THE EFFECT OF GLYCOSIDASES ON THE SURVIVAL OF RAT ERYTHROCYTES IN CIRCULATION[*,†]

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ABSTRACT

Enzymic removal of sialyl groups from mammalian erythrocytes resulted in their rapid sequestration from circulation subsequent to autologous transfusion. It has been demonstrated by many investigators that the terminal β-D-galactosyl group, exposed on red blood cell by *in vitro* desialosylation, is recognized by an autoimmune anti-galactosyl IgG and/or by a lectin-like receptor on monocytes and macrophages. It is demonstrated herein that the disaccharide structure β-D-Gal*p*-(1→3)-D-Gal*p*NAc (a) is masked in normal rat RBC, but exposed in asialo-RBC; (b) could be detected with fluorescently-labeled peanut agglutinin; (c) could be released from the asialo-RBC with an endo-*N*-acetyl-α-D-galactosaminidase; and (d) upon its removal by treatment with the endo-*N*-acetyl-α-D-galactosaminidase, enhances the survival of the asialo-RBC in circulation.

INTRODUCTION

Enzymic removal of sialyl groups from mammalian erythrocytes (RBC) resulted in their rapid sequestration from circulation[1]. With the demonstration that the sialic acid content of RBCs decreases with their *in vivo* aging in circulation[2], it has been postulated that a removal of sialyl groups might be involved in *in vivo* aging of RBC, with consequent sequestration of the RBC when the sialic acid content dropped by 10–15% to a threshold value[3]. Evidence from a number of studies in our laboratory has shown that in both artificial removal of sialyl groups from RBC with sialidase from *Vibrio cholerae,* aRBC, and *in vivo* aging of RBC in circulation, the loss of sialic acid groups is accompanied by the unmasking of the penultimate β-D-galactosyl residues. This is analogous to the Ashwell and Morell system for the sequestration of asialo plasma glycoproteins by hepatocytes[4]. In

contrast, we have shown that asialo RBC involve interaction with liver Kupffer cells and spleen monocytes[5].

We believe that the *in vivo* aging of RBC in circulation involves the desialosylation of glycophorin A, the most abundant sialoglyconjugate on human RBC surface. The hypothesis is supported by the isolation[6] of a "senescence factor glycopeptide" (SFG) from the tryptic digest of intact human RBC. This glyco-peptide, free of all sialic acid groups, had all the properties expected of the "senescence factor" with the capacity to inhibit erythrophagocytosis of senescent RBCs by autologous human blood monocytes in *nanomolar* concentrations, in contrast to D-galactose alone, which inhibited at *millimolar* concentrations, and β-D-galactosyl groups bound to bovine serum albumin which inhibited at *micromolar* concentrations[7].

The well known susceptibility of glycophorin A to tryptic digestion[8,9], and the method of purification devised[6] for the isolation of SFG would point to glyco-phorin A as the putative parent molecule of SFG on human and other mammalian RBC surfaces. Nonetheless, it could be argued that SFG still was contaminated with a potent inhibitor of erythrophagocytosis of an as yet unidentified origin. The objective of this communication is to provide yet another and more direct approach to elucidate the role of glycophorin-like structures, recently characterized[10] in rat RBC, in the sequestration of asialo RBC. Here, we focus on the disaccharide structure, β-D-Gal*p*-(1→3)-D-Gal*p*NAc, and demonstrate that: (a) It is masked in normal RBC, but exposed in asialo RBC (aRBC); (b) it can be detected with fluorescein isothiocyanate-labeled peanut agglutinin (FITC-PNA); (c) it can be released from the aRBC with endo-*N*-acetyl-α-D-galactosaminidase (endo-glycosidase)[11,12]; and (d) upon its release with the endoglycosidase treatment, the survival of the aRBC in circulation is enhanced[13,14]. As previously, the Sprague-Dawley rat was used as the experimental model.

EXPERIMENTAL

Materials. — The following enzymes were obtained from the sources indicated: β-D-galactosidase from bovine testes was a gift from Dr. G. W. Jourdian, University of Michigan[15]; *V. cholerae* sialidase was obtained from Worthington Biochemical Corp.; and endo-*N*-acetyl-α-D-galactosaminidase was prepared in our laboratory from *Clostridium perfringens*[11,12]. FITC-PNA, was obtained from Sigma Chemical Co. (St. Louis, MO); and Sprague–Dawley rats from Charles River Breeding Laboratories, Inc. (Wilmington, MA). $Na_2{}^{51}CrO_4$ used for *in vitro* labeling of RBC was purchased from E. R. Squibb and Sons, Inc. (Princeton, NJ).
Treatment of rat RBC with enzymes to determine their effect on the survival of RBC in circulation

Incubation of rat RBC with glycosidases. — Whole blood was obtained in a heparinized syringe by cardiac puncture of an approximately 400 g, male Sprague–Dawley rat. The blood was centrifuged, and the plasma and buffy coat were dis-

carded. The remaining packed cells were washed three times with isotonic saline solution. Four equivolume samples of RBC were incubated with the following reagents made up in isotonic saline solution at pH 5.5: (a) Isotonic saline control, (b) sialidase from *V. cholerae* (0.2 units/mL of packed cells), (c) sialidase from *V. cholerae* (0.2 units/mL of packed cells) and endoglycosidase (0.15 units/mL of packed cells), and (d) sialidase from *V. cholerae* (0.2 units/mL of packed cells) and β-D-galactosidase from bovine testes (0.15 units/mL of packed cells). The incubations were carried out for 1 h at 37°.

Quantitative determination of FITC–PNA adsorption on enzymically treated rat RBC. — Upon completion of the incubation period, the samples were centrifuged and the supernatant discarded. The samples then were washed twice with phosphate-buffered saline solution (pH 7.3) (PBS). Aliquots of packed cells containing 1×10^7 erythrocytes were removed from each of the samples and diluted to a volume of 100 μL with PBS. To these dilute red cell suspensions was added a 1:20 dilution of FITC–PNA of 0.1% solution in PBS (100 μL). The resulting 200-μL samples in triplicate were incubated for 1 h at 4°, and then centrifuged. Equal volumes of supernatant were removed from each sample and diluted to a total volume of 3 mL with isotonic saline solution adjusted to pH 9.0 to enhance the fluorescence. The fluorescence was measured with a Turner Model 430 spectro-fluorometer at excitation and emission settings of 498 nm and 534 nm, respectively. The measured fluorescence is that of unadsorbed fluorescent lectin. The determination was made possible by comparison to a set of calibration standards of the 1:20 dilution of 0.1% FITC–PNA in isotonic saline solution adjusted to pH 9.0. The amount of adsorbed FITC–PNA then was calculated by subtraction of the amount not adsorbed from the total FITC–PNA added to the sample. This procedure was adopted in order to shorten the assay. Previous experiments indicated that all the adsorbed FITC–PNA could be quantitatively eluted with 0.4M lactose. Results are shown in Table I.

Survival of radioactively-labeled and enzymically treated rat RBC in circulation. — Comparable enzymically-treated RBCs (Samples a–d), 4.0–6.5×10^9 RBC, were washed and incubated for 0.5 h at 37° with $Na_2^{51}CrO_4$ (50 μCi) to label the RBC. The resulting incubation mixtures were centrifuged to remove the unincorporated $Na_2^{51}CrO_4$ and washed until no more perceptible radioactivity remained in the washes; ~95% of the radioactivity was incorporated into the RBC. Each pellet of RBCs was suspended in saline solution (~1 mL) and the RBC suspension (2 mL) injected intraperitoneally into each rat[13,14]. Enough RBC incubation mixtures of a–d were prepared to inject two rats per treatment. After 24 h, blood (20 μL) was removed from the tail of each rat and suspended in saline solution (480 μL). Similar blood samples were removed at daily intervals, and all samples were stored at 4°. The radioactivity was counted simultaneously at the end of the experiment in a Packard gamma-counter. The per cent of radioactivity remaining in the blood samples was computed as a percentage of that found at the end of the first 24 h. The results are shown in Fig. 1.

TABLE I

FITC–PNA ADSORPTION[a] AS A PERCENTAGE OF THAT OBSERVED FOR ASIALO RAT-ERYTHROCYTES

Sample	Isotonic saline solution	Sialidase	Sialidase plus endoglycosidase	Sialidase plus β-D-galactosidase
n	3	3	3	3
x (%)	0	100	12.4	55.0
σ_x			10.2	35.3

[a]Determined by subtraction, see Experimental section.

RESULTS

In view of the marked difference in the molecular extinction coefficient of sialic acid in the TBA assay[16], the disaccharide in the modified Morgan–Elson assay[17], and D-galactose with the galactose–dehydrogenase assay[18], it was impractical to photometrically monitor the action, on the aRBC of the endo-glyco-sidase and bovine testes β-D-galactosidase. Instead, the process was monitored by determining the amount of FITC–PNA that was adsorbed on the aRBC[19]. The peanut agglutinin was chosen deliberately to monitor the changes on RBC surface resulting from the enzymic treatments. This lectin focuses our attention only on the D-galactosyl group (of the many exposed by removal of sialyl groups from RBC) that is present as the disaccharide residue, β-D-Galp-(1→3)-D-GalpNAc[20]. Normal rat RBCs do not adsorb significant amounts of FITC–PNA; after desialosylation,
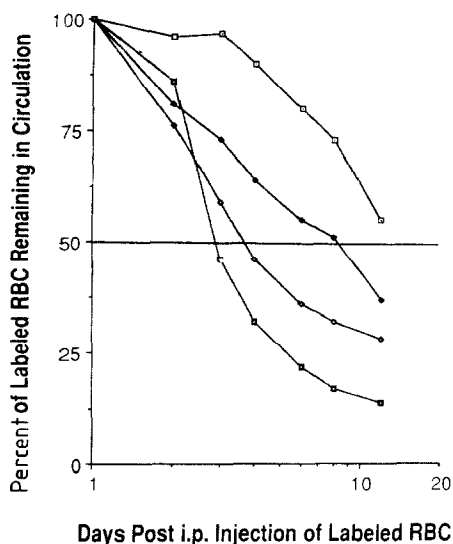

Days Post i.p. Injection of Labeled RBC

Fig. 1. Survival of enzymically treated rat RBC in circulation: (-⊡-⊡-⊡) Control RBC, in isotonic RBC at pH 5.5; (-□-□-□-) sialidase-treated RBC; (-◆-◆-◆-) sialidase- plus endoglycosidase-treated RBC; and (-◇-◇-◇-) sialidase- plus β-D-galactosidase-treated RBC.

they are very reactive, and the amount of lectin adsorbed is proportional to the number of aRBCs present. Under our standardized conditions, we arbitrarily assigned the amount of FITC–PNA bound to RBC as 100%, and then determined the effect of the two enzymes, β-D-galactosidase from bovine testes and the *Cl. perfringens* endoglycosidase, on the extent of FITC–PNA that still could bind, giving us a figure of 55 and 12%, respectively (Table I). Within the limitations of the data presented and under the conditions of this assay at pH 5.5 and 37°, it is apparent that the endoglycosidase is more effective than the β-D-galactosidase in removing the PNA reactive sites.

We have demonstrated previously[13,14] that intraperitoneal transfusion is an extremely useful procedure for the transfer of relatively large numbers of RBCs. Normal untreated RBCs are recovered almost quantitatively in the general circulation within 22 h, whereas aRBCs appear preponderantly in the liver with only a few cells remaining in the peritoneal cavity. By use of the same procedure, it was possible to monitor the survival of the enzyme-treated and -untreated RBCs remaining in circulation over a 12-day period. The mean results from two experiments are shown in Fig. 1, from which one may readily compare (a) the 50% survival of RBCs in circulation, and (b) the per cent of RBCs surviving after 12 days, namely, 55 ±10% for the untreated control RBCs, 12 ±4% for the sialidase-treated RBCs, 37 ±10% for the sialidase- plus endoglycosidase-, and 28 ±9% for the sialidase- plus β-D-galactosidase-treated RBCs, respectively.

DISCUSSION

The mechanism whereby RBCs are removed at the end of their life-span in circulation has been an intriguing question for many years. Briefly, the evidence favoring a role for the sialoglycoconjugates in RBC senescence and sequestration comes from the following six lines of investigation[21]: (a) Analysis of the carbohydrate composition of young and old RBCs, (b) use of asialo-RBC as a model for RBC-senescence, (c) identification of the interaction of immunoglobulin with senescent RBCs and aRBCs, (d) studies on the inhibition of interaction of senescent RBCs with macrophages and immunoglobulins, (e) correlations derived from a study of the RBC sialoglycoconjugates in the anemias, and (f) isolation of a putative senescence factor glycopeptide.

The circumstantial evidence thus collected implicates the involvement of a change in the chemical structure of the RBC membrane, as well as a change in its physical properties. A number of investigators have come to the same conclusion, but ascribe[22] the nature of the chemical change either to the involvement of Band 3, the exposure of a terminal α-D-galactosyl group[23], or to the β-D-Gal$p$-(1→3)-D-Gal$p$NAc group of a glycophorin-like molecule[21]. Our evidence in support of a glycophorin-like molecule can be summarized as follows: (a) The presence of more galactose oxidase and sialyltransferase receptor sites in senescent as compared to young RBCs (ref. 3); (b) the ability to isolate[6] from senescent, but not young,

RBCs a "senescence-factor glycopeptide[11] (SFG); (c) indications[6] from those properties of SFG utilized for its isolation as well as its reactivity with spleen mono-cytes, that SFG is biologically active; (d) the destruction of SFG's interaction with spleen monocytes after its treatment with β-D-galactosidase, attesting to the significant role of galactosyl residues in this biological activity[6]; (e) the ability of SFG to inhibit erythrophagocytosis at *nanomolar* concentrations, as compared to D-galactose at *millimolar* and D-galactosyl groups bound to BSA at *micromolar* concentrations[7]; and (f) the ability of injected SFG from human senescent RBC to inhibit the uptake of mouse senescent RBC at the end of the RBC life-span[24].

The aforementioned data are compatible with the hypothesis that a glyco-phorin-like molecule, devoid of sialic acid groups, could be the putative recognition factor on senescent RBCs. Since the homogeneity of SFG has not as yet been established[6], it could be argued that it is not necessarily a component of the glyco-phorin molecule that is involved, but another highly potent "senescence factor", which would still contaminate the glycophorin glycopeptides that were isolated from the RBC-tryptic digest[6] and used for the inhibition studies[7]. We have succeeded in showing, however, that the reduced survivability of aRBCs can be restored by removing the disaccharide groups. This "rejuvenation" of aRBCs by treatment with the endoglycosidase indicates that only the disaccharide groups exposed by the desialosylation of RBCs are detected by the reticulo-endothelial system.

Work in our laboratories and those of others has demonstrated significant differences in the behavior of asialo and senescent RBCs[21]. We attributed some of these differences primarily to the amount of sialic acid removed from the RBC. The observation that we can reverse the sequestration of aRBC by treatment with the endoglycosidase now provides us with a valuable tool to further explore this problem and eludicate the mechanism whereby senescent RBCs are recognized and sequestered.

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