Basic immunobiology

1

Platelet toll-like receptor 4 functions to present lipopolysaccharide to the leukocytes of the RES

Semple JW, Aslam R, Speck ER, Freedman J
Toronto Platelet Immunobiology Group, Toronto, Canada

The innate immune system is critical for initiation of adaptive immunity and recently, it has been demonstrated by several laboratories that platelets stimulate innate immune system responses by the expression of various toll-like receptors. Toll-like receptors (TLR) comprise a family of transmembrane proteins characterized by multiple copies of leucine-rich repeats in the extracellular domain and an IL-1 receptor motif in their cytoplasmic domain. The TLR family is a phylogenetically conserved mediator of innate immunity that is essential for microbial recognition and the stimulation of adaptive immune responses. Recently, our laboratory demonstrated that TLR4 expression on platelets was responsible for lipopolysaccharide (LPS)-mediated thrombocytopenia and tumour necrosis factor-alpha production *in vivo* (Aslam et al. Blood, 2006). To understand the mechanism of how platelet TLR4 mediated LPS-induced TNF-alpha production, platelets from wild type (WT) and TLR4 knockout mice were incubated with various concentrations of LPS *in vitro*, washed extensively and transfused into WT mice. Results suggest that only the WT platelets could stimulate TNF production *in vivo*. *In vitro* experiments confirmed that platelet TLR4 presented LPS to leukocytes of the RES and stimulated them to become activated and produce TNF. These results suggest that platelets, via TLR, act as initial sentinels of the innate immune system by quickly presenting bacterial products such as LPS to the RES.

2

A variable number of tandem repeats polymorphism influences the transcriptional activity of the neonatal Fc receptor a-chain promoter

Sachs UJH[1,2], Socher I[1], Braeunlich CG[1], Bein G[1], Kroll H[3], Santoso S[1]
[1]Institute for Clinical Immunology and Transfusion Medicine, Justus Liebig University, Giessen, Germany [2]Rudolf-Virchow-Center for Experimental Biomedicine, University of Würzburg, Würzburg, Germany [3]German Red Cross Blood Service NSTOB, Institute for Transfusion Medicine, Dessau, Germany

The neonatal Fc receptor, FcRn, plays a central role in IgG transport across placental barriers. Genetic variations of FcRn-dependent transport across the placenta may influence antibody-mediated pathologies of the foetus and newborn. Sequencing analysis of 20 unrelated individuals demonstrated no missense mutation within the five exons of the FcRn gene. However, a variable number of tandem repeats (VNTR) region within the FcRn promoter was observed, consisting of five different alleles (VNTR1–VNTR5). Alleles with two (VNTR2) and three (VNTR3) repeats were found to be most common in Caucasians (7.5 and 92.0%, respectively). Real-time PCR revealed that monocytes from VNTR3 homozygous individuals expressed 1.66fold more FcRn transcript than do monocytes from VNTR2/VNTR3 heterozygous individuals ($P = 0.002$). In reporter plasmid assays, the VNTR3 allele supported the transcription of a reporter gene twice as effective as did the VNTR2 allele ($P = 0.003$). Finally, under acidic conditions, monocytes from VNTR3 homozygous individuals showed an increased binding to polyvalent human IgG when compared to monocytes from VNTR2/VNTR3 heterozygous individuals ($P = 0.021$). These data indicate that a VNTR promoter polymorphism influences the expression of the FcRn receptor, leading to different IgG binding capacities.

3

Monoclonal antibodies that mimic antibodies found in drug-induced immune thrombocytopenia (DITP): Implications for the pathogenesis of DITP

Bougie DW, Birenbaum J, Ahl S, Aster RH
The Blood Research Institute, Blood Center of Wisconsin, WI, USA

DITP is characterized by an unusual type of antibody that reacts with specific epitopes on platelet membrane glycoproteins only in the presence of soluble drug. The molecular basis for this interaction has not been defined, but it is generally thought that the sensitizing drug first reacts non-covalently with the target glycoprotein to create the epitope recognized by antibody, through one of several possible mechanisms. The antibody then binds secondarily to this target. Utilizing mice immunized with glycoprotein (GP) IIb/IIIa/quinine (Qn) complexes, we recently succeeded in producing two murine monoclonal antibodies (mAb) that mimic those found in patients with Qn-induced immune thrombocytopenia and appear to provide a unique tool with which to characterize how drugs cause binding of an otherwise innocuous antibody to a cell membrane GP and cause cell destruction. Each of the mAbs binds reversibly to the GPIIb component of the GPIIb/IIIa complex at 50 nM Qn, a concentration much lower than is achieved pharmacologically, and are not inhibited by a ten-thousand-fold excess of Qn (0.5 mM). Quinidine (Qd) the diastereoisomer of Qn, supports only weak antibody binding at a concentration of 50 μM. The affinity of the mAbs for Qn was determined from the signal (resonance units) produced when Qn reacts with mAb immobilized on a Biacore chip. The stoichiometry of the reaction is 2:1 (Qn to mAb) and the KA is approximately 1.7×10^7 1/M (Qn) and 1×10^4 1/M (Qd). Studies to determine the affinity of Qn and Qd for the target GPIIb/IIIa complex are in progress, but it is known that Qn binds only weakly to protein (KA about 2×10^3 1/M). Thus, at pharmacological concentrations (10^{-5}M), Qn can bind to the mAb, but there is very little binding of Qn to GPIIb/IIIa. Accordingly, the findings favour the possibility that Qn reacts first with the CDR region of the mAb and modifies it so that it acquires specificity for an epitope on GPIIb/IIIa, enabling antibody to react with platelets. Further studies are required to determine whether human antibodies that cause DITP also contain high affinity binding sites for the sensitizing drug and whether the initial reaction in drug-induced antibody binding involves drug-antibody interaction.

4

Molecular characterization of the variable domains of an αIIBβ3 specific IgMκ platelet cold agglutinin in a follicular lymphoma patient with treatment refractory thrombocytopenia: evidence of idiotypic overlap with other αIIBβ3 integrin antibodies

Jennings N[1], Harmer I[1], Campbell K[1], Smith G[1], Stafford ??[1], Metcalfe P[2], Benton A[3], Marsh J[3], Ouwehand W[1]
[1]Department of Haematology, University of Cambridge and National Blood Service, Cambridge, UK [2]National Institute for Biological Standards and Control, Potters Bar, Herts, UK [3]St. George's Hospital Medical School, London, UK

Cold agglutinins (CA) were originally defined as IgMκ antibodies capable of agglutinating erythrocytes at temperatures below 37°C and cold haemagglutinin disease may ensue in patients with high titre cold agglutinins at low environmental temperature. Platelet CA is rare and have been poorly characterised. We present what we believe to be the first proven case of a pathological, platelet reactive, CA. This antibody was identified in a patient with follicular lymphoma (FL) who had profound, refractory, thrombocytopenia that resolved in response to Rituximab. Serological and molecular studies of this IgMκ that recognizes αIIbβ3 shows that binding is temperature dependent but independent of the integrin activation state. The V_H1-02 and V_λ3-20 encoded variable domains of the heavy and light chains have characteristics typical of a low affinity autoantibody. Maldi-Tof analysis of the platelet reactive IgM showed unequivocally that the IgM was derived from the FL cone. Interestingly we observed idiotypic cross-reactivity between this IgM paraprotein, several other FL idiotypes and our β3-leucine 33 (HPA-1a) specific human recombinant antibody. Our results suggest that binding of the latter and other αIIbβ3 specific monoclonal antibodies induces a subtle conformational change that enhances platelet binding of the patient IgM.

5

Harmful antibodies can be blocked by mutant antibodies devoid of effector functions

Michaelsen TE[1], Brekke OH[2], Sandin RH[1], Sparstad T[1], Sandlie I[3]
[1]Norwegian Institute of Public Health, Division Infectious Disease Control, Oslo, Norway [2]Invitrogen Dynal Bead Based separations, Oslo, Norway [3]Institute of Molecular Bioscience, University of Oslo, Norway

Some antibodies can be harmful for patients based on specificity and effector activities of the antibodies. Examples are Rhesus antibodies transported across the placenta, leading to fatal hemolytic disease of the fetus. Exeno-transplantation is hampered by the presence of Gal-Gal epitopes of the transplant combined with anti Gal-Gal antibodies in all human sera. Platelet antibodies can induce ITP (immune thrombocytopenic purpura) or NAIT (Neonatal alloimmune thrombocytopenia). In these and other cases, mutant antibodies with the same antibody specificity, but without antibody effector functions, can be able to neutralize the harmful antibodies by competition, provided that they have high enough antigen binding affinity. We have constructed two mutant human IgG3 antibodies, HM5 and HM5/R435H (the arginine in position 435 is mutated to histidine), which have no effector functions, but maintained binding activity. The HM5/R435H is binding to Protein A that have overlapping binding motif with FcRn responsible for the high half-life of IgG and is also involved in placenta transfer. We have expressed the HM5/R435H as a model NIP antibody, employing an expression vector able to accommodate all other antibody specificities of interest. The HM5 and HM5/R435H NIP-specific antibodies were able to inhibit the antibody dependent complement mediated cytotoxicity of corresponding IgG1wt as well as IgG3wt NIP-specific antibodies in a dose dependent manner.

6

Fibrinogen-independent platelet aggregation *in vivo* and *in vitro*: a novel pathway of thrombosis and haemostasis

Yang H[1,2], Reheman A[1,3], Chen P[1,2], Hynes R[4], Freedman J[1,3], Wagner D[5], Ni H[1,2,3]
[1]The Toronto Platelet Immunobiology Group, Department of Laboratory Medicine, St. Michael's Hospital, Toronto, Ontario, Canada [2]Canadian Blood Services, Canada [3]Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada [4]Department of Biology, Massachusetts Institute of Technology, Howard Hughes Medical Institute and Center for Cancer Research, Boston, Massachusetts, USA [5]Department of Pathology, The CBR Institute for Biomedical Research, Harvard Medical School, Boston, Massachusetts, USA

Platelet adhesion and aggregation at the site of vascular injury play a key role in the arrest of bleeding and thrombus formation. Fibrinogen (Fg) has been considered an essential molecule required to bridge platelet aggregates for four decades. However, we recently found that platelet-rich thrombi still formed in mice lacking either Fg (Fg-/-) or both Fg and VWF (Fg/VWF-/-) (Ni et al., JCI 2000;106:385–392). To explore the potential mechanisms of Fg-independent platelet aggregation, we studied platelet aggregation *in vitro* in platelet rich plasma (PRP). We found no platelet aggregation in Fg-/- or Fg/VWF-/- PRP induced by adenosine diphosphate (ADP) in the presence of anticoagulant reagents, including divalent cation chelators (ACD, sodium citrate, and EDTA), and thrombin inhibitors (heparin, hirudin, PPACK). Since no fibrin formation occurs in Fg/VWF-/- plasma, we then induced Fg/VWF-/- platelet aggregation in non-anticoagulated platelet poor plasma (PPP). Surprisingly, robust aggregation occurred after ADP treatment, but no aggregation occurred in β3 integrin-deficient (β3-/-) platelets in the same non-anticoagulated Fg/VWF-/- plasma. Furthermore, when fluorescently labelled β3-/- platelets were injected into Fg/VWF-/- mice, β3-/- platelets did not incorporate into Fg/VWF-/- thrombi under confocal intravital microscopy. The inability of β3-/- platelets to aggregate was not due to a deficiency in other adhesive receptors, since no reduction of GPIb, β1 integrins, or P-selectin were observed on β3-/- platelets. Thus, β3 integrin is essential for this Fg/VWF-independent platelet aggregation. To explore the possible ligand(s) and its location(s) (i.e. plasma or platelet), we isolated Fg/VWF-/- platelets via gel-filtration and induced aggregation with ADP, thrombin, and thrombin receptor activation peptide (TRAP) in 1 mM Ca^{2+} PIPES buffer (with and without plasma). Both thrombin and TRAP, but not ADP, which do not induce granule release, induced platelet aggregation in PIPES buffer. However, platelet aggregation in PIPES buffer was only 50–60% of that observed with plasma. This suggested that both plasma and platelet released ligand(s) contribute to this platelet aggregation. Since plasma fibronectin is a candidate ligand, we further studied aggregation in fibronectin-depleted plasma. Interestingly, fibronectin depletion did not affect this platelet aggregation. Thus, other plasma ligand(s) likely exists. We concluded that Fg is not an indispensable molecule required for platelet aggregation. Other alternative ligands of β3 integrin from either the plasma or platelet granules are capable of mediating platelet aggregation independent of both Fg and VWF under more physiologically relevant conditions.

7

The value of laboratory investigation in diagnosis of immune DITP

Hughes AS, Crawford JH, Lown JG, Cannell PK
Department of Haematology, Royal Perth Hospital, Perth, Western Australia, Australia

Drug-induced immune thrombocytopenia (DITP) is commonly caused by antibody specific platelet destruction. A definitive diagnosis is provided by recurrent thrombocytopenia on drug re-exposure though this may produce haemorrhage. Cessation and avoidance of the suspect drug is recommended. Detection of drug-specific platelet antibodies in patient serum is a useful DITP diagnostic tool. A 5-year-review (Jan 2000–Mar 2005) of drug specific antibody testing (excluding heparin) at our institution was performed. Sixty-seven suspected DITP episodes for 66 patients (hospital and referred patient samples) testing 77 drugs by a modified solid phase red cell adherence method (36 quinine, 11 GPIIb/IIIa inhibitors, 20 antibiotics, 10 other) were reviewed. Initial patient drug test results were 33 negative, 9 indeterminate (8 re-tested positive on later sample) and 25 positive. Published DITP diagnostic criteria were modified to include antibody test results and patients were classed as definite, probable, possible or unlikely DITP. Forty-one DITP events had clinical information enabling correlation with laboratory results and classification using the modified criteria. Twenty-one of 23 patients with positive laboratory antibody tests were categorised as definite DITP. Thirteen of 18 patients with negative tests were classified as unlikely DITP. Of the other five patients with negative drug antibody tests, three were classified as possible (multiple medications untested), and two as probable DITP. Documentation of DITP as an adverse drug reaction was sub-optimal (detailed in 31.6% of medical records and 52.4% letters to referring doctor). A DITP investigation and reporting plan was implemented. This used a flow chart to direct clinical information and guide antibody testing. Cases were categorised according to the modified criteria. Definite or probable DITP cases resulted in implementation of an Action Plan, which included direct notification of the patient and referring doctor of test results. Our experience with this new system is reported. Twenty-two patients were investigated for suspected DITP over 10 months (May 2005–Feb 2006). Eight definite, one probable, three possible and 10 unlikely DITP cases were identified. Suspect drugs were tested by a range of methods. The implementation of an Action Plan was effective in raising DITP awareness and this resulted in all hospital patients having DITP clearly recorded in medical notes. We found that despite present limitations, drug specific platelet antibody testing can be incorporated into DITP diagnostic criteria. It also demonstrates the importance of clinical information in guiding laboratory testing to provide meaningful test results.

8

The *FCGR2A* gene polymorphism: no evidence for an influence on mortality in the elderly

Flesch BK[1], Nikolaus S[2], El Mokhtari NE[3], Schreiber S[4], Nebel A[4]
[1]Institute of Transfusion Medicine, University Hospital Schleswig-Holstein, Kiel, Germany [2]Hospital for General Internal Medicine, University Hospital Schleswig-Holstein, Kiel, Germany [3]Department of Cardiology, University Hospital Schleswig-Holstein, Kiel, Germany [4]Institute of Clinical Molecular Biology, University Hospital Schleswig-Holstein, Kiel, Germany

Background: Fc receptors for immunoglobulin G (FcγR) are potent links between the cellular and the humoural immune response. The functional single nucleotide polymorphism (SNP) rs1801274 (His131Arg) in the *FCGR2A* gene, which encodes the FcγRIIa influences the efficiency of hIgG2-binding, the main isotype produced in response to encapsulated bacteria like *Streptococcus pneumoniae* and *Haemophilus influenzae*. In contrast to the receptor with the His131 allele, FcγRIIa-Arg131 binds hIgG2 poorly and carriers of this variant have been shown to be much more susceptible to succumb to bacterial pneumonia or meningitis. Since bacteremic pneumonia is one of the leading causes of death in elderly individuals, we hypothesized that the Arg131 variant could be a major mortality factor in the old.
Methods: We analysed the *FCGR2A*-His131Arg polymorphism (rs1801274) with a TaqMan® SNP Genotyping Assay in a group of 408 German centenarians and two samples of younger Germans aged 60–75 and 18–49 years, respectively.
Results: No statistically significant differences were observed between the three age groups, neither at the allele nor at the genotype level.
Conclusion: Apparently, the advantage of FcγRIIa–His131in the defence of life threatening infectious diseases like bacteremic pneumonia leaves the ability to attain old age largely unaffected.

9

Variability of IVIg inhibition of autoantibody-mediated phagocytosis

Léonard S, Pierard I, Michaelsen TE, Izui S, Masson PL, Coutelier J-P
Unit of Experimental Medicine, Christian de Duve Institute of Cellular Pathology, Universite Catholique de Louvain, Brussels, Belgium

Both human and experimental autoantibody-mediated autoimmune thrombocytopenia and anaemia may be attenuated by infusion of total IgG (IVIg). One of the major mechanisms that can explain this effect of IVIg is a blockade of Fc receptors expressed on phagocytic cells. IVIg efficacy may vary widely from one patient to another. Using a mouse experimental model of *in vitro* phagocytosis by macrophages, with autoantibody-coated erythrocytes as target cells, we analysed the possible causes for such a variability. Our results indicated that the efficacy of the phagocytosis inhibition depends upon both IVIg characteristics, such as the isotype and the extent of polymerization of the immunoglobulin used for the treatment, and host factors, including the genetic background of the mice and the state of macrophage activation that can be influenced by concomitant viral infection. The development of such an *in vitro* assay for the phagocytic activity of macrophages might improve the selection of patients susceptible to benefit from IVIg treatment.

10

Animal model for testing *in vivo* survival of human platelet concentrates and analysing a role of platelet surface molecules in regulation of platelet survival

Leytin V, Allen D, Freedman J
Division of Transfusion Medicine, Department of Laboratory Medicine, St. Michael's Hospital, Toronto, Ontario, M5B 1W8, CanadaDepartment of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada

Recently, we described a rabbit model for analysing *in vivo* kinetics of human platelet concentrates (PCs) (Leytin et al., Transfusion 2002;42:711; Transfusion 2003;43:983).

Platelet surface glycoprotein (GP) Ibα and P-selectin (CD62) can be involved in regulation of post-transfusion PC clearance, mediating adhesive interactions of platelets with counter-receptors on leukocytes and endothelial cells. In the present work, we used our validated rabbit model for studying the implication of CD62 and GPIbα expression in post-transfusion PC viability. Platelet activation *in vitro* was determined by flow cytometry using anti-CD62 and anti-GPIbα antibodies. PC viability *in vivo* was evaluated using rabbits with inhibited reticuloendothelial system, as measured by 0.5 hour ($R_{0.5}$), 24 hour (R_{24}) and total (R_{Σ}) platelet recoveries, and survival time (ST). Correlations between *in vitro* assays of platelet activation and *in vivo* viability of conventional (Day 2–5) and outdated (Day 7–8) PCs stored at 22°C, and refrigerated PCs were analysed. We found that the binding of anti-CD62 to the platelet surface was significantly increased and of anti-GPIbα decreased in outdated and refrigerated PCs compared to conventional PCs. Inverse correlation was observed between *in vitro* anti-CD62-binding and the fast ($R_{0.5}$), but not with delayed (R_{24} and ST), platelet clearance. In contrast, anti-GPIbα-binding showed direct correlation with delayed but not with fast platelet clearance. Overall (R_{Σ}) clearance correlated better with anti-GPIbα- than with anti-CD62-binding. We also demonstrated that CD62 is shed from the platelet surface after transfusion, whereas GPIbα remains unchanged on the surface of circulating human platelets. These data suggest that CD62 exposure on the platelet surface during PC storage triggers fast CD62-mediated PC clearance, whereas *in vitro* GPIbα down modulation is involved in delayed GPIbα-mediated PC clearance.