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Carbohydrate Specificity and Opsonin Dependency of the Interaction between Human Senescent Red Cells and Autologous Monocytes

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The previously-demonstrated galactose and/or *N*-acetylgalactosamine specificity of senescent human erythrocyte adhesion to, and phagocytosis by, autologous monocytes was further investigated by the use of natural and synthetic compounds with well known structures.

The results demonstrate that 1) the inhibitory effect of the galactosyl compounds does not strictly depend on their anomeric conformation, 2) both *N*-linked and *O*-linked carbohydrates are inhibitors, 3) the inhibition occurs whether the carrier is peptidic or lipidic, 4) the inhibitory effect is dependent on the carrier size.

Interaction between erythrocytes and autologous monocytes is also opsonin-dependent, since 2-deoxyglucose inhibits phagocytosis of the senescent red cells, and thermolabile opsonins are present in autologous plasma. These results suggest that complement components may be involved in such interaction.

In order to be recognized and phagocytosed by Kupffer cells in the liver or by splenic or bone marrow macrophages, the human senescent red blood cell (S-RBC) must carry specific surface alterations that are recognized by the phagocytic cell. While treatment of red cells with sialidase shortened cell survival [1-4] it did not result in the rapid clearance of young desialylated cells [4] as might have been expected if loss of sialic acid were the primary signal for cell removal. Indeed, the half-life of intact S-RBC is significantly shorter than that of sialidase-treated young red blood cells (Y-RBC) with a

Abbreviations: S-RBC, senescent red blood cells; Y-RBC, young red blood cells; CMP, cow caseinomacroglycopeptides; AS-CMP, sialidase-treated CMP; CMP-Pr, pronase-treated CMP; AS-CMP-Pr, sialidase- and pronase-treated CMP.

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similar sialic acid content [4]. Other investigations in humans provided evidence for interactions between S-RBC and monocyte-macrophages mediated by immunoglobulin IgG bound to specific membrane antigens of the S-RBC [5-7]. However, the interaction between red cells and phagocytic cells appears to involve recognition of sugar residues, as assessed in rats [8] and in humans [9-11]. In the latter, by the use of monosaccharides, neoglycoproteins and peanut lectin-positive glycoconjugates obtained after erythrocyte trypsinisation, we have shown that this interaction is inhibited by galactosyl and/or *N*-acetylgalactosaminyl derivatives [10, 11]. Taken together, all these data suggest that both "lectin-like" mechanisms and opsonins might be involved in the interaction between human S-RBC and autologous monocytes in culture.

The purpose of the present work is 1) to define the kind of galactose and/or *N*-acetylgalactosamine stereospecificity involved in the interaction between senescent human red blood cells and autologous monocytes in culture, using various α and β galactosyl derivatives of well defined carbohydrate structure, and 2) to obtain further evidence of the opsonin dependency of this interaction. To our knowledge, this is the first investigation of the possible role of complement components in the interaction between human S-RBC and monocytes in culture. The only work published in this connection is the recent study suggesting that S-RBC bear significantly more C3d than young cells [12].

Materials and Methods

Blood

Human blood was obtained from healthy donors of the Seine Saint-Denis Blood Transfusion Center in the Paris area, collected into heparinized tubes and processed within 15 min.

Fractionation of Erythrocytes According to their Age in Vivo

S-RBC were separated from Y-RBC within 15 min after venipuncture on the basis of differences in density as previously described [13]: whole blood was centrifuged at $2000 \times g$ for 120 min at 15°C and the plasma removed. The top and the bottom 5% of the erythrocyte layers were collected by aspiration. The effectiveness of this age-dependent fractionation was checked by measuring the pyruvate kinase activity in these two fractions. The specific activity of the Y-RBC was 2.1 ± 0.5 times that of the S-RBC.

Monocytes

Mononuclear cells were isolated from peripheral blood by centrifugation over Ficoll Hypaque as described by Böyum [14], and adjusted to a concentration of 2×10^6 cells/ml of Medium 199 (Eurobio, France) supplemented with 20% foetal calf serum. Aliquots (1 ml) were layered into 16 mm flat bottomed wells (24 wells/plate, Costar, Cambridge, MA, USA) containing 12 × 12 mm sterile glass coverslips, and incubated for 2 h at 37°C in humidified air with 5% CO_2. Non-adherent cells were removed by four washings with Medium 199. Adherence monocytes were cultured in the Medium 199 containing 2 μmol/ml glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 20% foetal calf

serum. 95% of these cells were identified as monocytes by staining with acridine, phagocytosis of latex particles or morphological criteria after staining by May-Grünwald Giemsa.

Visual Evaluation of Binding and Phagocytosis

After a culture period of 2-20 h the medium was gently aspirated and 0.5 ml Medium 199, with or without inhibitor, was added to each well. Three minutes later, 0.1 ml Y-RBC or S-RBC suspension (10^7 cells) was added. Cells were co-cultured for 3 h at 37°C under a 5% CO_2 humidified atmosphere. The glass coverslips were washed twice with Hank's medium (Eurobio, France) to remove unbound RBC, fixed with methanol, then dried and stained with May-Grünwald Giemsa reagent. In several duplicate experiments, RBC that had been bound but not internalized were lysed with 140 mM NH_4Cl-Tris buffer pH 7.4 before fixing. Random fields of the coverslips were examined by light microscopy (1000 × magnification) under oil, and at least 500 monocytes were scored for binding and ingestion of RBC. All experiments were run in duplicate for each blood donor and the results are expressed as the average of several different donors. The percentage of monocytes which bound or phagocytosed one or more RBC was determined as the number of RBC bound or internalized by 100 monocytes.

Inhibition

For the inhibition experiments, monocytes in culture were pre-incubated for 5 min with the following compounds: *p*-nitrophenyl β-galactoside (PNP-β-Gal), *p*-nitrophenyl α-galactoside (PNP-α-Gal), *p*-nitrophenyl β-glucoside (PNP-β-Glc), methyl α-glucoside (Me-α-Glc), methyl β-galactoside (Me-β-Gal), methyl α-galactoside (Me-α-Gal), stachyose, glucocerebroside, fetuin, asialofetuin (all from Sigma Chemica Co, St Louis, MO, USA); 2-deoxyglucose (Merck, Darmstadt, W. Germany); cow caseino-macroglycopeptides which were either intact (CMP) [15] or treated with sialidase (AS-CMP), pronase (CMP-Pr), or both (AS-CMP-Pr), which were a gift from Dr. Neeser; G_{M1}-ganglioside (a gift from Dr. N. Bauman); bi-antennary glycan from desialylated bovine serum transferrin (a gift from Dr. Bauvy); and Galβ1-3Galβ1-4Glc-Albumin (3 mol sugar/mol albumin), a gift from Dr. Michalski. The structures of the carbohydrate moieties of some of these compounds are shown in Fig. 1.

Inhibitory activity was expressed as the % of inhibition calculated from the following equations:
1) % inhibition of phagocytosis: (P-PI)/P × 100 in which P = % S-RBC phagocytosed by monocytes without added inhibitor and PI = % S-RBC phagocytosed in the presence of inhibitor,
2) % inhibition of adhesion = (A-AI)/A × 100 in which A = % of S-RBC adhering to monocytes without inhibitor, and AI = % of S-RBC adhering to monocytes in the presence of inhibitor.

Plasmatic Factors

In some experiments, the interaction between monocytes in culture and either washed Y-RBC or S-RBC was compared to that between cultured monocytes and either unwashed or washed RBC pre-incubated for 30 min at 37°C in whole autologous plasma before

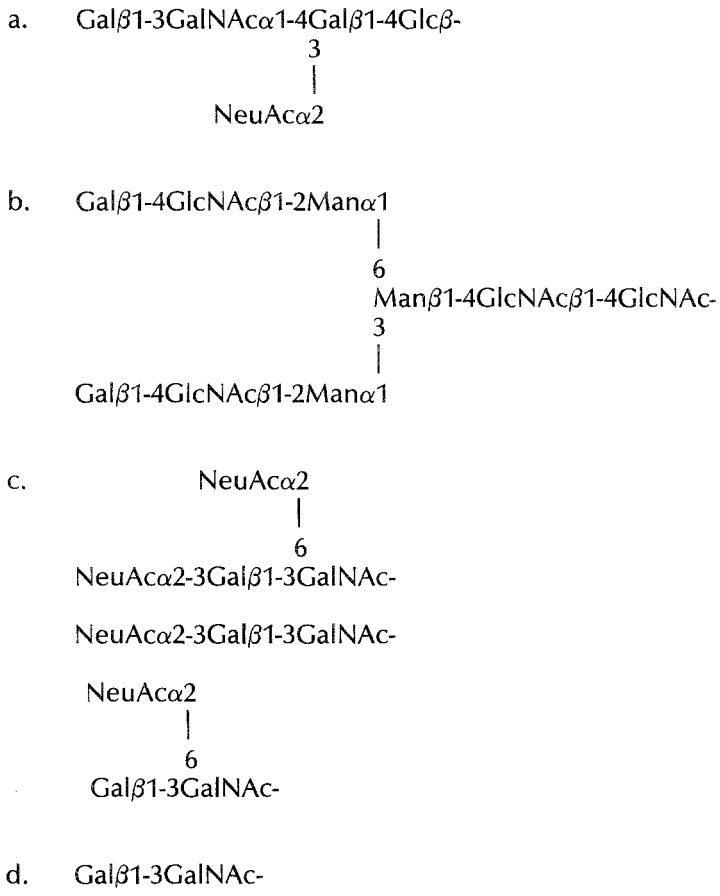

Figure 1. Oligosaccharide structures of a) G_{M1}-ganglioside, b) desialylated bovine serum transferrin, c) CMP, d) AS-CMP.

the assay. The interactions between monocytes in culture and Y-RBC or S-RBC preincubated for 30 min at 37°C in whole autologous plasma were also compared to those observed between monocytes and RBC pre-incubated either in heat-inactivated plasma alone, or in heat-inactivated plasma supplemented with pure C3 at the concentration of 2 mg/ml (CNTS-Orsay, France). Heat-inactivated plasma was obtained by treatment of the plasma for 30 min at 56°C.

Results were expressed as the % inhibition of phagocytosis and of adhesion observed:
a) for washed RBC compared either to unwashed RBC or to RBC pre-incubated in whole plasma,
b) for RBC pre-incubated in heat-inactivated plasma compared either to RBC pre-incubated in whole plasma or in heat-inactivated plasma supplemented with C3.

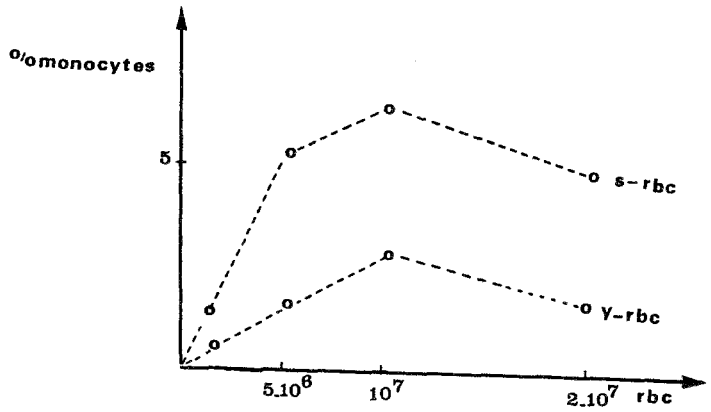

Figure 2. Per cent monocytes involved in binding or phagocytosis of RBC.

Results

Normal Values

In each experiment freshly drawn young and senescent RBC were used, just separated according to density in autologous plasma in order to avoid *in vitro* alteration of the cells. Co-culture of RBC with monocytes was performed for 3 h, in order to minimize *in vitro* changes of the cells, which might occur during longer period of co-culture. Maximum amount of binding and phagocytosis occurred when a mean of 10^7 RBC were applied to 2×10^5 monocytes (Fig. 2). This ratio was thus used in all the experiments described; under these experimental conditions the percentage of monocytes that had bound or phagocytozed autologous S-RBC was significantly higher than those which had bound or phagocytozed the corresponding Y-RBC. Significantly more S-RBC were bound or phagocytozed by monocytes than Y-RBC (Table 1, Fig. 2).

Galactose and/or N-Acetylgalactosamine Stereospecificity

Among the sugars most commonly found in glycoconjugates on the RBC surface, we have previously shown that D-galactose and *N*-acetyl-D-galactosamine inhibited both the adhesion and phagocytosis of human senescent RBC by autologous monocytes [9-11]. Thus, the inhibitory effect of different galactosyl derivatives was tested here; these compounds were tested at different concentrations, in order to determine the minimum concentration giving maximum inhibition. Indeed, increasing concentration of the active compounds resulted in an increase of inhibition of phagocytosis (Fig. 3).

The results reported in Table 2 are obtained for each group of compounds at a concentration higher or equal to that producing the maximum inhibition for the best inhibitor in each group of compounds. Under these experimental conditions, no significant inhibition of adhesion to, or phagocytosis by, autologous monocytes was observed with

Table 1. Light microscope observation of human young and senescent red blood cell (RBC) adhesion to, and phagocytosis by, autologous monocytes in culture. Results are expressed as means ± SEM of 22 coupled experiments.

RBC	RBC per 100 monocytes involved in	
	adhesion	phagocytosis
Young (Y)	4.6 ± 1.4	1.7 ± 0.3
Senescent (S)	10.8 ± 2.5	5.4 ± 0.9
p of the Y-S difference	<0.01	<0.001
	% monocytes involved in	
	adhesion	phagocytosis
Young (Y)	2 ± 0.31	1.75 ± 0.30
Senescent (S)	6.00 ± 1.20	5.00 ± 0.5
p of the difference	<0.01	<0.001

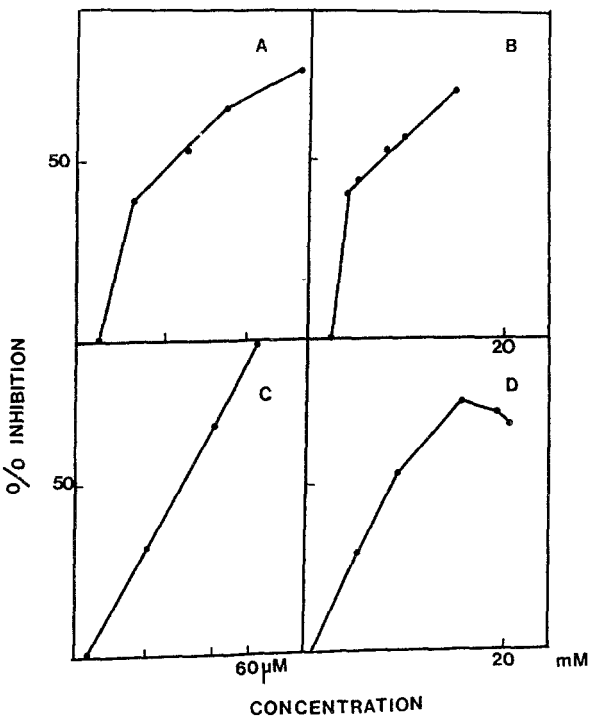

Figure 3. Inhibition of human senescent RBC phagocytosis by autologous monocytes in culture by A) AS-CMP, B) PNP-β-Gal, C) G_{M1}-ganglioside, D) Me-β-Gal.

Table 2. Carbohydrate specificity of human senescent RBC phagocytosis by, and adhesion to, autologous monocytes. Results are expressed as the mean ± s.

Carbohydrate	Concentration	% of inhibition of		No. of
		phagocytosis	adhesion	experiments
PNP-β-Gal	15 mM	73 ± 25	68 ± 35	5
PNP-α-Gal	15 mM	75 ± 8	75 ± 26	3
PNP-β-Glc	15 mM	34 ± 31	33 ± 33	3
Me-β-Gal	22 mM	62 ± 9	69 ± 5	3
Me-α-Gal	22 mM	47 ± 33	50 ± 26	9
Me-α-Glc	22 mM	0	0	3
Stachyose	22 mM	54 ± 22	39 ± 18	7
CMP	51 μM	0	0	4
AS-CMP	51 μM	67 ± 27	62 ± 37	5
AS-CMP-Pr	51 μM	25 ± 30	34 ± 30	7
CMP-Pr	102 μM	0	0	7
Bi-antennary glycan	37 μM	83 ± 24	52 ± 21	3
G_{M1}-ganglioside	67 μM	100	59 ± 39	4
Glucocerebroside	100 μM	15 ± 15	13 ± 13	4
Fetuin	3.5 μM	35 ± 25	34 ± 25	7
Asialofetuin	1.8 μM	62 ± 30	56 ± 20	7

Me-α-Glc, CMP and CMP-Pr. Mean inhibition of less than 50% was observed for PNP-β-Glc, AS-CMP-Pr, fetuin and glucocerebroside; the variability of these results reflects the natural heterogeneity of the cells from different donors. Indeed, similar values of inhibition were observed by the use of the cells from a single donor tested at different periods with a given compound. However, PNP-β-Gal and PNP-α-Gal, Me-β-Gal, AS-CMP, asialofetuin, G_{M1}-ganglioside and bi-antennary glycan from desialylated bovine serum transferrin significantly inhibited both the adhesion and phagocytosis of S-RBC by autologous monocytes in culture. Me-α-Gal caused significant inhibition of the binding of washed S-RBC to monocytes, and stachyose caused inhibition of their internalization (Table 2).

Opsonin Dependence and Involvement of Plasmatic Factors

The involvement of opsonins in the interaction between S-RBC and monocytes in culture was confirmed by the significant inhibitory effect of 30 mM 2-deoxyglucose on phagocytosis (Table 3), since 2-deoxyglucose is known to depress IgG- and/or C3-dependent phagocytosis [16]. Furthermore, the results clearly show that washing the S-RBC significantly reduced their interaction with monocytes (Table 3); this interaction was significantly enhanced by pre-incubation of the washed S-RBC for 30 min at 37°C with either autologous plasma or heat-inactivated plasma supplemented with C3 at nearly physiological concentration. However, this significant enhancement was no longer observed when the washed S-RBC were pre-incubated in heat-inactivated plasma instead of whole plasma (Table 3). It should be pointed out that unwashed S-RBC and S-RBC pre-incubated in whole autologous plasma were phagocytozed by autologous monocytes in a statistically similar manner (Table 3). When some galactosyl derivatives were tested for their effect on phagocytosis of unwashed S-RBC by monocytes it was observed that stachyose, G_{M1}-ganglioside, bi-antennary glycan from

Table 3. Involvement of plasmatic factors and opsonin dependency of human senescent RBC phagocytosis by autologous monocytes. Results are expressed as the ratios of phagocytosis or binding of the S-RBC treated in the different indicated manners × 100, and are the means ± s.

	% of inhibition of		No. of
	phagocytosis	adhesion	experiments
2-Deoxyglucose (30 mM)	65 ± 37	41 ± 29	5
Washed S-RBC / Unwashed S-RBC	49 ± 13	41 ± 19	6
Washed S-RBC / S-RBC preincubated in plasma	56 ± 31	66 ± 21	6
S-RBC preincubated in h.i.p.[a] / S-RBC preincubated in plasma	56 ± 26	50 ± 33	8
S-RBC preincubated in h.i.p.[a] / S-RBC preincubated in h.i.p.[a]+C3	57 ± 24	43 ± 23	3

[a] h.i.p.=heat-inactivated plasma.

desialylated bovine serum transferrin, asialofetuin and Galβ1-3Galβ1-4Glc-Albumin inhibited significantly their phagocytosis in a dose dependent manner.

Maximum inhibition was observed with 22 mM stachyose (62 ± 25%; n = 2), 4 μM asialofetuin (69 ± 2%; n = 6), 67 μM G_{M1}-ganglioside (82 ± 15%; n = 3), 37 μM bi-antennary glycan from desialylated bovine serum transferrin (83 ± 25%; n = 3), and 18 μM Galβ1-3Galβ1-4Glc-Albumin (95 ± 7%; n = 2). Fetuin, glucocerebroside and Me-α-Glc were devoid of significant inhibitory effect.

As regards Y-RBC, it was interesting to observe that washing of these cells also significantly reduced their interaction with monocytes (Table 4), although this interaction was similar whether Y-RBC were pre-incubated in whole or heat-inactivated plasma. However, pre-incubation of the Y-RBC in heat-inactivated plasma supplemented with C3 at nearly physiological concentration enhanced the phagocytosis of the Y-RBC compared with that of Y-RBC pre-incubated in heat-inactivated plasma alone, indicating that under these experimental conditions complement components are also able to opsonize Y-RBC (Table 4).

Discussion

Galactose and/or N-Acetylgalactosamine Specificity

The results reported in this study complete our previous work which showed significant inhibition of *in vitro* interaction between S-RBC and autologous monocytes by free sugars such as galactose and *N*-acetylgalactosamine at the millimolar level, by

Table 4. Involvement of plasmatic factors in the phagocytosis of human young RBC by autologous monocytes. Results are expressed as the ratios of phagocytosis or binding of the Y-RBC treated in the different indicated manners × 100, and are the means ± s.

	% of inhibition of		No. of
	phagocytosis	adhesion	experiments
Washed Y-RBC / Unwashed Y-RBC	48 ± 37	62 ± 32	8
Y-RBC preincubated in h.i.p.[a] / Y-RBC preincubated in plasma	0	0	2
Y-RBC preincubated in h.i.p.[a] / Y-RBC preincubated in h.i.p,[a]+C3	64 ± 30	17 ± 7	4

[a]h.i.p. = heat-inactivated plasma.

neoglycoproteins such as Galβ-albumin and GalNAcβ-albumin at the micromolar level and by senescent tryptic glycopeptides bearing PNA^+ sites at the nanomolar level [11]. In the present study, we observed for the first time that binding and phagocytosis of the S-RBC were significantly inhibited by asialofetuin and not by fetuin; by PNP-β-Gal as well as by PNP-α-Gal but not by PNP-β-Glc; by Me-β-Gal but not by Me-β-Glc; by AS-CMP but not by CMP or AS-CMP-Pr; by G_{M1}-ganglioside but not by glucocerebroside; and by bi-antennary glycan from desialylated bovine serum transferrin. These results further demonstrate that galactosyl compounds are involved in the interaction between S-RBC and autologous monocytes. As regards the α-galactosyl compounds tested, their inhibitory action is significant; thus it can be concluded that this effect does not strictly depend on the anomeric conformation of the galactosyl residues. As G_{M1}-ganglioside and AS-CMP, which have *O*-linked carbohydrate chains (Fig. 1) are proved to be inhibitors, unlike CMP, it can be concluded that *O*-linked β-galactosides are inhibitors, and that inhibition occurs whether the carrier is lipidic or peptidic. Furthermore, as AS-CMP is inhibitory and as its corresponding pronase-treated compound AS-CMP-Pr is devoid of a significant inhibitory effect, it can be concluded that the size and multivalence of the galactosyl structure has a role in its inhibitory effect. As the bi-antennary glycan tested, which is an *N*-linked carbohydrate compound of the *N*-acetyllactosaminic type (Fig. 1) is also an inhibitor, it may be concluded that both *N*-linked and *O*-linked carbohydrate compounds are inhibitory.

These results imply either that different types of galactosyl structures may be involved in the complex interaction leading to the binding and phagocytosis of S-RBC by monocytes in culture, or that broad galactosyl specificity is involved in one of the steps in this interaction. The fact that the inhibition levels of phagocytosis almost always stay at 60-80% argue in favor of the hypothesis of the involvement of different types of galactosyl structures in the interaction.

Opsonin Dependence

When washed S-RBC were preincubated for 30 min at 37°C in autologous plasma, a source of both antibodies and complement, significantly more were internalized by monocytes than were washed S-RBC. Furthermore, the phagocytosis of unwashed S-RBC was similar to that of S-RBC pre-incubated in whole autologous plasma. The opsonin dependence of this interaction was further assessed by observing the inhibitory effect of 2-deoxyglucose, known to depress IgG and/or C3b-dependent phagocytosis [16]. It should be pointed out that phagocytosis of unwashed S-RBC is also inhibited by α and β, *N*- or *O*-linked galactosyl derivatives such as asialofetuin, G_{M1}-ganglioside, stachyose, bi-antennary glycan from bovine serum transferrin, and Galβ1-3Galβ1-4Glc-albumin. On the other hand, when washed S-RBC were pre-incubated in heat-inactivated instead of whole plasma, binding and phagocytosis no longer differed from that observed with washed S-RBC. As the naturally occurring opsonins which bound S-RBC under these experimental conditions are thermolabile, it may be suggested that they are components of the complement system.

When the washed S-RBC were pre-incubated in heat-inactivated plasma supplemented by pure C3, at a nearly physiological concentration (in these conditions C3 is cleaved leading to the formation of C3bi) [17], binding and phagocytosis were similar to those observed when S-RBC were pre-incubated in whole plasma. Consequently, S-RBC might be able to bind, *in vitro*, autologous plasma-opsonizing complement components, thus enhancing the binding and ingestion of these cells by monocytes. Under our experimental conditions, Y-RBC were also opsonized in whole plasma, but as heat-inactivation of plasma did not reduce Y-RBC monocyte interaction, any involvement of thermolabile complement components can be excluded. Nevertheless, as supplementation of heat-inactivated plasma by pure C3, enhanced both monocyte/Y-RBC and monocyte/S-RBC interaction, it can be concluded that Y-RBC are able to bind opsonin C3 components under certain experimental conditions (Table 4). It is important to note that in all the experiments, the absolute levels of binding and ingestion of Y-RBC by monocytes were, on average, 2-3 times lower than those observed for the S-RBC treated under similar conditions (Table 1). This result strongly suggests that the absolute number of opsonin binding sites is significantly smaller on the young RBC surface membrane and increases during ageing.

The occurrence of ~ 100 specifically-bound IgG molecules per S-RBC, and their involvement in the interaction with monocytes has already been demonstrated [5, 6, 7]. The results described in this study imply that the complement components might also play a role in this interaction, for instance by enhancing IgG-dependent phagocytosis by monocytes. Antibody dependent enhancement of the alternative pathway might also occur [18, 19]. The effect of membrane sialic acid reduction on human S-RBC clearance remains unclear, the possible relationship between this reduction and increased complement deposition through activation of the alternative pathway [20] may also be envisaged.

In conclusion, our results show that several types of galactosyl structures present on *O*- or *N*-glycoside chains in both α or β forms inhibit the interaction between S-RBC and monocytes in culture. They also confirm the opsonin dependence of this interaction and demonstrated the thermolability of some of the S-RBC opsonins, suggesting that complement components might be involved.

Further studies are necessary to elucidate; 1) at what step(s) in the S-RBC/monocyte interaction the galactosyl structures are involved, 2) the mechanisms of action of the complement components, and 3) the possible relationship between the opsonin(s) and the galactosyl structures.

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