Abstract. Human natural anti-α-galactoside (anti-Gal) and anti-β-glucoside (ABG) antibodies were previously reported to recognize the serine- and threonine-rich peptide sequences (STPS) of albumin-associated O-glycoproteins (AOP1 and AOP2) as surrogate antigens, forming anti-Gal/ABG-AOP1/AOP2-albumin triplet immune complexes in plasma. Since antibodies in these triplets still possessed unoccupied binding sites, the presence of triplets on human platelets that abound in surface O-glycoproteins was examined. Upon treatment with α-galactosides and β-glucosides, normal platelets freshly isolated from young healthy individuals released triplets identical with plasma triplets according to ELISA results. The resulting denuded platelets, unless pre-treated with fibrinogen or the O-glycan-binding lectin jacalin, recaptured these sugar-extracted triplets in the absence of antibody-specific sugars, suggesting that the triplet antibodies recognized the STPS of O-glycosylated receptors on platelets. Molecular weight of the dominant jacalin-binding subunit on triplet-free platelet membrane was 116 kDa, close to the ~120 kDa reported for the IIb subunit of the most abundant fibrinogen-binding platelet O-glycoprotein, GPIIb/IIIa. Denuded, but not native, platelets underwent slow spontaneous aggregation and rapid ADP-mediated GPIIb/IIIa-dependent aggregation according to spectrophotometric assay. Pre-treatment of denuded platelets with jacalin significantly reduced their ADP-mediated aggregation. Amyloid β (Aβ-42 monomer) was reported to bind triplet O-glycoproteins through their STPS but not to albumin or the antibodies. This peptide bound to the triplets on normal platelets and to surface membrane O-glycoproteins on denuded platelets, suggesting that the surface O-glycoproteins on the normal platelets were engaged and masked by the triplets. The ABG-specific sugar glucose denuded the platelets at concentrations typically reached in diabetic sera, since anti-Gal specific or ABG-specific sugar released the triplets of both the antibodies from the platelets. In conclusion, the present study offered rationale for the presence of anti-Gal/ABG-O-glycoprotein-albumin triplets on normal platelets, for the role of triplets in platelet physiology amidst circulating platelet-activating factors such as ADP, and for platelet vulnerability during diabetes.

Introduction

A fraction of circulating human albumin molecules are non-covalently attached to either of the two recently discovered albumin-associated O-glycoproteins (AOP1:107 kDa and AOP2: 98 kDa) that are heavily O-glycosylated (1). Most, if not all, plasma anti-α-galactoside (anti-Gal) and anti-β-glucoside (ABG) antibodies are bound to albumin-associated AOP1 or AOP2 to form antibody-AOP1/AOP2-albumin triplets (1) due to recognition of the serine- and threonine-rich peptide sequences (STPS) on the O-glycoproteins as surrogate ligands by either antibody (2). As a result, plasma anti-Gal and ABG samples prepared by affinity chromatography (APAG and APABG respectively) mostly consisted of triplets aforementioned (1). Whilst purified AOP1 and AOP2 occupy all available binding sites on either antibody, albumin-bound AOP1 or AOP2 can occupy only some of these sites during triplet formation, possibly due to steric hindrance (1). Utilizing the free binding sites available on their antibodies, triplets can bind to affinity chromatography matrices containing antibody-specific ligands immobilized on them (1) and to activated human macrophages (unpublished findings). This is possibly because the ligands available on these two systems are more accessible compared with those on albumin-bound
AOP1 or AOP2. Since amyloid β (Aβ-42) recognizes STPS, isolated AOP1 and AOP2, their complexes with albumin or the complete triplet, but not purified albumin, can bind to this peptide (3).

Factors that prevent platelet-activating blood factors such as ADP and fibrinogen from causing aberrant platelet activation remain poorly understood. Notably, glycoprotein GPIIb/IIa, which is the most abundantly expressed protein on the surface of platelets, serves as a fibrinogen receptor and acts as a link in the ADP-mediated platelet activation cascade and aggregation (4). GPIIb/IIa is heavily O-glycosylated and therefore rich in STPS, especially on its IIb subunit (4). Furthermore, the levels of immunoglobulins and albumin carried by platelets at any given time are reported to vary in parallel (5). Platelets are also major carriers of Aβ-42 (6), which had been reported to bind triplet O-glycoproteins in plasma (3). The aim of the present study was to investigate the presence of anti-Gal/ABG-AOP1/AOP2-albumin triplets, anchored using the unutilized binding sites of their antibodies, on the surface of normal human platelets using the STPS of O-glycoprotein(s) on platelet surface membrane as ligands. In addition, the consequence of denuding the platelets by removal of their triplet complexes were examined. Although hyperglycemia is the hallmark of diabetes, the molecular mechanism underlying the pathophysiological changes induced by hyperglycemia, including vascular diseases, platelet dysfunction, platelet-leukocyte adhesion and increased susceptibility to Alzheimer’s disease, remain elusive. In this context, the role of high concentrations of glucose, which is an ABG ligand, assumes clinical relevance, as diabetes has been reported to increase platelet aggregation (7). Therefore, the results of the present study may help to determine whether a shield containing a natural immune complex, which carries the platelet-bound Aβ-42, prevents platelet aggregation and masks reactive surface proteins on the platelet. This may be a defence system used by the body against diabetes-driven platelet malfunctions, such as premature aggregation and platelet-leukocyte adhesion, which can lead to vascular diseases.

Materials and methods

Reagents, proteins and conjugates. Fluorescein isothiocyanate (FITC), methyl α-D-mannoside (MaM), methyl α-D-galactoside (MaG), cellobiose (4-O-β-D-Glucopyra nosyl-D-glucose), orthophenylene diamine (OPD), horseradish peroxidase (HRP; cat. no. P-8375), neuraminidase from Clostridium perfringens, soybean trypsin inhibitor, galactose, Tween-20, Coomassie brilliant blue (G-250 and R-250), soluble guar galactomannan, fibrinogen, ADP and rabbit antibodies to human albumin (cat. no. A3293) were purchased from Sigma Aldrich; Merck KGaA, HiLyte™ Fluor-488-labelled amyloid β peptide Aβ-42 monomer (cat. no. AS-60479) was purchased from AnaSpec, Inc. Polystyrene 96-well microplates (Maxisorb BREAKAPART and Polysorb BREAKAPART) were purchased from Nunc; Thermo Fisher Scientific, Inc. and antibodies to human IgA (cat. no. Q0332), IgG (cat. no. Q0331) and IgM (cat. no. Q0333) raised in rabbit, were from Dako, Agilent Technologies, Inc. Jacalin was prepared from jackfruit (Artocarpus integri folia) seeds according to the method described by Suresh Kumar et al (8). Using affinity chromatography, lectins concanavalin A (ConA) was prepared from the seeds of Canavalia ensiformis and peanut agglutinin (PNA) was prepared from peanuts (1). Trypsin inhibitor-cellobiose (TIC) and trypsin inhibitor-melibiose (TIM) were prepared by coupling cellobiose or melibiose (Sigma Aldrich; Merck KGaA) to soybean trypsin inhibitor by reductive amination (9). Yeast glycoprotein was isolated from Saccharomyces cere visiae (yeast type-II; Sigma Aldrich; Merck KGaA). Briefly, yeast (5 g) was extracted to PBS by three successive -20˚C to +2˚C freeze-thaw cycles. After homogenization and sonication at 25˚C (six 30-sec bouts at 6 micron amplitude in SONIPREP probe sonicator; MSE (UK) Ltd.), the yeast protein samples were resolved by electrophoresis in 7% polyacrylamide gel and the two slowest-moving proteins were electroeluted together (10). Previously reported procedures were used to prepare affinity-purified samples of plasma anti-Gal (APAG)- and ABG (APABG)-containing triplets using cross-linked guar galactomannan gel (11) and cellulose (12) in the affinity matrices, respectively. APAG and APABG were triplet complexes containing anti-Gal and ABG antibodies, respectively (1). The source of these samples were outdated human plasma obtained between 1 March and 31 August, 2017 following approval from the Institutional Ethics Committee, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram (approval no. IEC/674). Plasma was prepared from blood of 50 healthy voluntary donors (male, 40; female, 10) aged 22-45 years (32±6 years) selected without gender bias at the Department of Transfusion Medicine of the Sree Chitra Tirunal Institute for Medical Sciences and Technology. Donors with high blood pressure, ongoing infections or dependence on any narcotics were excluded. Plasma samples declared outdated by the Institute after storage at -80˚C for ≥1 years were thawed at 4˚C and used within 3 days thereafter. Purified samples of AOP1, AOP2, anti-Gal, ABG and human serum albumin (HSA), without contamination by each other due to non-covalent interactions, were isolated by the alkaline electrophoretic separation of APAG or APABG from plasma or platelets into their individual components (1). Electrophoretically purified samples of albumin, anti-Gal, AOP1 and AOP2 were labelled with FITC by treating with FITC (150 µg per mg protein) in 250 mM carbonate-bicarbonate buffer, pH 9.0 overnight followed by dialysis against PBS at 4˚C (13).

The number of platelets in PBS medium were counted using the ABX Pentra 60 C+ haematology analyser (Horiba Medical), which counts platelets using focused flow impedance measurement through a double hydrodynamic sequential system. To prepare HRP conjugates of proteins (antibodies against the following human proteins: Albumin, IgG, IgM and IgA, and lectins ConA, PNA and jacalin), HRP was oxidized using sodium periodate (1 b), dialyzed overnight against sodium carbonate buffer pH 9.5 at 25˚C and treated with the protein in a 2:3 ratio of HRP and protein for 2 h at 4˚C before the product was changed to PBS medium by dialysis at 4˚C.

Preparation of native and denuded (triplet-free) platelets. Fresh blood samples were collected from 50 (male, 30;
female, 20) healthy volunteers aged 23-50 years (32±6 years), using the procedure described by Jennings and Philips (14) after obtaining informed consent in writing in accordance with the guidelines of the Institutional Ethics Committee (permission no. IEC/1072). Blood samples collected in anti-coagulant (heparin)-treated tubes (10 ml) were centrifuged at 150 x g for 5 min at 25°C. The supernatant was then mixed with 100 mM EDTA (1:1) to prevent platelet activation and centrifuged at 25°C, first at 230 x g for 10 min to remove erythrocytes and again at 4,530 x g for 15 min. The final supernatant was removed and the pellet was collected as platelets. This platelet pellet was suspended in 300 µl PBS and one half diluted to 300 µl with PBS was incubated for 2 h at 25°C with a mixture of MoG and cellobiose (both 15 mM), which are sugars specific for anti-Gal and ABG respectively. The triplets extracted from the platelets were collected as the supernatant after centrifugation at 4,530 x g for 15 min at 25°C. The pellet was washed twice with PBS and collected as denuded (triplet-free) platelets. The second half of platelets that was treated similarly but with the non-specific sugar MoM (30 mM), yielded native non-denuded platelets.

Resolution of triplet components by alkaline PAGE. Electrophoresis of triplets extracted from the platelets after incubation with MoG and cellobiose was performed in 6% polyacrylamide gel tubes in Tris-glycine pH 8.3 buffer at 4°C with 50 µg triplet per tube. One gel was stained with Coomassie Brilliant blue G-250 for 1 h at 25°C. Bands of O-glycoproteins AOP1 and AOP2, albumin and antibodies (anti-Gal and ABG that move together) were compared with the electrophoretic mobility of components of plasma triplets (mixture of APAG and APABG; 25 µg each) (1). Corresponding segments of unstained gels were cut and each protein was electroeluted separately. The two O-glycoprotein bands were also eluted together where required to obtain AOP, which is a mixture of AOP1 and AOP2 in the ratio in which they occur on the platelet-bound triplets.

ELISA for ligand binding and inhibition assays General procedures. Macromolecules [thryoglobulin (Tg), TIC, TIM, guar galactomannan or yeast glycoproteins] were coated on Nunc MAXISORB microplate wells (maximum 1 µg in 200 µl PBS) by incubation at 37°C for 3 h or overnight at 4°C. The wells were washed with PBS containing 0.05% Tween-20 (PBST) and blocked by incubation for 30 min at 37°C with PBS containing 0.5% Tween-20. After another wash with PBST, the wells were incubated for 2 h at 4°C with 200 µl PBST containing the primary reactants (sugar-extracted platelet-bound triplets or antibodies separated from them) pre-incubated with or without specific sugars. Wells were washed with PBST again and treated with HRP-labelled secondary reactants (anti-immunoglobulin antibodies for antibody assay or anti-albumin antibody for triplet assay). After washing the plates again three times with PBST, bound HRP was measured by adding 200 µl OPD (0.5 mg/ml) dissolved in 0.1 M citrate-phosphate buffer (pH 5.0) containing 0.03% H₂O₂ for 15 min at 25°C, followed by the addition of 50 µl 12.5% H₂SO₄ to stop the reaction. Absorbance was measured at 490 nm using an ELx800™ ELISA reader (BioTek Instruments, Inc.).

**Anti-Gal and ABG assay.** To measure anti-Gal or ABG in the platelet-derived triplets, 100 ng immunoglobulin was pre-incubated with the anti-Gal-specific sugar MoG, ABG-specific sugar cellobiose (each 25 mM) or no sugar in 200 µl PBS for 2 h at 4°C. This was inoculated into ELISA microplate wells coated with Tg or TIC (1 µg per well) for 2 h at 4°C and washed as described above. The levels of bound antibody were determined by treatment with a mixture of equal amounts of HRP-conjugated antibodies against human IgG, IgA and IgM (0.3 µg total antibody in 200 µl PBST; 2 h at 4°C), washing and measurement of bound HRP as aforementioned.

**Triplet assay.** To assay sugar-extracted platelet-bound triplets, 300 µl platelet suspension (7.5x10⁷ cells) in PBS was incubated for 2 h at 25°C with the non-specific sugar MoM, the ABG-specific sugars cellobiose, glucose or the anti-Gal-specific sugar MoG (15 mM each). Following centrifugation at 4,500 x g for 15 min at 25°C, 250 µl supernatant containing the triplets extracted from the platelets was dialyzed against PBS at 4°C (6 h, 3 times) to remove the sugars. Afterwards, 1:100 dilution of this dialysate (200 µl) was added into the microplate wells coated with guar galactomannan (1 µg per well) to capture anti-Gal complexes or with yeast glycoproteins to capture the ABG complexes. After incubation for 2 h at 4°C, wells were washed with PBST and bound complexes were determined by incubation with rabbit anti-human albumin-HRP (0.75 µg antibody in 200 µl PBST; 2 h at 4°C). Bound HRP was measured using ELISA as aforementioned.

**Assay of triplet distribution in platelets and plasma.** From the platelet samples harvested as aforementioned, 8x10⁷ cells in 320 µl PBS were treated with a mixture of MoG and cellobiose (15 mM each) at 25°C for 2 h. The mixture was centrifuged at 4,500 x g for 15 min at 25°C and the supernatant (250 µl) was dialyzed against PBS at 4°C (two 6-h dialysis cycles). This sample and cell-free plasma from the same donor (supernatant after removing all cells by centrifugation at 5,000 x g for 15 min at 25°C) were diluted 100X in PBST before 200 µl was added into microplate wells coated with TIM or TIC (1 µg per well) to estimate the levels of triplets from anti-Gal and ABG, respectively, using rabbit anti-human albumin-HRP. Bound HRP was measured using ELISA as aforementioned.

**Assay of triplet binding to fibrinogen-treated denuded platelets.** Platelets (8.75x10⁷ cells in 350 µl PBS) were incubated with 1 µM fibrinogen at 25°C for 2 h. After centrifugation at 4,500 x g for 15 min at 25°C and removal of the supernatant, the pellet was washed twice with PBS and incubated in 300 µl PBS containing 2 µg triplets released from normal platelets (using MoG and cellobiose, both 25 mM) for 4 h at 25°C. Unbound triplets (200 µl supernatant collected after centrifugation at 4,500 x g for 15 min at 25°C) was added to wells coated with a mixture of TIM and TIC (0.5 µg each). After 2 h incubation at 4°C and washing, the level of triplets was assayed using incubation with rabbit anti-human albumin-HRP (0.75 µg antibody in 200 µl PBST). Bound HRP was measured using ELISA as aforementioned.
SDS electrophoresis, western blotting and lectin staining of platelet surface proteins. Native platelets from normal blood (10 ml samples) prepared as aforementioned were lysed in cold hypotonic buffer [2 h in 7.5 mM phosphate buffer (pH 7.3) containing 10 mM EDTA; 600 µl]. The lysate was centrifuged at 100,000 x g for 1 h at 4°C to retrieve a fraction enriched with platelet surface proteins as the pellet. Bradford protein assay was used to measure protein concentration and SDS-PAGE (7.5%) of this sample was performed as described by Laemmli (15), loading ~30 µg protein per lane. Separated proteins were transferred onto PVDF membranes using protocols previously described by Towbin et al (16). Strips 3-4 mm in width were cut out from the transfer membrane, blocked with PBS containing 0.2% Tween 20 at 25°C for 2 h. The strips were then treated with HRP-conjugated lectin (concanavalin A-HRP; 150 µg lectin per ml); PNA-HRP; 75 µg lectin per ml before and after neuraminidase treatment (100X dilution in PBS of 1.5 U per ml; 1 h at 37°C), and PNA-HRP as above in presence of lactose control (25 mM) after prior neuraminidase treatment of the strip or jacalin-HRP (0.75 µg per ml) for 2 h at 4°C. The strips were then washed twice with 0.05% PBST and once with PBS alone, before being stained with freshly prepared 4-chloronaphthol solution (1 mg in 0.4 ml methanol mixed with 1.6 ml PBS containing 0.05% H2O2). As the protein bands appeared, the strips were washed in PBS. To assess relative O-glycan content in the protein bands, jacalin binding and amido black (0.1% in 4% ethanol, 1% acetic acid) binding responses to them were compared by digital scanning using ImageJ 1.53e software (National Institutes of Health).

Platelet aggregation assay. In total, 6.3x10⁷ denuded or non-denuded platelets were treated with 20 µM ADP in 250 µl PBS at 25°C and absorbance was measured after 2 min at 405 nm using an ELx800™ ELISA reader (BioTek Instruments, Inc.). A reduction in absorbance was considered as a measure of aggregation.

Fluorescence assay. FITC-labelled proteins and HiLyte-Fluor-488-labelled amyloid β in free or platelet-bound form were assayed by measuring the fluorescence of their solutions or suspensions of 300 µl PBS in Nunc Polysorb BREAKAPART microplates using a FLx800™ fluorescence reader (BioTek Instruments, Inc.) with the excitation wavelength of 485 nm and emission wavelength 528 nm (485/528 nm). To confirm the identity of plasma triplets and platelet-bound triplets, attachment of FITC-labelled anti-Gal and triplets to denuded platelets was studied. To prepare de novo plasma triplet samples 600 ng anti-Gal in 200 µl PBS was treated for 2 h at 4°C with 25 mM anti-Gal specific sugar MαG or the non-specific sugar MoM and added to a pre-incubated (2 h at 4°C) mixture of AOP1, AOP2 and FITC-labeled albumin (200 ng each in 200 µl PBS) and incubated again for 2 h at 4°C. Denuded or native platelet suspensions (7.5x10⁷ cells in 300 µl PBS) were mixed with either of the following: i) Glycoprotein-free anti-Gal-FITC (600 ng in 400 µl PBS) pre-incubated for 2 h at 4°C with 25 mM MoG (specific) or MoM (non-specific) (1); or ii) de novo plasma triplets as aforementioned (400 µl). The mixture was incubated for 2 h at 4°C and centrifuged at 4,500 x g for 15 min at 4°C to remove the supernatant. Pellet was washed twice with PBS and re-suspended in 320 µl PBS. Cell-bound fluorescence was measured using a 300 µl suspension at 485/528 nm.

To characterize the cell surface molecule recognized by triplets for anchoring on platelets, denuded platelets (6.3x10⁷ cells in 250 µl PBS) were incubated with 50 ng jacalin or heat-inactivated (95°C for 2 min) jacalin for 1 h at 4°C before the supernatant was removed by centrifugation at 4,500 x g for 15 min at 4°C. Platelets in the pellet were washed twice with PBS and incubated at 25°C for 3 h in 400 µl PBS containing either 600 ng glycoprotein-free anti-Gal-FITC or 400 µl de novo triplet reconstituted from components of platelet-derived samples, with FITC-labelled albumin. After washing three times in PBS by centrifugation at 4,500 x g at 25°C for 15 min each, the pelleted was re-suspended in 320 µl PBS before cell-bound fluorescence was measured at 485/528 nm.

To measure amyloid β binding to platelets, denuded or native platelets in 320 µl PBS (8x10⁶ cells) were incubated for 2 h with 75 ng HiLyte™ Fluor-488-labelled amyloid β at 25°C. Following centrifugation at 4,500 x g for 15 min at 25°C fluorescence in the supernatant was measured at 485/528 nm in a 300 µl aliquot. The pellet was washed twice with PBS and incubated for 1 h at 25°C with a mixture of anti-Gal- and ABG-specific sugars MoG and cellubiose (15 mM each) in 350 µl PBS before the triplet-bound amyloid β released from specific sugars was measured using 300 µl of supernatant at 485/528 nm.

Density gradient ultracentrifugation (DGUC). Plasma made cell-free by centrifugation at 5,000 x g for 30 min at 4°C, AOP1-FITC or AOP2-FITC was incubated with or without sugar molecules for 2 h at 4°C before the density was increased to 1.24 g/cm³ by adding solid KBr. The resulting solution (1.1 ml in 1.3 ml tubes) was centrifuged in a Hitachi CS 15 GXII microcentrifuge (Hitachi, Ltd.) at 535,000 x g for 15 min at 4°C. The middle (400 µl) and bottom (300 µl) layers were separately saved and used for fluorescence assay.

Statistical analysis. Data are presented as the mean ± SD unless otherwise specified. Statistical significance as judged from two-tailed P-values, following analysis of the results using Microsoft Excel 2010 (Microsoft Corporation) and GraphPad Prism 5 (GraphPad Software, Inc.), was determined by using unpaired student's t-test except for Figs. 2, 4 and 7. For Fig. 2, one-way ANOVA followed by Dunnett's test and for Fig. 4, one way ANOVA followed by Tukey's post hoc test were employed. For Fig. 7, the data was analyzed using two-way ANOVA followed by Sidak’s post hoc tests, by using native/denuded triplets as one factor and ADP/Jn as the second factor. P<0.05 was considered to indicate a statistically significant difference.

Results

Albumin and either of the two O-glycosylated proteins are attached through anti-Gal or ABG to platelets. Following alkaline polyacrylamide gel electrophoresis, protein samples extracted from platelets using a mixture of 15 mM each of MoG (specific for anti-Gal) and cellubiose (specific for ABG), exhibited bands with mobilities identical to those of a mixture of equal amounts of APAG and APABG (triplets of anti-Gal...
A mixture of guar galactomannan gel (11) and cellulose (12) could capture all the immunoglobulins out of 0.5 ml PBST, suggesting that anti-Gal and ABG were the respective affinity matrices for anti-Gal and ABG. Electroeluted immunoglobulins also contained ABG which were released by specific sugars from the platelets was confirmed by capturing them on microwells coated with jacalin and probing the bound proteins using HRP-labelled anti-albumin as performed in the case of plasma triplets previously (1). Albumin has no direct association with anti-Gal or ABG, whilst O-glycoproteins identical in mobility and O-glycan content with those of AOP1 and AOP2 of plasma triplets were present in the released proteins. These results suggest that the proteins released by antibody-specific sugars from platelets contained O-glycoprotein molecules that bridged between anti-Gal or ABG on one side and albumin on the other to form triplets of the same structure as that of plasma anti-Gal/ABG-AOP1/AOP2-albumin triplets (1). Since these triplets possessed binding sites that remained free on their antibodies and were capable of binding to ligand-bearing matrices and cells, the aforementioned results suggested that platelet membranes carry receptor molecules that possessed ligands for anti-Gal and ABG, which could capture the triplets by utilizing their free binding sites. Notably, glucose (15 mM)
released nearly as many triplets from the platelets as the same concentration of MrG or cellobiose did (Fig. 2). Since this level of serum glucose in circulation is frequently observed under a diabetic setting this finding suggests that the consequences of depriving platelets of their triplets accompany diabetes.

Although anti-Gal and ABG share the affinity for STPS (1), their specificities towards small sugars are distinct, irrespective of if they were isolated from plasma triplets or platelet-bound triplets (Fig. 1). Nevertheless, the same amounts of anti-Gal triplets and ABG triplets were released by either the ABG-specific sugar or the anti-Gal-specific sugar (Fig. 2).

To verify the observations involving the small sugar-mediated release of free albumin and O-glycoproteins from triplets, the differential distribution of AOP1-FITC or AOP2-FITC, added to sugar-treated and untreated plasma, was examined. Following DGUC of 1.1 ml KBr-treated plasma, undissociated triplets were found predominantly in the bottom 300 µl, which was mostly due to the presence of immunoglobulins (1). However, in the case of plasma treated with antibody-specific sugar, the albumin-AOP1 and albumin-AOP2 complexes liberated from triplets migrated from the antibody-rich bottom layer to the antibody-free and albumin-rich middle layer (400 µl), apparently due to the high buoyancy of albumin, although free AOP1/AOP2 mostly occupied the bottom layer under these conditions (1). The results in Fig. 3 show that the majority of AOP1-FITC and AOP2-FITC added to PBS or untreated plasma remains in the bottom 300 µl of the 1.1 ml sample subjected to DGUC. However, AOP1-FITC and AOP2-FITC, added to plasma that was pre-treated with anti-Gal- and ABG-specific sugars before DGUC, migrated mostly to the middle layer, suggesting that free albumin that was ready to interact with free AOP1/AOP2 or their FITC derivatives was released in this case.

Release of fresh free albumin is also accompanied by the release of free AOP1 and AOP2, which were bound to the albumin. Free AOP1 and AOP2 could react with either antibody in a similar manner to the small sugar ligands (1), which possibly explains the phenomenon of sugar specific to only one antibody ably releasing triplets of both antibodies (Fig. 2).

Platelet-bound and plasma triplets are identical. Platelets deprived of their attached triplets following treatment with a mixture of anti-Gal- and ABG-specific sugars (denuded platelets) were treated with FITC-labelled anti-Gal or with plasma triplets reconstituted using FITC-labelled albumin, anti-Gal and

Figure 3. Specific sugars release free albumin from triplets. Plasma diluted with PBS to 1 mg protein per ml was treated with cellobiose and methyl α-D-galactoside (25 mM each), incubated for 3 h at 4°C and again for 3 h at 4°C after addition of AOP1-FITC or AOP2-FITC to a final concentration of 1 mg per ml. After density gradient ultracentrifugation, total fluorescence in middle (400 µl) and bottom (300 µl) layers was measured. "P<0.0001. Data are presented as the mean ± SD of six donors; AOP, albumin-associated O-glycoprotein.

Figure 4. Platelet-bound and plasma triplets are identical. Attachment of FITC-labeled anti-Gal or anti-Gal triplets from plasma to native and denuded platelets was monitored by fluorescence assay. Fluorescence attached to denuded platelets treated with labeled anti-Gal or triplet, incubated in advance with specific or non-specific sugar, was measured. Data are presented as the mean ± SD of eight donors. "P<0.0001. AOP refers to a mixture of AOP1 and AOP2. Gal, α-galactoside; MrM, methyl α-D-mannoside; MrG, methyl α-D-galactoside; HSA, human serum albumin.
a mixture of AOP1 and AOP2. Plasma anti-Gal alone, in addition to reconstituted plasma triplets, was captured by denuded platelets but not in the presence of the specific sugars. By contrast, native platelets captured significantly less of both free anti-Gal and its corresponding reconstituted triplets (Fig. 4). Since reconstituted plasma triplets added to the denuded platelets contained FITC-labelled albumin and their attachment to these platelets was inhibited by antibody-specific sugars, these results suggest that antibody-O-glycoprotein-albumin triplets anchored onto the platelets using the remaining free binding sites available on their antibodies.

As triplets from cell-free plasma could substitute for platelet-bound triplets and the O-glycoproteins contained within the triplets from the plasma and platelets were equal, both in terms of alkaline gel electrophoretic mobility and in O-glycan content, it was hypothesized that the triplets of the same composition and structure exist in free form in the plasma or bound to platelets. These triplets have been previously found to be consisted of albumin and either anti-Gal or ABG linked by AOP1 or AOP2 as the bridging molecule (1).

**Table I. Triplet distribution in blood (n=8).**

| Triplet antibody | Plasma (ng albumin/mm³) | Platelet (ng albumin/mm³) |
|------------------|-------------------------|---------------------------|
| Anti-Gal         | 52.8±1.61               | 62.9±1.41*                |
| ABG              | 58.6±1.72               | 66.7±1.21*                |

*P<0.0001 vs. Plasma. Distribution of triplets in plasma and platelets, expressed as albumin content measured using anti-albumin-HRP response in ELISA for triplets of either antibody separately. Response of the respective plasma triplets (100 ng) was used as standard.

**Figure 5. Antibody-O-glycoprotein-albumin triplet and anti-Gal bind to O-glycosylated membrane glycoproteins on the surface of denuded platelets.** (A) Denuded platelets with or without their O-glycoproteins blocked using jacalin were treated with FITC-labeled anti-Gal or its corresponding triplet reconstituted by mixing with HSA and AOP. "*"P<0.001. (B) From plasma triplets added to denuded platelets which had been pre-treated with or without fibrinogen, the level of unbound triplets was assayed by ELISA. "**"P<0.0001 for fibrinogen-treated vs. untreated denuded platelets. Data are presented as the mean ± SD of six donors. AOP, mixture of AOP1 and AOP2; Jn, jacalin; HSA, human serum albumin; for fibrinogen-treated vs. untreated denuded platelets. F, fibrinogen; Gal, α-galactoside.

Anti-Gal and its triplets recognize the O-glycosylated region of their platelet surface glycoprotein receptor. Jacalin was used to block the O-glycosylated regions of proteins on the surface of denuded platelets. FITC-labelled anti-Gal and FITC-labelled triplets of anti-Gal (reconstituted de novo using anti-Gal, AOP1, AOP2 and FITC-labelled albumin) were prepared from components of sugar-extracted triplets of platelets. Neither FITC-labelled anti-Gal nor FITC-labelled triplets could bind to the denuded platelets that were pre-treated with jacalin. Heat-inactivated jacalin, however, did not effect this blocking of...
rebinding of anti-Gal or triplet (Fig. 5A). Since ABG shares the same STPS specificity with anti-Gal, the aforementioned results suggest that the platelet surface O-glycosylated protein(s) may serve as ligands for the unoccupied binding sites of anti-Gal- and ABG-derived triplets.

Subsequently, binding of triplets to the denuded platelets was also assessed by determining the percentage of the remaining unbound triplets in the supernatant of a limited quantity of triplets added to the denuded platelets in suspension. Pre-treatment of denuded platelets with fibrinogen (1 µM) resulted in the complete blocking of triplet binding to denuded platelets (Fig. 5B). Presence of 1 µM fibrinogen did not affect the ELISA response of the triplet sample used (data not shown).

Western blotting of proteins from these membranes revealed a number of concanavalin A-binding N-glycosylated proteins, but only one O-glycosylated subunit (arrow) was detected by jacalin, which recognizes the core-1 type O-linked oligosaccharides regardless of the presence of terminal sialic acid moieties on them (Fig. 6). The presence of the aforementioned oligosaccharides on this subunit was confirmed by the sugar-dependent binding of peanut agglutinin to its desialylated form. Furthermore, the ratio of responses of individual protein subunit bands to jacalin-HRP and amido black, represented by the areas under the peaks generated by ImageJ analysis (Table II), were 0.8095 for the protein band recognized by jacalin and a mean of 0.0209 (SD: 2.38%) for four of the other prominent protein bands in Fig. 6 (lane 1). The marked jacalin reactivity of the strongly jacalin-reactive band, despite lectin-carbohydrate interactions being much weaker [association constant (K_a) ~1.67x10^5 M^-1 for jacalin binding to fetuin] (19) than antigen-antibody interactions (K_a in the range 1x10^6-10^8 M^-1) (20), suggesting its high O-glycan content. The size of this dominant O-glycoprotein on the platelet surface was determined to be 116 kDa (Fig. 6), a value close to the reported molecular weights of 120 (21), 118 (22) and 125±15 kDa (23) for the GPIIb subunit, implying the possibility that GPIIb/IIIa acts as the dominant, if not the sole, platelet ligand for anti-Gal and ABG in the triplets.

Triplet-free platelets are more prone to spontaneous aggregation whilst ADP-induced aggregation is suppressed by prior jacalin treatment. Aggregation of platelets in suspension was monitored in terms of reduction in the absorbance of the suspension at 405 nm. Denuded platelets showed a significantly increased tendency for spontaneous aggregation compared with native platelets (Fig. 7). However, ADP addition caused
an insignificant increase in the aggregation of native platelets, though it caused a significant increase in the aggregation of denuded platelets (Fig. 7). Treatment with jacalin prior to ADP addition did not affect the ADP-induced aggregation of native platelets (Fig. 7), but it significantly reduced the aggregation of denuded platelets. This protection by jacalin of denuded platelets from ADP-mediated aggregation involved the binding by the lectin to O-glycan-bearing receptors on the latter, since the heat-inactivated version of jacalin could not offer this protection (Fig. 7). There was no difference between the extent of aggregation of jacalin-treated denuded platelets and their native counterparts (Fig. 7), whilst there was significantly greater aggregation by denuded platelets compared with native platelets when inactivated jacalin was used (Fig. 7). These results suggested that an O-glycosylated membrane component that is crucial for ADP-mediated platelet aggregation got exposed after the platelets were denuded.

Amyloid β present on the circulating platelets is bound to the O-glycoproteins of adhering triplets rather than to GPIIb/IIIa. It was recently demonstrated that AOP1 and AOP2 in free form, in complex with albumin or in the anti-Gal/ABG-AOP1/AOP2-albumin triplet complex can capture amyloid β (Aβ-42) by offering the STPS on these O-glycoproteins as ligands for the peptide (3). The results in Fig. 8 demonstrated that native and denuded platelets captured comparable quantities of amyloid β presented to them, suggesting that the newly exposed O-glycoproteins on denuded platelets are capable of capturing amyloid β. However, upon treatment of the resulting amyloid β-bearing platelets with sugars that can release the triplets, the amount of amyloid β released into the supernatant by the denuded platelets was significantly lower compared with that by the native platelets (Fig. 8). This suggests that on native platelets, amyloid β are bound to the triplets using the STPS on their AOP1 and AOP2 so that they could be released along with the triplets by antibody-specific sugars. By contrast, amyloid β bound to the denuded platelets could have utilized the STPS on membrane O-glycoproteins that were newly exposed following the release of triplets, such that the antibody-specific sugar could not elute the peptide.

Discussion

The weaker binding of AOP1/AOP2 compared with albumin or immunoglobulins to Coomassie blue G-250 in the present study could be attributed to the abnormally high contents (≈55% by weight) of glycans in these O-glycoproteins (1). Being highly hydrophilic, glycans can occupy the surfaces of glycoprotein molecules, but have little affinity for hydrophobic dyes. By contrast, albumin is not glycosylated whereas immunoglobulins are poorly glycosylated (24), making them potent Coomassie blue dye binders. This means that the minimal quantities of triplet proteins loaded for SDS-PAGE for visualizing the AOP1/AOP2 bands are sufficient to produce intense albumin and immunoglobulin bands. Supporting this, Osset et al (25) previously demonstrated that carbohydrate moieties can block Coomassie brilliant blue binding to proteins and that dye binding to bovine pancreatic ribonuclease increased with de-O-glycosylation. In addition, the presence of sugars was shown to significantly reduce the absorbance of proteins in a Bradford protein assay using Coomassie brilliant blue G-250 (26). This may explain why the AOP1 and AOP2 bands were poorly stained even when triplet protein quantity that produced intense albumin and immunoglobulin bands was loaded in electrophoresis gels.

One possible reason for sugar specific for either antibody being able to release triplets of both antibodies from the platelets is that a small sugar specific for one antibody may occupy all of its binding sites, resulting in the detachment of its triplets from the platelets. This in turn releases albumin-bound O-glycoproteins from the triplet antibody. If the latter event also resulted in the temporary destabilization of the O-glycoprotein-albumin complex, free O-glycoproteins would be generated. Free O-glycoproteins, unlike those complexed with albumin, resemble low molecular weight antibody-specific sugars in that they occupy all available binding sites of either antibody without steric hindrance (1). This would result in the release of triplets of the other antibody. The colligative effect of any sugar molecule per se as the reason for the altered distribution upon DGUC of FITC-labelled AOP1/AOP2 added to sugar-treated plasma had been previously ruled out using sugars not specific to either antibody (1). O-Glycoprotein-free albumin that is already present in the plasma before sugar treatment did not appear to combine with the FITC-labelled AOP1 and AOP2, since their distribution was not altered in the presence of sugar-free plasma. A possible reason for the labelled AOP1/AOP2 to combine with albumin liberated from the triplets is that the albumin molecules not involved in triplet formation are likely to be engaged by ≥ one of the other albumin-binding biomolecules in the plasma, unlike the nascent albumin liberated from triplets by antibody-specific sugars.

The results aforementioned demonstrated that STPS that bear the O-glycans in the O-glycoproteins on the platelet surface got blocked upon jacin treatment, since this lectin recognizes O-glycans. O-Glycan-bearing STPS also appear to be true ligands for anti-Gal, since terminal α-linked galactose, the monosaccharide ligand for this antibody, is absent in humans and O-glycans per se are not ligands for anti-Gal (27), unlike for jacin. Jacalin-mediated blocking of anti-Gal access to STPS was demonstrated in the case of O-glycoproteins in lipoprotein (a) [Lp(a)] (28) and in plasma AOP1 and AOP2 (1). STPS underlying the O-glycans has been confirmed to be anti-Gal and ABG ligands, since the de-O-glycosylation of AOP1, AOP2 and Lp(a) without destruction of peptide sequences only enhanced the binding of the antibodies (1,28). Since ABG shares the same STPS specificity as anti-Gal, the aforementioned results suggest that platelet surface O-glycosylated protein(s) may serve as receptors for the unoccupied binding sites of anti-Gal- and ABG-derived triplets. Since fibrinogen recognizes the GPIIb/IIIa integrin present on the platelet surface (4,21), failure of fibrinogen-treated denuded platelets to capture triplets pointed to GPIIb/IIIa as a possible receptor for anti-Gal and ABG antibodies present in triplets. In support of this hypothesis, GPIIb/IIIa is the dominant O-glycosylated protein expressed on the platelet membrane (21-23). The requirement for an O-glycosylated site for the binding of anti-Gal or triplet to platelets found in the present study also supports this hypothesis. Although plasma fibrinogen concentration (8-9 μM) far exceeds the concentration used in the present study for blocking triplet attachment, circulating platelets can remain bound to triplets.
This suggests that although fibrinogen and triplets may bind to the same receptor(s) on the denuded platelets, competitive displacement of bound triplets by fibrinogen is not extensive.

The primary event in ADP-mediated platelet aggregation is binding to the two G-protein-coupled receptors, P2Y1 and P2Y12, on the platelet surface (29). Although no data on the dependence of this binding on O-glycosylated platelet surface molecules are available, involvement of GPIIb/IIIa in subsequent events after ADP-mediated platelet aggregation has been reported (21). In addition to explaining the jacalin-mediated inhibition of the ADP-induced aggregation of denuded platelets, the present study also suggested the possibility that GPIIb/IIIa that is heavily O-glycosylated and becomes exposed following denudation serves as a major platelet membrane receptor for triplets. Compared with AOP1 and AOP2, albumin that was electrophoretically separated from these O-glycoproteins was inert towards amyloid β (3). In addition, though it was previously reported that amyloid β could bind to an albumin sample (30), this sample also contained AOP1 and AOP2 (3). Amyloid β binding to AOP1 and AOP2 was consistent with the reported binding of this peptide to the STPS-rich GPIIb/IIIa (31). Since GPIIb/IIIa is the most abundant O-glycoprotein on the platelet surface and shares the amyloid β-binding property of AOP1 and AOP2, the present study also suggested a possible role for GPIIb/IIIa in triplet adhesion to platelets. This is because GPIIb/IIIa-bound amyloid β, which cannot be extracted from platelets using the anti-Gal- or ABG-specific sugars, can be formed only on denuded platelets. This observation suggests that GPIIb/IIIa is otherwise engaged and is inaccessible to amyloid β on native platelets until the triplets have been removed.

Beyond their role in clotting and homeostasis of blood, platelets have also been found to adhere onto leukocytes, resulting in the release of leukocyte and platelet constituents (32). This damages vascular walls and may be implicated in diseases, including myocardial infarction (32) and ischemic stroke (33). A clinically important phenomenon, largely unexplained on molecular level, is the increased susceptibility of the platelets of patients with diabetes to activation and aggregation (7,34). This has been hypothesized to contribute to widespread vascular damage (35). Furthermore, a role for platelets as carriers of amyloid β, thereby acting as a circulating sink for this peptide to reduce its concentration in the brain, has been previously recognized (5), although the exact receptor for this peptide on platelets remains unknown.

The availability of anti-Gal- and ABG-containing triplets in the blood appears to be determined mostly by the synthesis and/or plasma availability of these antibodies, since ~36% plasma albumin were found to be combined with AOP1 or AOP2 in plasma (unpublished data), ensuring a large excess of albumin-O-glycoprotein complexes over the antibodies. Identification of a platelet surface molecule that can be recognized by the antibodies in anti-Gal/ABG-AOP1/AOP2-albumin triplets is important for understanding the contribution of the latter towards platelet function. Complete blocking of triplet attachment to denuded platelets by jacalin, which specifically binds to O-glycoproteins and keeps them engaged, suggests the O-glycoprotein abundance of the triplet receptor on platelet surface. Furthermore, since the most jacalin-reactive O-glycosylated molecule identified in the present study on the platelet membrane was a subunit with an estimated molecular weight of 116 kDa, which was comparable to the reported molecular weights of the GPIIb subunit, GPIIb/IIIa appeared to be the most probable ligand on the triplets. Another well-known O-glycosylated platelet membrane protein, GPIbα, has a reported molecular weight of ±135 kDa (36) and is far less abundant than GPIIb/IIIa which is expressed at a density of ~80,000 molecules per platelet, considered the highest in any known cell type (37). This could explain the absence of any detectable jacalin-binding protein other than the 116-kDa subunit in the western blots of the platelet membrane. Other evidence implicating GPIIb/IIIa to be the ligand for triplet attachment includes the following: i) resistance of native triplet-containing platelets to ADP-mediated aggregation, even when all platelet components and plasma factors were available; ii) blocking of the ADP-mediated aggregation of denuded platelets by pre-bound jacalin, which for reasons aforementioned should be bound predominantly to GPIIb/IIIa, an essential intermediate in ADP-mediated platelet activation and aggregation (21,27,28); and iii) complete blocking of triplet binding to denuded platelets by pre-incubation with fibrinogen, which binds to GPIIb/IIIa. Therefore, whilst direct evidence remains lacking, GPIIb/IIIa is most likely to be a ligand for anti-Gal/ABG-AOP1/AOP2-albumin triplets on platelets.

In addition to serving as the plasma sink for this peptide to limit its availability in the brain (5), platelets have also been reported to act as carriers of amyloid β to perivascular cells of the brain following vascular damage (31). Although GPIIb/IIIa has been reported to serve as a receptor for amyloid β on platelets (31), the present results indicate that this may be true only of denuded platelets or of isolated platelet membranes. In addition, in native circulating platelets anti-Gal/ABG-AOP1/AOP2-albumin triplets mediate amyloid β binding. Supporting this conclusion are previous reports that diabetes is the most common predisposing factor for Alzheimer’s disease (AD) (38,39). Although glucose is nearly as efficient as cellobiose as a ligand for ABG, it does not dissociate the ABG-triplet at normoglycemic levels (~4.7 mM) (1). However, concentrations of glucose in diabetes may reach up to 5–6 times higher than normal, which is inhibitory to ABG binding and can dissociate triplets containing ABG and anti-Gal (12), as shown in the present study. The denuded platelets were capable of capturing amyloid β, presumably through the newly exposed cell surface O-glycoprotein vacated by triplet antibodies. However, they are more prone to aggregation and are therefore less stable, resulting in substantial attenuation of their amyloid β-arresting activity. A previous report that blood samples from patients with AD exhibited a 39.57% increase in platelet aggregates and a 53.3% increase in leukocyte-platelet complexes (40) also supports this conclusion. The myriad of platelet-mediated vascular injuries found alongside diabetes also implicates the triplet-mediated protection of platelets. Platelet-dependent thrombosis was found to be proportional to blood glucose levels in coronary artery disease (35). Majority of the tissue toxicity associated with diabetes has been attributed to oxidative and inflammatory stress due to the enhanced production of mitochondrial superoxide dismutase, resulting in reduced mitochondrial function and cell viability (41). However, short-term hyperglycaemia in
patients with diabetes has been reported to be sufficient in causing vascular occlusion through platelet activation (34,42), suggesting that events that occur earlier than oxidative stress induction can account for vascular damage. In addition, the cascade of inflammatory events leading to cerebral amyloid angiopathy preceding AD has been reported to be triggered by amyloid β binding to the exposed GPIIb/IIa on platelets that adhere to vessel walls (31).

Platelet-leukocyte adhesion facilitated by platelet surface GpIIb/IIIa is a trigger for the synthesis and release of inflammatory factors, such as leukotrienes and thromboxane A2, by leukocytes and platelets (43). These factors have been implicated in acute myocardial infarction (44) and stroke (33). Since the protective cover for GPIIb/IIIa have been implicated in acute myocardial infarction (44) and stroke (33). Since the protective cover for GPIIb/IIIa presumably provided by triplets is diminished during hyperglycaemia, the platelets become susceptible to leukocyte adhesion. Results from the present study may explain in molecular terms the contribution of diabetes towards the GPIIIb/IIa-mediated pathophysiology of the aforementioned disorders.

In summary, the results of the present study revealed the molecular basis for ABG/anti-Gal antibody-AOP1/AOP2-albumin triplets anchoring onto platelets, for the absence of platelet aggregation under normal conditions and for platelets serving as an amyloid β sink in the circulation. These data may offer an alternative direction for the investigation of diabetes-mediated platelet vulnerability, platelet-leukocyte adhesion and the contribution of diabetes towards AD. The main limitation of the present study is that the data indicating GPIIb/IIa as the main O-glycoprotein anchor on platelets for triplets remains indirect. Further investigations are therefore required to confirm this using additional experiments, such as blocking of triplet attachment to platelets using antibodies to suspected receptors and variations in triplet binding to platelets with GPIIb/IIa O-glycosylation levels.

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Competing interests

The authors declare that they have no competing interests.

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