Glycosylation Restores Survival of Chilled Blood Platelets

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Cooling of blood platelets clusters the von Willebrand factor receptor complex. Macrophage $\alpha_M\beta_2$ integrins bind to the GPIbα subunit of the clustered complex, resulting in rapid clearance of transfused, cooled platelets. This precludes refrigeration of platelets for transfusion, but the current practice of room temperature storage has major drawbacks. We document that $\alpha_M\beta_2$ is a lectin that recognizes exposed β-*N*-acetylglucosamine residues of N-linked glycans on GPIbα. Enzymatic galactosylation of chilled platelets blocks $\alpha_M\beta_2$ recognition, prolonging the circulation of functional cooled platelets. Platelet-associated galactosyltransferase produces efficient galactosylation when uridine diphosphate–galactose is added, affording a potentially simple method for storing platelets in the cold.

In 2001, blood centers performed over 12 million phlebotomies to procure platelets for transfusion into platelet-deficient patients at risk for bleeding. The fact that refrigerated platelets do not circulate after transfusion and therefore require room temperature storage complicates this potentially life-saving procedure. Platelets stored at room temperature lose hemostatic function, as assessed by in vitro assays. Room-temperature storage also accommodates bacterial growth, and bacterial sepsis is currently the major risk factor for transfusion-transmitted disease. The risk of bacterial infections transmitted through platelet concentrate transfusion is estimated to be 50 times higher than that of refrigerated red blood cell products (*1*, *2*) Thus, regulatory agencies limit platelet storage to 5 days, and this short shelf life severely compromises platelet inventories, creating chronic shortages (*3*, *4*).

We recently defined the clearance mechanism of chilled platelets (*5*). Cooling irreversibly reorganizes the von Willebrand factor receptor [the $(GPIb_{\alpha\beta}IX)_2V$ complex] into clusters on the platelet surface. The integrin receptor $\alpha_M\beta_2$, (complement receptor type 3/Mac-1) of hepatic macrophages recognizes clustered GPIbα, and the macrophages ingest the platelets. However, refrigerated and rewarmed platelets do not clear rapidly from the circulation of mice lacking $\alpha_M\beta_2$ receptors. The circulating platelets are not activated, and they function normally in response to injury in vivo. In addition, the binding of activated von Willebrand factor to the GPIb complex of warm or cold platelets in vitro is indistinguishable. Therefore, we suggested that the hemostatic and cold-induced clearance functions of the GPIb complex are separable and that modification of the latter might accommodate storage of platelets in cold without impairment of platelet function. Here, we provide evidence that $\alpha_M\beta_2$ recognizes GPIbα on cooled platelets through a lectin-mediated interaction with exposed β-*N*-acetylglucosamine (β-GlcNAc) residues on N-linked oligosaccharides. We demonstrate that this interaction between GPIbα and macrophages can be blocked by galactosylation of exposed β-GlcNAc residues, such that glycan-modified platelets circulate even better after cooling than room temperature platelets. We also discovered that sufficient galactosyl transferase activity is associated with platelets such that GPIbα can be modified by simple incubation in the presence of the donor substrate UDP-galactose (UDP-Gal).

The I domain of $\alpha_M\beta_2$ binds to platelet GPIbα (*6*), but $\alpha_M\beta_2$ also contains a lectin domain implicated in innate immunity with specificity for β-glucans (*7*). Rabbit erythrocytes bind to neutrophil $\alpha_M\beta_2$ via its lectin domain–triggering ingestion (*8*, *9*). We hypothesized that the clearance of chilled platelets could involve a similar mechanism. Therefore, we looked for inhibitory effects of monosaccharides on the phagocytosis of chilled platelets by differentiated THP-1 cells expressing $\alpha_M\beta_2$. D-glucose, methyl-β-D-glucose but not methyl-α-D-glucose or -galactose, and methyl-α/β-D-mannose inhibit

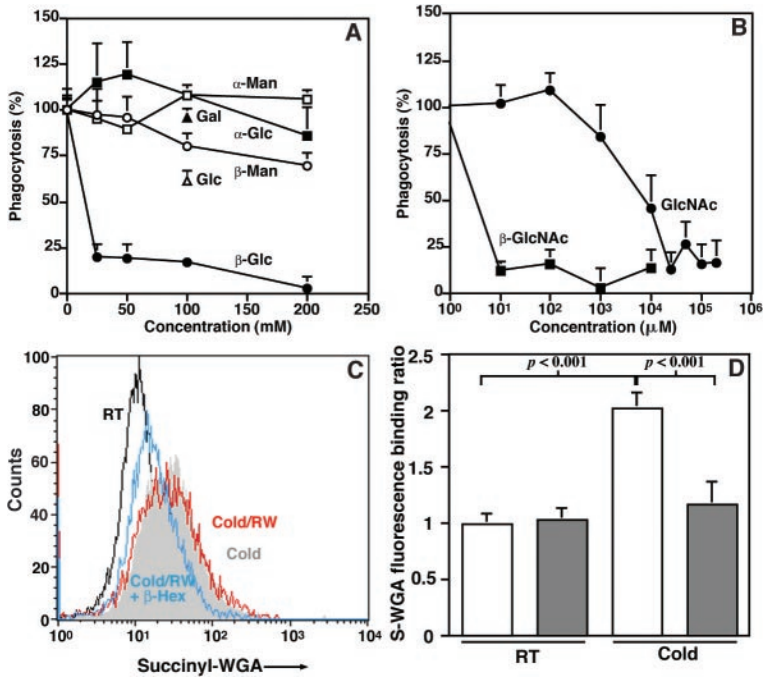

Fig. 1. Identification of β-GlcNAc as the lectin target for αMβ2. (**A** and **B**) Effect of monosaccharides on the ingestion of chilled platelets by THP-1 cells. D-Glucose (Glc), D-Galactose (Gal), methyl-α/β-mannoside (α/β-Man), methyl-α/β-glucoside (α/β-Glc), N-acetyl D-Glucosamine (GlcNAc) or β-GlcNAc were added at the indicated concentrations. The extent of chilled platelets ingestion without the added monosaccharides was defined as 100%. Each point represents the mean ± SD of five to six experiments in triplicate. (**C** and **D**). Effects of temperature and β-hexosaminidase (β-Hex) on binding of S-WGA to platelets. The results shown in the fluorescence-activated cell sorter (FACS) tracing in (C) are representative of six or more experiments. Bars in (D) depict means ± SD of four separate experiments. Open bars, non-treated room temperature (RT) or chilled (Cold) platelets; solid bars, β-Hex–treated platelets.

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phagocytosis of chilled platelets, implying that the β-glucan recognizing lectin of the $\alpha_M\beta_2$ integrin interacts with chilled platelets (Fig. 1A). High concentrations of N-acetyl-D-glucosamine are also inhibitory; 10 μM β-*N*-acetyl-D-glucosamine (β-GlcNAc) efficiently blocks phagocytosis (Fig. 1B). These results suggest that the interaction of the $\alpha_M\beta_2$ integrin with platelets is through lectin-mediated recognition. The effective inhibition by low β-GlcNAc concentrations indicates that the $\alpha_M\beta_2$-lectin recognizes exposed β-GlcNAc residues on chilled platelets. Thus, partial interference with the platelet $\alpha_M\beta_2$-lectin interaction might be sufficient to prevent recognition. The monosaccharides tested did not induce platelet aggregation detectable by light scattering or α-granule secretion, as measured by P-selectin up-regulation. They also did not influence aggregation responses or secretion induced by thrombin or ristocetin (data not shown). Sialic acid and galactose residues cover β-GlcNAc residues on most cell surface glycans including platelets (*10*–*12*), but our experiments indicate that macrophage $\alpha_M\beta_2$ recognizes oligosaccharides, presumably with exposed β-GlcNAc residues on GPIbα on the chilled platelet surface.

We used a panel of lectins with known specificities to identify further the carbohydrate ligands recognized on chilled platelets. Chilling induces a twofold increase in the binding to human platelets of succinyl–wheat germ agglutinin (S-WGA), a lectin specific for β-GlcNAc (Fig. 1C). Fluorescein isothiocyanate (FITC)-labeled lectins Ricinus communis I (RCA I), Maackia Amurensis (MAA), or Sambucus Nigra (SNA), specific for β-galactose (β-Gal), 2-3 sialic acid, or 2-6 sialic acid, respectively, showed no differences in binding to chilled or room temperature platelets (data not shown). Binding of these lectins to platelets is consistent with the known presence of glycans containing terminal sialic acids (*10*–*12*) and with previous documantation of RCA I binding to platelets (*13*). The enhanced binding of S-WGA after chilling of platelets cannot be reversed by warming of chilled platelets to 37°C (Fig. 1C). Thus, chilling exposes β-GlcNAc residues, a conclusion further substantiated by the demonstration that β-hexosaminidase, which hydrolyzes terminal β-GlcNAc as well as β-D-*N*-acetylgalactosamine (β-GalNAc) residues from oligosaccharides (*14*), diminishes S-WGA binding to cold platelets equivalent to S-WGA room temperature binding levels. Treatment of room temperature platelets with β-hexosaminidase had no effect on S-WGA binding (Fig. 1D). Although β-hexosaminidase digestion inhibits S-WGA binding to chilled platelets, it does not block the phagocytosis of chilled platelets by THP-1 cells (Fig. 1, C and D). In contrast, THP-1 cells also avidly ingest β-hexosaminidase–treated platelets kept at room temperature (fig. S1), suggesting that the removal of exposed β-GlcNAc residues unmasks underlying mannose residues recognized by phagocytic macrophage mannose receptors (*15*).

Exposed β-GlcNAc residues should serve as substrate for a β-1,4-galactosyltransferase enzyme that catalyzes the linkage Galβ-1GlcNAcβ1-R. In support of this prediction, masking of β-GlcNAc residues by enzymatic galactosylation inhibited S-WGA binding to cold platelets, phagocytosis of chilled platelets by THP-1 cells, and the rapid clearance of chilled platelets after transfusion into mice (Fig. 2, A to C). The enzymatic galactosylation, achieved with bovine β-1,4-galactosyltransferase and its donor substrate UDP-Gal, decreases S-WGA binding to chilled human platelets to levels equivalent to room temperature platelets. Conversely, the binding of the galactose-specific RCA I lectin increases by about twofold after galactosylation (Fig. 2A). UDP-glucose and UDP alone had no effect on S-WGA or RCA I binding to chilled or room temperature human platelets.

We found that the enzymatic galactosylation of human and mouse platelets is efficient without addition of exogenous β-1,4-galactosyltransferase (Fig. 2A). Alone, the addition of the donor substrate UDP-Gal reduces S-WGA binding and increases RCA I binding to chilled platelets, inhibits phagocytosis of chilled platelets by THP-1 cells in vitro, and prolongs the circulation of chilled platelets in mice. An explanation for this unexpected finding is that platelets reportedly slowly release endogenous galactosyltransferase activity (*16*). A least one form of β-1,4-galactosyltransferases, β4Gal T1, is present in human plasma on washed human platelets and in the supernatant fluids of washed platelets (Fig. 2D). Galactosyltransferases may associate specifically with the platelet surface (*17*, *18*). Alternatively, the activity may be plasma-derived and leak out of the platelet's open canalicular system. In either case, modification of platelet glycans responsible for cold-mediated platelet clearance is possible by simple addition of the sugar-nucleotide donor substrate, UDP-Gal.

Importantly, both chilled and nonchilled platelets show the same increase in RCA I binding after galactosylation, implying that β-GlcNAc residues are exposed on the platelet surface independent of temperature. However, chilling is a requirement for recognition of β-GlcNAc residues by S-WGA and by the $\alpha_M\beta_2$ integrin. We have previously demonstrated that chilling of platelets induces an

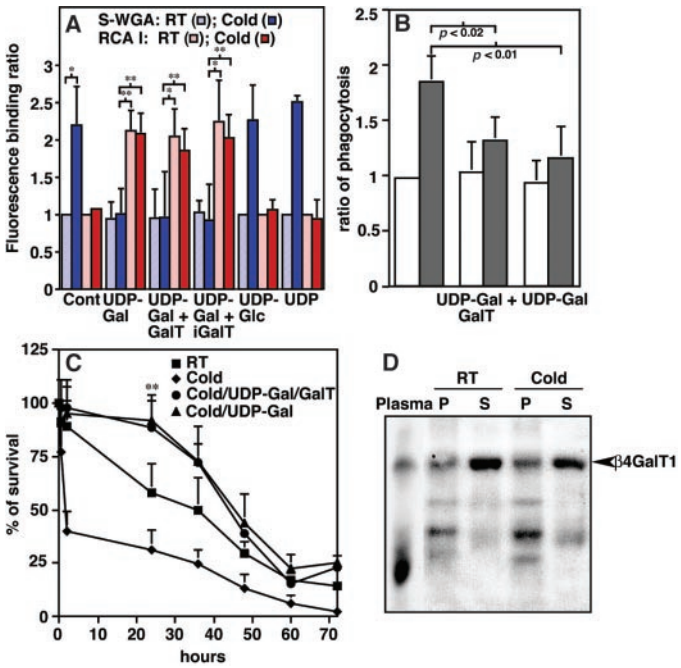

Fig. 2. Enzymatic galactosylation of platelets restores survival. (**A**) Effects on S-WGA or RCA I binding to room temperature (shaded bars) and chilled (solid bars) human platelets. The donor substrates UDP-galactose and UDP-glucose are indicated as UDP-Gal and UDP-Glc, respectively; heat inactivated bovine β-1,4-galactosyltransferase is indicated as iGalT. S-WGA or RCA I binding was compared to corresponding room temperature binding values. Mean ± SD, $n = 5$; $^*P < 0.02$, $^{**}P < 0.01$. (**B**) Effect on the phagocytosis of room temperature (open bars) or chilled (solid bars) human platelets by THP-1 cells. Mean ± SD, $n = 5$. (**C**) Effects on survival of chilled murine platelets transfused into mice. The survival of room temperature (RT) platelets is also shown. The *P* values represent comparisons between room temperature and UDP-Gal–treated chilled platelets. Recoveries were 71.8 ± 12.5% for room temperature platelets, 62.5 ± 14.4% for chilled platelets, 72.5 ± 12.1% for chilled, UDP-Gal-treated platelets with the addition of bovine β-1,4-galactosyltransferase (GalT), and 76.61 ± 9.67% for platelets treated with UDP-Gal only. Mean ± SD, $n = 21$ using RT, Cold and Cold + GalT platelets; $n = 12$ using Cold w/o GalT platelets; $^{**}P < 0.01$. (**D**) Detection of β-1,4-galactosyltransferase (β4Gal T1) in human plasma and in association with platelets. Isolated platelets were chilled (Cold) or maintained at room temperature (RT) for 2 hours and separated by centrifugation. Plasma, platelets (P), and the corresponding supernatant (S) were displayed by SDS-PAGE, transferred to membranes and blotted against a monoclonal antibody to human β4Gal T1. The results are representative of three experiments.

irreversible clustering of GPIbα (*5*). Generally, lectin binding is of low affinity, and multivalent interactions with high density of carbohydrate ligands increases binding avidity. It is possible that the local densities of exposed β-GlcNAc on the surface of nonchilled platelets are too low for recognition, but cold-induced clustering of GPIbα provides the necessary density for binding to S-WGA or the $\alpha_M\beta_2$ integrin lectin domain. We confirmed by S-WGA and RCA I binding flow cytometry that UDP-Gal transfers galactose onto murine platelets in the presence or absence of added galactosyl transferase (fig. S2), and we documented that chilled, galactosylated murine platelets circulate and initially survive significantly better than untreated room temperature platelets (Fig. 2C). Although the earliest recoveries (<2 min) did not differ between transfused room temperature, chilled, and chilled and galactosylated platelets, galactosylation abolished an initial platelet loss of about 20% consistently observed with room temperature platelets.

Galactosylation of murine and human platelets did not impair their functionality in vitro, as measured by aggregation and P-selectin exposure induced by collagen related peptide (CRP) or thrombin at concentrations ranging from maximally effective to three orders of magnitude lower (Fig. 3). The aggregation responses of unmodified and galactosylated chilled human platelets to a range of ristocetin concentrations, a test of the interaction between GPIb and activated von Willebrand factor (VWF), were indistinguishable or slightly better. This result is consistent with structural information concerning this interaction (*19*). The attachment points for N-linked glycans on GPIbα are outside the binding pocket for VWF. Moreover, mutant GPIbα molecules lacking N-linked glycans bind VWF tightly.

The cold-induced increase in binding of human platelets to $\alpha_M\beta_2$ integrin (*5*) and to S-WGA occurs rapidly (within minutes). The enhanced binding of S-WGA to chilled platelets remained stable for up to 12 days of refrigerated storage in autologous plasma (Fig. 4A). RCA I binding remained equivalent to room temperature levels under the same conditions (Fig. 4B). Galactosylation doubled the binding of RCA I lectin to platelets and reduced S-WGA binding to baseline room temperature levels. The increase in RCA I and decrease in S-WGA binding were identical whether galactosylation proceeded or followed storage of the platelets in autologous plasma for up to 12 days (Fig. 4, A and B). These results show that galactosylation of platelets to inhibit lectin binding is possible before or after refrigeration and that the glycan modification is stable during storage for up to 12 days. Platelets stored at room temperature rapidly lose responsiveness to aggregating agents; this loss does not occur with refrigeration (*20*). Accordingly, refrigerated platelets with or without galactosylation, before or after storage, retained aggregation responsiveness to thrombin for up to 12 days of cold storage.

We localized the exposed β-GlcNAc residues mediating $\alpha_M\beta_2$ lectin domain recognition of GPIbα N-glycans. The extracellular domain of GPIbα contains 60% of total platelet carbohydrate content in the form of N- and O-glycosidically linked carbohydrate chains (*12*, *21*, *22*). Binding of peroxidase-labeled WGA to GPIbα is easily detectable in displays of total platelet proteins resolved by SDS–polyacrylamide gel electrophoresis (SDS-PAGE), demonstrating that GPIbα contains the bulk of the β-GlcNAc residues on platelets, and binding of WGA to GPIbα is observable in GPIbα immunoprecipitates. UDP-Gal with or without added galactosyltransferase diminishes S-WGA binding to GPIbα, whereas RCA I binding to GPIbα increases (Fig. 5A). These results show that galactosylation specifically covers exposed β-GlcNAc residues on GPIbα. Removal of the N-terminal 282 residues of GPIbα from human platelet surfaces using the snake venom protease mocarhagin (*23*), which inhibited phagocytosis of human platelets by THP-1 cells in vitro (*5*), reduces S-WGA binding to chilled platelets to levels nearly equivalent to S-WGA room temperature binding levels (Fig. 5B). WGA binds predominantly to the N-terminus of GPIbα released by mocarhagin into platelet supernatant fluids as a polypeptide band of 45 kD recognizable by the monoclonal antibody SZ2 specific for that domain (Fig. 5C). The glycans of this domain are N-linked (*22*). A small portion of GPIbα remains intact after mocarhagin treatment, possibly because the open canalicular system of the platelet sequesters it. Peroxidase-conjugated WGA weakly recognizes the re-

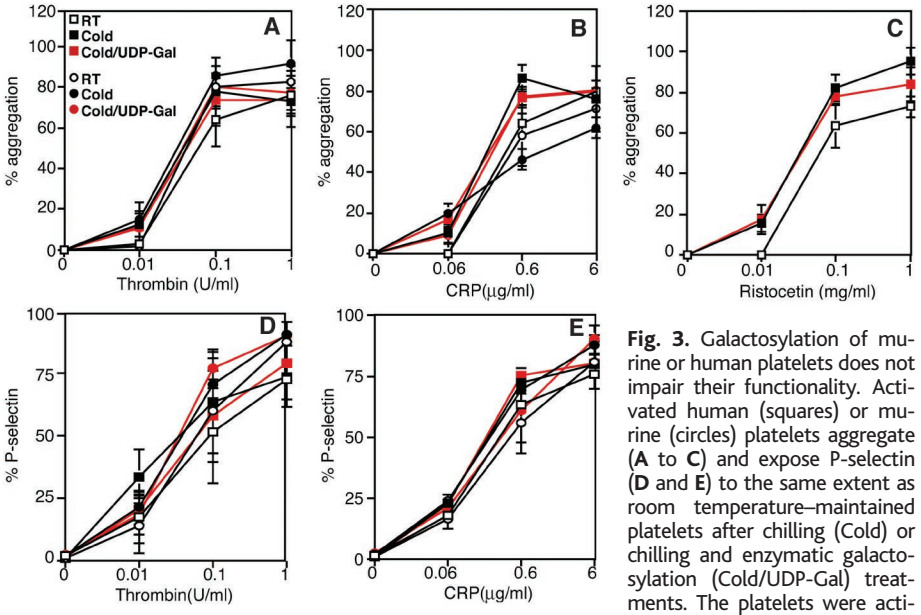

Fig. 3. Galactosylation of murine or human platelets does not impair their functionality. Activated human (squares) or murine (circles) platelets aggregate (**A** to **C**) and expose P-selectin (**D** and **E**) to the same extent as room temperature–maintained platelets after chilling (Cold) or chilling and enzymatic galactosylation (Cold/UDP-Gal) treatments. The platelets were activated with thrombin or the collagen related peptide (CRP), as indicated. Agglutination was induced by adding ristocetin to human platelet rich plasma.

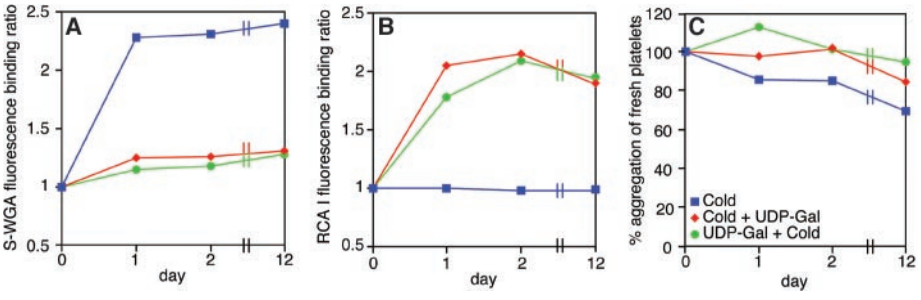

Fig 4. Galactosylation of human platelets can be accomplished before or after 12 days of storage at 4°C and does not impair platelet function. Platelet rich plasma left untreated (Cold) or platelets galactosylated in plasma before (UDP-Gal + Cold) or after the extended storage for 1, 2, or 12 days (Cold + UDP-Gal) are compared. Galactose transfer to the platelet surface was verified using (**A**) S-WGA or (**B**) RCA I binding. (**C**) Chilled or chilled and galactosylated platelets aggregate in vitro in response to 1 U/ml thrombin after being stored for 12 days at 4°C. The graphs represent the mean values of two independent experiments.

sidual platelet associated GPIbα C-terminus after mocarhagin cleavage, identifiable with monoclonal antibody WM23 (*24*) (Fig. 5C).

We have shown that the lectin domain of the $\alpha_M\beta_2$ integrin on phagocytic cells recognizes terminal β-GlcNAc residues on platelet GPIbα's N-linked glycans clustered by chilling and that this recognition results in phagocytosis and clearance of platelets exposed to cold temperatures. We have also demonstrated that enzymatic galactosylation stably masks the exposed platelet GlcNAc residues

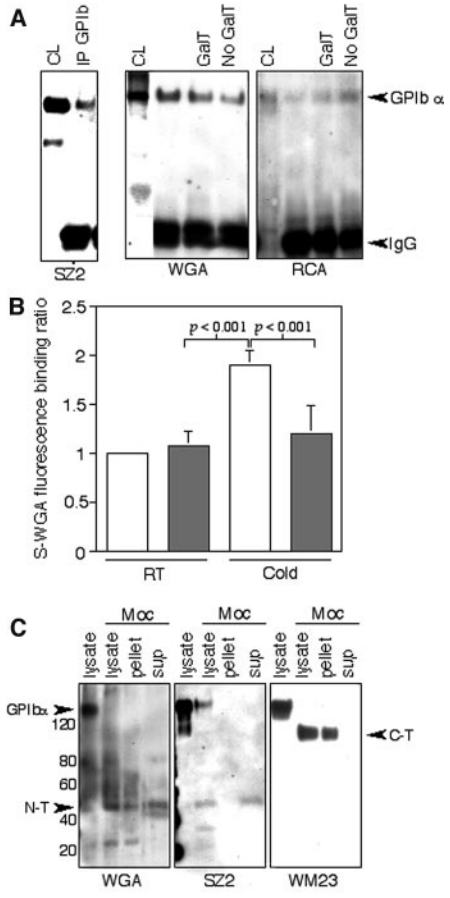

Fig. 5. Evidence that WGA-reactive β-glycans reside on GPIbα. (**A**) Co-migration of peroxidase–S-WGA and GPIbα-reactive monoclonal antibody SZ2 on SDS-PAGE of human platelet lysates, and immunoprecipitation of WGA-reactive protein by the GPIbα-reactive monoclonal antibody WM23. WGA also reacts with known β-GlcNAc residues on immunoglobulin G (IgG) (*30*). The results are representative of three experiments. (**B**) Mocarhagin treatment of platelets eliminates S-WGA binding after chilling. The open bars represent values of untreated platelets; the closed bars represent values mocarhagin-treated platelets. Mean ± SD, $n = 4$. (**C**) Peroxidase-labeled WGA recognizes the 45 kD N-terminus of GPIbα. Release of the GPIbα N-terminal by mocarhagin from platelets and detection of the fragment with WGA and with SZ2. The right-hand panel shows detection of the residual C-terminal of GPIbα with WM23. The results are representative of four experiments.

to inhibit the recognition and clearance mechanism (fig. S3). This inhibition appears anomalous at face value, because the hepatic asialoglycoprotein receptor is a well-characterized mediator of the removal of desialylated proteins with exposed galactose residues (*25*) and even of proteins with galactose residues covered with sialic acid (*26*). However, lectin recognition of glycans presumably requires more than the mere presence of a particular target sugar. For example, we investigated the interaction of human polymorphonuclear leukocytes with rabbit erythrocytes known to express abundant surface β-GlcNAc residues. As previously reported (*9*), we found that 10 mM GlcNAc inhibited phagocytosis of the erythrocytes by the leukocytes; we also observed that 10 μM β-GlcNAc inhibited this interaction, strongly implicating β-glycans in the recognition mechanism. However, addition of UDP-Gal and bovine β-1,4-galactosyltransferase to rabbit erythrocytes under conditions that modify platelet glycans had no detectable effect on the phagocytosis of the erythrocytes (fig. S4A). Basal S-WGA binding to these cells was an order of magnitude greater than to platelets, suggesting that the high density of β-GlcNAc residues on rabbit erythrocytes leads to recognition and phagocytosis by $\alpha_M\beta_2$ integrin, whereas clustering of a much smaller number of exposed β-GlcNAc residues on chilled platelets leads to their recognition by the phagocyte $\alpha_M\beta_2$ integrin. Consistent with this conclusion, we found that chilling a mixture of human platelets and polymorphonuclear leukocytes with rabbit erythrocytes led to selective binding of the platelets to the leukocytes (fig. S4B). Other evidence in favor of this interpretation: Sialyltransferases ST3Gal-IV and ST6Gal-I can mask galactose residues recognized by the asialoglycoprotein receptor, but only ST3Gal-IV deficiency enhances the clearance of von Willebrand factor and diminishes platelet counts in mice (*27*). The level of expression of exposed galactose residues on the ST3Gal-IV-deficient platelets, documented by RCA I binding, is an order of magnitude greater than we observed after galactose modification of human or murine platelets (*27*) (fig. S2).

UDP-Gal is a normal constituent of human cells, and micromolar free UDP-Gal concentrations are detectable in body fluids (*28*). Endogenous galactose production in humans is 2 grams per day (*29*). Most sialyltransferase-deficient animals have no major phenotypic abnormalities, and we observed no ill effects on infusing 12.5 μg of UDP-Gal into mice during platelet transfusion experiments that lasted up to a week. Therefore, the simple addition of UDP-Gal to platelets before or after refrigeration is probably safe and offers a compelling strategy to secure the circulation of chilled platelets. If glycan modification safely prolongs the circulation of refrigerated platelets in humans, it could contribute to better control of platelet inventories, diminution of bacterial contamination, and increased ability to provide platelets of improved functional quality to patients needing platelet transfusion.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/301/5639/1531/DC1
Materials and Methods
Figs. S1 to S4
References

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