlcNAc. On the other hand, D-galactose and GalNAc inhibited both

Table 1. Adhesion and phagocytosis of human RBC by autologous monocytes as determined by the ^{51}Cr assay and visual microscopic assay

RBC assay	% of RBC involved	
	Adhesion	Phagocytosis
^{51}Cr		
Y-RBC	4.00 \pm 0.50	1.63 \pm 0.20
S-RBC	8.45 \pm 0.80	5.71 \pm 0.75
<i>P</i> of the Y-S difference	<0.001	<0.001
% of monocytes involved		
Visual		
Y-RBC	2.00 \pm 0.31	2.00 \pm 0.30
S-RBC	6.00 \pm 1.20	5.00 \pm 0.50
<i>P</i> of the Y-S difference	<0.01	<0.001

Results in each assay are expressed as the mean \pm SEM of 22 coupled experiments. Y, young; S, senescent.

the adhesion and phagocytosis of human senescent RBC by autologous monocytes (Table 2).

With D-galactose, GalNAc, or lactose, increasing concentrations resulted in an increase of inhibition of phagocytosis. For sugar concentrations up to 20 mM, the curves were almost linear and their slopes were not significantly different as assessed by statistical analysis (Fig. 1 A and B).

The results from six experiments indicate that adhesion and phagocytosis of senescent RBC previously incubated in buffer, either with or without galactose, are not significantly different. This suggests that the inhibitory effects of these carbohydrates are not mediated by an interaction with the RBC but with the monocytes.

No significant inhibition was observed with β -D-GlcNAc-BSA, α -D-Man-BSA, or α -L-Fuc-BSA. In contrast, β -D-Gal-BSA and β -D-GalNAc-BSA are able to inhibit significantly both the adhesion and phagocytosis of senescent erythrocytes by monocytes (Table 2). Increased concentrations of these two neoglycoproteins also led to an increase of inhibition (Fig. 1 C and D) that was greater than with the free sugars and was effective at micromolar concentrations (Table 2; Fig. 1 C and D).

Both the adhesion and erythrophagocytosis of senescent RBC were inhibited by TP-PNA⁺. Most significantly, tryptic glycopeptides prepared from 3×10^6 RBC showed almost no

Table 2. Inhibition of adhesion and phagocytosis of human senescent RBC by autologous monocytes as determined by the ^{51}Cr assay in the presence of monosaccharides and disaccharides, and monosaccharide derivatives of BSA

	Concentration, mM	% inhibition, mean \pm SEM		No. of experiments
		Adhesion	Phagocytosis	
Monosaccharides and disaccharides				
L-Fucose	25	0	0	6
D-Galactose	23	50 \pm 4	56 \pm 6	4
D-Glucose	23	0	0	4
D-Mannose	25	11 \pm 11	12 \pm 12	4
GalNAc	23	69 \pm 9	63 \pm 25	4
GlcNAc	25	23 \pm 30	14 \pm 14	5
Lactose	23	54 \pm 15	61 \pm 22	3
Melibiose	25	42 \pm 9	27 \pm 20	4
Monosaccharide derivatives of BSA				
β -D-Galactose	19	49 \pm 16	64 \pm 14	4
β -GalNAc	19	49 \pm 8	74 \pm 14	5
β -GlcNAc	23	0	4 \pm 4	5
α -D-Mannose	23	0	0	4
α -L-Fucose	25	0	0	3

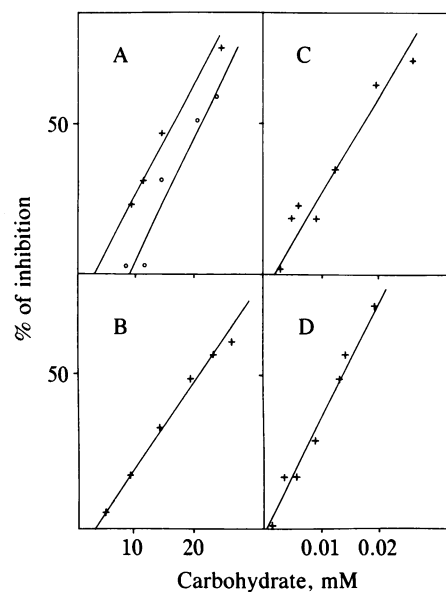


FIG. 1. Inhibition of phagocytosis of human senescent RBC by autologous monocytes at various concentrations of carbohydrate. (A) D-Galactose (○) and lactose (+). (B) GalNAc. (C) D-Gal-BSA. (D) D-GalNAc-BSA.

inhibitory activity if isolated from young RBC, whereas a 66% inhibition was observed when they were prepared from senescent RBC (Fig. 2, Table 3).

No statistically significant age-related difference was detected for the ^3H -labeling of TP from young and senescent RBC: $1.96 \pm 0.52 \times 10^6$ cpm per cell ($n = 5$) as compared with $2.08 \pm 0.39 \times 10^6$ ($n = 5$), respectively. Subsequent to affinity chromatography on PNA, however, the percentage of bound radioactivity was lower for TP-PNA⁺ from young RBC (0.6%) compared with that from senescent RBC (4.4%). From these results, the number of galactosyl/galactosaminyl residues oxidizable with galactose oxidase could be computed (22)—namely, $1.12 \times 10^4 \pm 0.45$ (residues) per young RBC (n

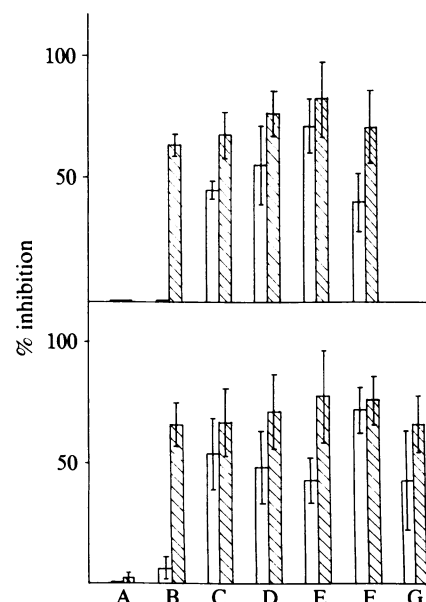


FIG. 2. Inhibition of adhesion (Upper) and phagocytosis (Lower) of human senescent RBC by autologous monocytes in the presence of TP-PNA⁺ isolated from young RBC □ or senescent RBC ▨. The number of RBC used to prepare TP-PNA⁺ were as follows: $<2 \times 10^6$ (bars A), 3×10^6 (bars B), 7×10^6 (bars C), 30×10^6 (bars D), 2.5×10^8 (bars E), 7.5×10^8 (bars F), 2×10^9 (bars G).

Table 3. Inhibition of adhesion and phagocytosis of human senescent RBC by autologous monocytes in the presence of TP-PNA⁺ isolated from young (Y) and senescent (S) RBC (⁵¹Cr assay)

Source of TP-PNA ⁺	³ H]Galactose, nM	% inhibition, mean ± SD		No. of experiments
		Adhesion	Phagocytosis	
3 × 10 ⁶ Y-RBC	0.6	0	7 ± 4	3
3 × 10 ⁶ S-RBC	4.6	44 ± 4	66 ± 10	4
18 × 10 ⁶ Y-RBC	3.4	71 ± 10	55 ± 12	20
18 × 10 ⁶ S-RBC	27.0	88 ± 8	74 ± 11	36

= 4) and $9.15 \times 10^4 \pm 0.82$ per senescent RBC ($n = 4$), respectively. These results are in close agreement with those previously reported (14). The data indicate that the inhibition of erythrophagocytosis by TP-PNA⁺ is obtained at a nanomolar level of galactosyl residues labeled by the galactose oxidase/borotritide method (Table 3).

DISCUSSION

To study the adhesion and phagocytosis of human RBC by autologous monocytes, two different assays were used—⁵¹Cr-labeled RBC and visual microscopic analysis. Both the conditions and the perspective of the two assays differ. Emphasis in the ⁵¹Cr assay is on the populations of RBC involved, whereas in the visual assay, it is on the recruitment of monocytes. Nonetheless, by both assays we are able to demonstrate a greater reactivity with the senescent than with the young RBC. These results confirm previous observations (25–29) and extend them by (i) studying a readily available human experimental model involving autologous RBC and blood monocytes and (ii) using unmodified young or senescent RBC rather than asialo-RBC as the target cells. Asialo-RBC, which have been used extensively in many studies on the mechanism of sequestration of senescent RBC from circulation, have played a prominent role in establishing the significance of sialoglycoconjugates in the maintenance of RBC in circulation. More careful work, however, has revealed significant differences in the behavior of asialo-RBC and senescent RBC (6, 19, 30).

Exploiting the ability of autologous monocytes to recognize human senescent RBC, it became feasible to further characterize the chemical nature of the “senescence factor” on the surface of RBC membranes. Of the sugars most commonly found as sialoglycoconjugates on RBC surface, only D-galactose and GalNAc showed significant inhibition of adhesion and phagocytosis, and this was at millimolar concentrations. Neoglycoproteins emphasize the role of the same two sugars and again demonstrate the sensitivity and specificity of the two assays. The neoglycoproteins inhibit at micromolar concentrations. These observations again confirm previous ones in many laboratories but, nonetheless, deal still with simple sugars and their synthetic products.

The demonstration that it is possible to isolate from RBC very potent inhibitors of both adhesion and phagocytosis that are capable of inhibiting at nanomolar concentrations is in keeping with our hypothesis that the sequestration of senescent RBC from circulation could be mediated through a specific recognition of molecular changes on the RBC cell surface and not through only a physical change in rigidity of cell membrane. Our hypothesis is further substantiated in these studies by the demonstration that the material isolated from senescent RBC is more potent than that from young RBC.

This potent inhibitor is obtained by tryptic digestion of RBC with subsequent specific adsorption and elution from immobilized PNA (14). The method of isolation emphasizes

the role of galactose and implicates the molecular origin of the glycopeptide as glycophorin or some other glycophorin-like minor glycoprotein rather than band 3, which is not susceptible to solubilization with trypsin (31). *In vivo* experiments demonstrate the effectiveness of injected TP-PNA⁺, prepared from human senescent RBC, to increase the survival of senescent mouse RBC (D.B., J.V., R. Vassy, L.G., and D.A., unpublished data).

The evidence accumulated so far suggests that the “active site” of the “senescence factor” recognized by the monocytes is the terminal nonreducing β-galactosyl residues (14). While we ascribe the major role to the terminal nonreducing β-galactosyl residues, we nonetheless are cognizant of the preliminary data (Table 2) which show differences in the abilities of lactose and melibiose to inhibit adhesion and phagocytosis of senescent RBC. Further study of these interactions will be necessary to shed light on the controversy surrounding the anomeric form of the galactosyl residues in the story of RBC senescence and sequestration (19, 32).

In these studies on the interaction of human senescent RBC with autologous monocytes and their inhibition by sugars and glycopeptides, we made no attempt to ascribe a specific role to the autoimmune IgG or the lectin-like receptor on the monocytes.

In conclusion, we have demonstrated: (i) autologous monocytes readily can distinguish between young and senescent RBC; (ii) adhesion and phagocytosis of senescent RBC can be inhibited by millimolar concentrations of D-galactose and GalNAc, by micromolar concentrations of the same two sugars covalently bound to BSA, and by nanomolar concentrations of glycopeptides isolated from RBC; (iii) the material isolated from senescent RBC is more effective than that isolated from young RBC, and (iv) the inhibiting activity is proportional to the nonreducing β-galactosyl residues identified with the glycopeptide isolated from the tryptic digest of RBC and purified by specific adsorption and elution from immobilized PNA.

It should be emphasized that the demonstration of a reactivity of senescent RBC with autologous human peripheral monocytes does not imply that they are necessarily involved in the normal physiological mechanism of sequestration of senescent RBC. The significance of this demonstration is that it has provided us with another tool that (i) distinguishes between young and senescent RBC, (ii) demonstrates a high degree of specificity for a component that can be isolated in greater quantities from senescent than from young RBC, (iii) has a specificity for terminal nonreducing β-galactosyl residues and (iv) by its application has detected a component derived from a tryptic digest of senescent human RBC that is capable of competing with the sequestration of senescent mouse RBC (unpublished data), thereby increasing their “survival” in circulation.

With the restrictions on the use of human subjects for *in vivo* studies, this tool now offers a useful experimental model for *in vitro* explorations of certain aspects of RBC senescence and sequestration.

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