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Experiences in the measurement of RBC-bound IgG as markers of cell age

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Abstract

An immunologically mediated pathway has been largely accepted to be one of the mechanisms involved in the clearance of senescent or prematurely damaged RBC. According to this pathway, RBC removal is mediated by binding of naturally occurring IgG to clustered integral membrane proteins, followed by complement deposition. The validation of an immunoenzymatic method for the detection of RBC-bound autologous IgG is presented. The use of RBC-bound IgG as an index related to red cell age was evaluated by measuring IgG binding in RBC treated with the clustering agent $ZnCl_2$, in density fractionated RBC and in a selected group of patients expected to have an altered RBC life span. The immunoenzymatic method for IgG detection resulted to be reproducible (CV = 3.4%). IgG binding to in vitro clustered RBC was found to be enhanced to a very great extent, about 20 times higher with respect to untreated RBC. A slight but significant increase (about 1.8-fold) in membrane-bound IgG was observed in the highest density fraction of normal RBC, which constituted 1% of the total cells. A significantly greater number of RBC-bound IgG was measured in splenectomized β-thalassemia intermedia patients and in subjects with secondary decreases in the C3 complement fraction concentration.

Keywords: Naturally occurring antibodies; Erythrocytes; Aging; β-Thalassemia intermedia; Complement

1. Introduction

It is well documented that, after a life span of 120 days, senescent erythrocytes (RBC) expose removal markers that account for their selective recognition by macrophages and clearance from the circulation. Different controversial hypotheses have been proposed in order to explain the mechanism of erythrophagocytosis and to find, among the many physical and biochemical alterations occurring during red cell aging, the primary senescent signal that marks the cells to be removed [1,2]. Some authors have suggested that the senescent marker derives from modifications of membrane proteins [3], others from desialylation of sialoglycoconjugates [4] or from exposure of internal leaflet lipids [5]. A currently popular hypothesis states that an immunologically mediated pathway is one of the mechanisms involved in the clearance of senescent or prematurely damaged RBC [6–8]. According to this pathway, RBC removal is mediated by binding of autologous, naturally occurring antibodies and complement to the RBC surface. The removal pathway is triggered by the clustering of integral membrane proteins, predominantly band 3. This is mainly caused by hemoglobin denaturation with formation of hemichromes that tightly bind to the cytoplasmic domain of band 3. As a result, band 3 undergoes a topographic redistribution within the membrane leading to integral protein cross-linking. The clustered sites are rapidly recognized by the immune system and opsonized by IgG antibodies and the C3 fraction of complement. IgG-complement complexes strongly induce phagocytosis by macrophages following their binding to Fc and complement receptors. Such a IgG-dependent recognition mechanism has found experimental evidence in physiologically senescent RBC, in vitro oxidized RBC and has also been demonstrated in enzyme-deficient RBC, malaria-infected RBC and in RBC with hereditary hemoglobin abnormalities and with hereditary spherocytosis [9,10].

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The availability of a reliable index related to the RBC age may be of relevant clinical utility when age-dependent biochemical parameters have to be carefully evaluated. RBC creatine and membrane protein ratio 4.1a/4.1b have so far been described as useful parameters for the assessment of red blood cell age [11,12]. Our objective was the validation of an immunoenzymatic method for the determination of RBC-bound autologous IgG to be used as an index related to red cell age. To this aim, membrane-bound IgG was measured in in vitro artificially aged RBC, in the highest density fraction of normal RBC and in a selected group of patients expected to have an altered erythrocyte life span.

2. Experimental

Fresh blood samples were obtained from normal blood donors ($n=31$), β-thalassemia intermedia patients ($n=9$ splenectomized, $n=3$ not splenectomized), β-thalassemia carriers ($n=14$) and subjects with secondary decrease in C3 complement fraction concentration ($n=18$). None of the thalassemic patients was ever submitted to transfusional treatment. Blood was collected in EDTA except for the experiments with clustering agents in which heparin was added as anticoagulant. Freshly drawn blood was passed through a cellulose column to remove leukocytes and platelets. After three washes with isotonic saline (1000 g for 5 min at +4 °C), RBC were suspended at 10% hematocrit in 10 mmol/l Hepes, 130 mmol/l NaCl, 10 mmol/l glucose, 2% (w/v) bovine serum albumin (BSA), pH 7.4. Suspended RBC were then supplemented with 15 μg/ml (final concentration) of goat anti-human IgG antibodies conjugated with alkaline phosphatase (GAH-IgG, γ-chain specific, affinity-purified monoclonal, A-3187 Sigma) and incubated overnight at +4 °C [13]. After washing to remove unbound antibodies, membrane ghosts were prepared by hypotonic lysis of the labelled RBC in 5 mmol/l sodium phosphate pH 8.0 (dilution 1:60). After a 30-min incubation on ice, three washes in the same lysis buffer were performed (6000 g for 30 min at +4 °C). In a typical experiment using 3.0 ml of suspended RBC from normal subject, about 400 μl of ghosts could be obtained. Ghosts were then solubilized in 1% (w/v) Triton X-100 and alkaline phosphatase (ALP) activity was measured at 404 nm using *p*-nitrophenyl phosphate as the substrate. Membrane protein concentration was determined by the bicinchoninic acid assay (from Pierce). Membrane-bound IgG was expressed as the number of ALP-conjugated GAH-IgG antibodies bound by treated RBC.

Clustering of RBC integral membrane proteins was induced as described by Turrini et al. [14]. Briefly, washed RBC were incubated at 10% hematocrit with increasing concentrations (up to 1 mmol/l) of $ZnCl_2$ and 1 mmol/l bis-sulfosuccinimidyl-suberate (BS3, from Sigma) for 15 min at room temperature. After washing, opsonization was performed at 33% hematocrit by incubation for 60 min at 37 °C in autologous serum. Membrane-bound IgG was measured after labelling washed RBC with ALP-conjugated GAH-IgG antibodies as described above.

Density-fractionated RBC were prepared using *Percoll* discontinuous preformed gradients. Leucocyte-free RBC obtained from normal blood donors were separated in five subpopulations of increasing density by modification of a previously reported procedure [15]. To this regard, composition of density gradients was specifically designed for each subject, to allow the separation of a very small highest density fraction containing not more than 1% of the total cells. A typical gradient was formed by superimposing four solutions at final *Percoll* concentration of 58%, 65%, 72% and 75% (v/v; *d*: 1.090–1.110 g/ml). Isolated RBC fractions were thoroughly washed with cold isotonic saline to remove any *Percoll* and analysed for cell-bound IgG as described above. Glycated hemoglobin (Hb A_{1c}) was measured in the fractionated RBC as a cell age marker to monitor RBC separation [15,16].

3. Result and discussion

The reproducibility of the immunoenzymatic assay for RBC-bound IgG was estimated by the method of duplicate analyses (Table 1). Analytical CVs of 0.9%, 1.2% and 3.4% were obtained with regard to protein concentration, ALP activity and IgG binding assay, respectively. For each parameter, the reproducibility was deemed to be quite satisfactory.

Stability at +4 °C of both blood ($n=8$) and RBC ($n=11$) samples was evaluated relatively to membrane-bound IgG (Fig. 1). Whole blood samples were found to be stable up to 3 days at +4 °C (data not shown) while prolongation of storage time up to 8 days resulted in a small but significant increase in the number of cell-bound IgG ($P<0.001$). Leucocyte-free RBC suspended in Hepes buffer resulted to be much more stable with respect to whole blood and no relevant differences in cell-bound IgG were noted over the same storage period. Better stability of RBC with respect to blood was probably due to the elimination of plasma containing autologous IgG, therefore unable to bind to red cells during in vitro aging.

Table 1
Reproducibility of the immunoenzymatic method for the measurement of RBC-bound IgG

Parameters	*N*	Mean	S.D.	CV, %
Total protein, mg/ml	26	3.22	0.03	0.9
ALP activity, ΔA/min	26	0.130	0.002	1.2
IgG binding, number of GAH-IgG/RBC	26	235	7.96	3.4

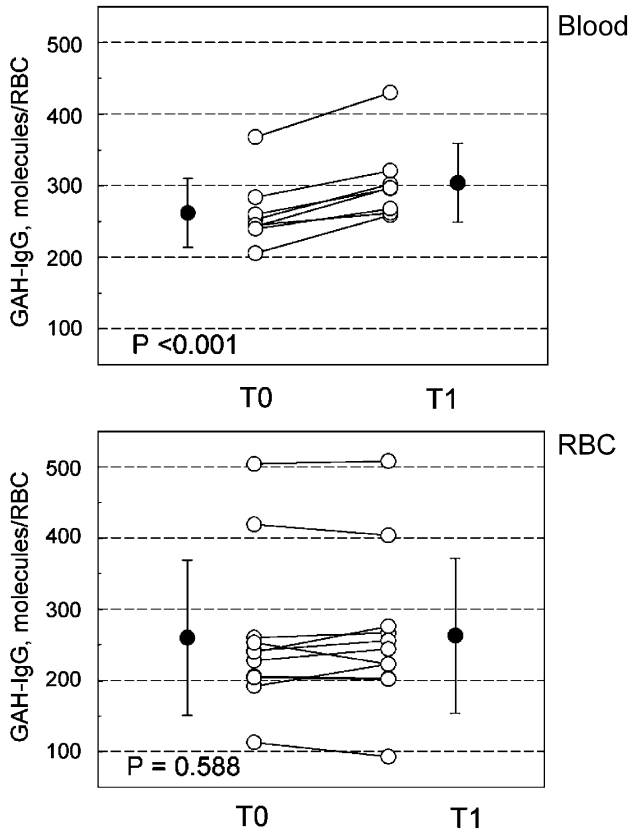

Fig. 1. Stability of whole blood and RBC samples stored at +4 °C with regards to membrane-bound IgG content. Statistical analysis was performed by means of *t*-test for paired samples. Dark circles represent mean values. T0 = day 0, T1 = day 8.

Experiments were conducted in which RBC were treated with clustering and cross-linking agents ($ZnCl_2$ and BS^3) known to give rise to integral protein clustering in RBC and to induce autologous IgG binding upon reincubation in their own serum. These experiments were aimed at modeling the senescent pathway in vitro in order to obtain RBC artificially enriched with autologous IgG. Antibody binding to in vitro clustered RBC was observed to increase in function of $ZnCl_2$ concentration with the highest binding reached when RBC were treated with 0.5 mmol/l $ZnCl_2$ (Fig. 2). At this concentration, IgG binding was found to be enhanced to a very great extent, about 20 times higher with respect to untreated RBC. When $ZnCl_2$ concentration exceeded 0.5 mmol/l concentration, IgG binding slightly decreased and was found to be 14 times higher with respect to untreated RBC at 1.0 mmol/l. Our results are in agreement with those obtained by Turrini et al. [14] who demonstrated RBC treated with the same reagents to be more susceptible to autologous IgG binding and indicated the ability of our immunoenzymatic method to detect RBC-bound IgG.

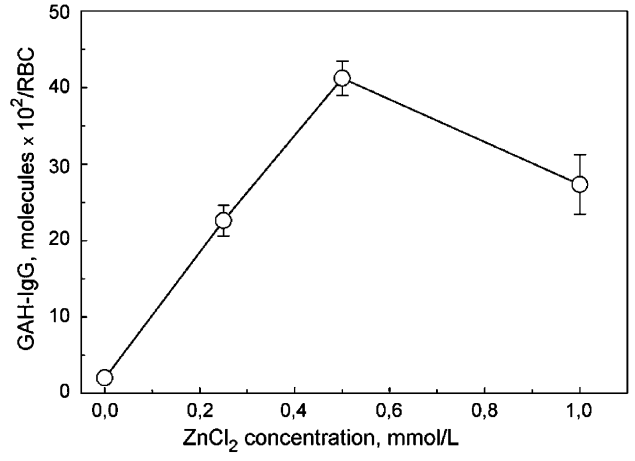

Fig. 2. Autologous IgG binding to RBC treated with increasing concentrations of $ZnCl_2$. Results represent mean values ± S.D. of three experiments.

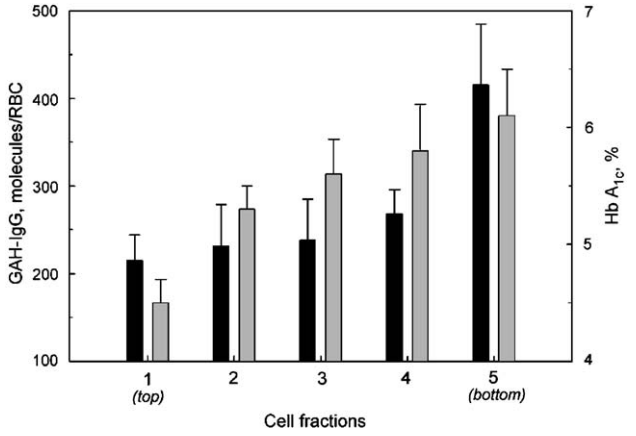

Fig. 3. IgG binding (dark bars) and Hb A_{1c} concentration (light bars) in density separated RBC. Data are from three normal subjects. Error bars show the S.D.

By using *Percoll* density gradients, normal blood was separated in five RBC fractions whose densest fraction was characterized to comprise no more than 1% of the total cells. This small proportion of highest density RBC has

Table 2
Autologous IgG binding measured in a selected group of patients

Subjects	*N*	Median	Confidence interval 25–75%	Min	Max	*P*
Normals (blood donors)	31	188	164–250	86	338	–
β-Thal carriers	14	247	203–278	93	350	0.038
β-Thal intermedia (after splenectomy)	9	325	267–365	174	498	0.002
C3 deficit	18	451	285–974	201	3136	<0.001

Data are expressed as number of ALP-conjugated GAH-IgG antibodies bound per RBC. Statistical analysis was performed on comparison between pathological and normal subjects by means of Mann–Whitney rank sum test. NS, not significant.

been demonstrated to be constituted by cells destined to survive only a few additional hours in circulation being rapidly recognized and removed when they are re-infused into the donor [17]. Density fractionated RBC were analysed for cell-bound IgG and Hb A_{1c} concentration (Fig. 3). A progressive enrichment in Hb A_{1c} level was measured in RBC fractions of increasing density as expected in density–age-based separation. In the restricted 1% highest density RBC population, a slight but significant increase (1.8-fold ± 0.2, $n=3$, $P=0.035$) in the number of membrane-bound IgG was observed with respect to unfractionated cells while in the other RBC fractions, IgG content remained roughly constant. Similar findings have also been previously reported by other authors [18,19] who found the 0.5% highest density fraction of normal RBC to contain 5- to 10-times more membrane-bound IgG than the other cell fractions of lower density. The more remarkable enrichment in IgG binding found by these authors is probably due to the even more restricted population selected, as well as to different experimental procedures.

A selected group of subjects comprising patients suffering from β-thalassemia syndromes and subjects with secondary decreases in C3 complement fraction were studied. Detailed results together with statistical significance are reported in Table 2. A significantly greater number of RBC-bound IgG was detected in splenectomized β-thalassemia intermedia patients and in C3 deficit subjects. A small increase in IgG binding was also noted in RBC from β-thalassemia carriers. Previous studies performed on β-thalassemia intermedia patients have shown higher amounts of RBC-bound IgG as well as membrane-bound hemichromes and clustered band 3 in splenectomized subjects [20,21]. These findings have been related to the accelerated clearance of β-thalassemia RBC that causes these cells to have a shorter life span than normals [22].

4. Conclusion

We describe a validation of a method for measuring RBC-bound IgG. Although still quite time-consuming, we think that this method is reproducible and robust. The application of this method to density-gradient isolated RBC subfractions and to selected clinical cases of patients with altered red cell survival proves that RBC-bound IgG can be a parameter related to cell age and a marker of membrane damage.

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