Chapter 2

REVIEW OF THE LITERATURE ON POST-TRANSFUSION AND MATERNAL RBC ALLOIMMUNIZATION

CONTENTS

2.1 A short history of post-transfusion and maternal RBC alloimmunization

Alloimmunization to RBC antigens, a consequence of blood transfusion or pregnancy, was recognized following the discovery of the Rhesus (now Rh) blood group system in 1939 by Philip Levine and Rufus Stetson. They reported a case of a woman who delivered a stillborn infant and suffered a severe haemolytic transfusion reaction (HTR) following transfusion with apparently ABO compatible blood from her husband. Her serum was found to contain an alloagglutinin (reacting at 37˚C) which agglutinated RBCs of her husband and those of 85% of blood donors. Levine and Stetson showed that this new antigen, which they did not name, was independent of the then known blood groups ABO, MN and P. They postulated that the cause of this case of haemolytic disease of the newborn (HDN) was a maternal antibody entering the fetal circulation leading to fetal RBC destruction (Levine & Stetson, 1939). In 1940, Landsteiner and Wiener made an antibody by injecting Rhesus monkey RBCs into rabbits and guinea pigs (Landsteiner & Wiener, 1941). The resulting antiserum (anti-Rh) agglutinated not only Rhesus monkey RBCs but also those of 85% Caucasians. This specificity appeared identical to that of antibodies in the sera of patients who suffered HTRs after receiving ABO-identical blood (Wiener & Peters, 1940). In 1941, Levine and co-workers reported that the antibody responsible for HDN had the same specificity as the anti-Rh produced by Landsteiner and Wiener, later shown to be anti-LW. Levine and Stetson were indeed describing anti-D although they identified it as anti-Rh in these earliest publications. In 1945, Coombs, Mourant and Race described the use of antihuman globulin (later known as the “Coombs’ test”) to identify “incomplete” antibodies. A year later, they used this test to detect Rh antibodies on RBCs of babies suffering from HDN (Coombs *et al.,* 1946). Thereafter, the versatility of the Coombs’ test in immunohaematology for the detection of post-transfusion and maternal RBC alloantibodies became evident.

2.2 Human blood group diversity and function

Human blood groups are unique, inherited polymorphic structures located on mostly non-polymorphic proteins, glycoproteins, and glycolipids on the extracellular surface of RBCs. Blood groups are detected by a specific alloantibody, implying that the antigens are immunogenic for individuals lacking the blood group. Currently, 33 blood group systems, which include a total of about 339 antigens, have been established by the International Society of Blood Transfusion (ISBT Committee on Terminology for Red Cell Surface Antigens, Cancun 2012). In addition,

antigens not yet fulfilling the requirements for classification into a system have been gathered into collections or series of high- and low-frequency antigens (Daniels *et al.,* 2009). In blood group nomenclature, antigens encoded by the same gene, or cluster of two or more closely linked homologous genes with virtually no recombination events occurring among them, are assigned to the same blood group system.

Each blood group system is genetically discrete from other blood group systems and accommodates from 1-50 antigens. The two most important blood group systems from the point of view of clinical transfusion medicine are ABO and Rh. Rh and MNS are the most complex systems, with 61 and 46 antigens respectively. Most blood group polymorphisms are the result of single nucleotide polymorphisms (SNPs) encoding amino acid substitutions in an extracellular domain of an RBC surface protein. All blood group systems represent a single gene, apart from Rh, Xg and Chido/Rodgers, which have two closely linked genes, and MNS with three genes. In null phenotypes, the whole protein is absent from the membrane usually as a result of a gene deletion or an inactivating mutation. Genes that encode all the blood group systems present on RBCs have been identified (Storry & Olsson, 2004; Storry *et al.*, 2011).

The development of DNA sequencing techniques, and then the polymerase chain reaction (PCR) has paved the way for the rapid molecular characterization of the genes encoding blood group antigens (Beiboer *et al.,* 2005). As the molecular basis of many blood group antigens has been determined (Reid & Lomas-Francis, 2004), it is now feasible to predict the blood group antigen profile of an individual by testing the DNA. Such molecular analyses can be used to overcome the limitations of haemagglutination in clinical transfusion practice e.g. typing of multiply transfused patients, determination of paternal *RHD* zygosity, fetal genotyping from amniocytes or maternal plasma to determine the risk for HDFN, typing of RBCs with a positive direct antiglobulin test (DAT), detection of altered D antigens (weak D or partial D) and screening donor units for antigens (Do^a, Do^b, Js^a, Kp^a, Co^a, Yt^a, etc.) for which there are no commercial reagents (Legler *et al.,* 2001; Reid, 2003; Harper *et al.,* 2004).

The RBC membrane protein structures bearing blood group antigens exhibit diverse functional heterogeneity. The following functions have been attributed to blood group antigens. Some are membrane transporters e.g. band 3 (the Diego antigen) provides an anion channel for HCO_3^- and Cl^- ions; the Kidd glycoprotein is a urea transporter; the Colton glycoprotein, aquaporin 1, is a water channel; and RhAG is probably a gas channel. The Lutheran, LW, and Indian (CD44)

glycoproteins are adhesion molecules while the Duffy glycoprotein is a chemokine receptor. The Cromer and Knops antigens are markers for decay accelerating factor (CD55) and complement receptor 1(CD35) respectively (Catron & Colin, 2001; Telen, 2005).

2.3 Pathophysiology of the post-transfusion alloimmune response

2.3.1 Blood transfusions may lead to either alloimmunization or tolerance induction

RBC transfusion for anaemia is both the oldest and the most widely employed transplantation procedure. Multiple allogeneic blood transfusions introduce a multitude of foreign antigens and living cells into the recipient that persist for a variable period of time. These can affect the immune response in two opposite ways, leading to either *alloimmunization* or to *tolerance induction*. Alloimmunization is reflected by the development of alloantibodies against RBC antigens (Lostumbo *et al.*, 1966), HLA antigens (Perkins *et al.,* 1966) and other cellularly expressed or soluble antigens; and by T cell activation leading to CD8 positive cytotoxic T cells.

T cell receptors specific for alloantigens develop in utero. Intrauterine transfusion of allogeneic blood before the 14th week of gestation may result in tolerance and (transient) establishment of low dose chimerism (Hayward *et al.*, 1998). The full capacity to produce immune antibodies develops slowly after birth. Although anti-HLA antibodies have been reported after non-leucocyte depleted whole blood transfusion (Bedford-Russel *et al.,* 1993), the immunization rate in preterm infants is very low to negligible due to functionally immature B cells (Marshall-Clarke, 2000).

The induction of tolerance is suggested by the enhanced graft survival in transfused versus non-transfused solid organ recipients (Opelz & Terasaki, 1978). Also, recipients of allogeneic blood transfusions have been reported to be at a greater risk of post-operative infections referred to as transfusion-related immunomodulation (TRIM). Moreover, allogeneic blood transfusions have been shown to lead to suppressive effects in immunologic function in recipients *ex vivo* i.e. a decrease in the CD4:CD8 ratio of circulating T cells, reduced natural killer cell function, defective antigen presentation, suppression of lymphocyte blastogenesis, and reduction in delayed type hypersensitivity (Blajchman & Bordin, 1994). In general, contaminating leucocytes are thought to play a pivotal role in the above immunomodulatory effects of blood transfusions; with leucocyte depletion preventing both HLA alloimmunization and tolerance induction (Merryman, 1989).

2.3.2 Pathways for immune recognition of alloantigens

Two recipient T cell recognition mechanisms have been shown to be critical for the initiation of alloimmunity. The *direct pathway* occurs when recipient T helper (Th) cells directly interact with major histocompatibility complex (MHC) class II molecules on donor antigen presenting cells (APCs). The T cell activation by this direct pathway is only exerted by allogeneic class II bearing cells, such as in fetomaternal transfusion and by leukocyte-containing blood products. Approximately 100 times more T cells can be activated by the direct pathway as compared to the *indirect pathway,* which reflects the normal immune response. Indirect recognition occurs when foreign (allogeneic donor) molecules are processed by recipient APCs and presented to self Th cells. Within the context of indirect allorecognition, T cells recognize protein antigens that are degraded or processed within APCs to peptides which combined with MHC molecules are transported to the cell surface and bound within the antigen-binding grooves of either MHC class I or class II molecules (Sayegh *et al.,* 1994). The spectrum of antigen processing ranges from the simple unfolding of conformational determinants to the proteolytic exposure of primary structure by pH-dependent enzymes (e.g. cathepsins). Generally, APCs process exogenously derived proteins via endosomal compartments that shunt the processed peptides to intracellular compartments rich in MHC class II molecules. This pathway is necessary for the activation of $CD4^+$ Th cells to provide helper factors for B cell activation and eventual IgG antibody production. Endogenous proteins (e.g. self-proteins or when infected with virally derived proteins), are generally processed by large-molecular-weight proteosomes within the cell cytosol and are subsequently transported to the luminal surface of the endoplasmic reticulum for loading onto MHC class I molecules and foreign molecules can be recognized by $CD8^+$ (cytotoxic) T cells. For both direct and indirect pathways, the loaded MHC molecules are expressed on the surface of the APC and are available for presentation to circulating T cells (Watts, 1997). However, after T cell recognition a second signal provided by costimulatory molecules is needed to activate the Th cells.

2.3.3 Role of costimulatory molecules

As depicted above, both antibody production and cytotoxic T cell development depend on the stimulation of the recipient antigen-specific $CD4^+$ (helper) T cells. The key requirements for CD4 stimulation are the simultaneous expression of at least two different signals (Schwartz, 1989). The first signal, occupancy of the clonotypic T-cell receptor (TCR), is provided by MHC-

peptide on the APCs. TCR binding leads to a cascade of events culminating in IL-2 expression but not IL-2 secretion (Mueller *et al.,* 1989). A second, costimulatory signal is required for the expression of the IL-2 gene with consequent secretion of IL-2 and cell proliferation (Mincheff & Merryman, 1990). On APC, the B7-1 protein, delivers a costimulatory signal through binding with the CD28 (positive) and CTLA-4 (negative) T-cell receptors, which regulate IL-2 secretion. Lack or impairment of this second signal has been shown to lead to T cell unresponsiveness or anergy (Nossal, 1989). In the case of allogeneic transfusions, the alloantigens on donor class II bearing APCs will be recognized by recipient T cells through the direct pathway (Pouteil-Noble *et al.,* 1991). The imunogenicity of those alloantigens will then be determined by the ability of the donor APCs to present the costimulatory signals to the recipient T cells. After 2 weeks' storage in vitro at 2 - 6˚C, APCs lose costimulatory molecules (Mincheff & Merryman 1990). Besides leukocytes, unmodified RBCs often contain large numbers of platelets which are rich in both cell surface and soluble CD40L (Henn *et al.*, 1998). This costimulatory molecule activates B cells and is critical for IgM-to-IgG class switching (Grewal & Flavell, 1996). WBC reduction removes class II bearing donor APCs (e.g. dendritic cells, B-cells, and monocytes) and reduces HLA immunization in particular in case of platelet transfusions, provided that the residual white cell count is less than $1 – 5 \times 10^6$ per unit (Claas *et al.,* 1981, Sirchia *et al.,* 1986; van Marwijk *et al.,* 1991; Oksanen *et al.,* 1991; Saarinen *et al.,* 1993; Blumberg *et al.*, 2003). However, WBC removal has no demonstrable effect on the formation of RBC alloantibodies (Schonewille *et al.,* 2005), indicating that these are mainly elicited through the indirect antigen presentation pathway.

2.3.4 Type 1 and type 2 immune responses

Immunologic responses can become polarized to favour cells and cytokines of Type 1 (Th1) or Type 2 (Th2) responses (Mosmann & Sad, 1996). Type 1 responses involve cytokines such as γ-interferon (IFN-γ), IL-12, and IL-2, enhancing cellular immune responses such as delayed type hypersensitivity. Type 2 responses involve cytokines such as IL-4, IL-5 and IL-10 and enhance humoral immune responses, particularly those involving specific IgG subclasses, as well as IgA and IgE (Romagnani, 1996). Allogeneic leukocyte-containing blood transfusions have been shown to elicit immune deviation favouring Type 2 responses and downregulation of Type 1 responses (Kirkley *et al.,* 1995). This immunological mechanism could account for the unfavourable associations of allogeneic transfusions with the development of alloantibodies to RBCs, WBCs, platelets, and plasma proteins; presumed increased tumour recurrence; and post-

operative bacterial infection, and favourable associations with reduced spontaneous abortions and increased tolerance of solid organ allografts (Blumberg & Heal, 1996).

2.4 Nature of RBC alloantibodies

Alloantibodies against RBC antigens may be *"naturally occurring"* or *"immune"* in nature. Naturally occurring antibodies are most often IgM class, reacting at a temperature optimum below 37˚C, but may be partly IgG and are found in individuals who have never been transfused with RBCs or who have not been pregnant with a fetus carrying the relevant RBC antigen. Natural antibodies are not present at birth, but arise early in life presumably due to cross-reactivity with ingested antigens. Immune antibodies are most often IgG but may be IgM or a mixture of IgG and IgM; they may sometimes have an IgA component. An antibody is considered to be *clinically significant* if examples with that specificity are known to have caused HTRs, HDFN or unacceptably short survival of the transfused RBCs (Walker, 1993). RhD is by far the most immunogenic antigen followed by K and c. Development of alloantibodies may compromise the care of chronically transfused patients since the deleterious effects of RBC alloimmunization, including delayed HTRs and HDFN, are increased (Moise, 1993). Antibodies may appear as early as 7 - 10 days after transfusion in primary immunization and within 2 - 7 days in a secondary response. The optimal screening times for the detection of post-transfusion RBC alloimmunization are not known and depend on the nature of the antigen, dose and recipient immunocompetence, although testing after 2 - 4 weeks and 3 - 6 months have been suggested. Schonewille *et al.* (2006) reported that anti-Jk^a and anti-Jk^b were predominant antibodies found in patients tested within one month, whereas anti-K and anti-Fy^a were most encountered after more than 3 months following blood transfusion.

Antibodies against HLA class I may cause confusion in RBC immunohaematologic testing. HLA class I antigens are widely distributed and, in general, can be detected on all nucleated cells. In peripheral blood, platelets and RBCs (which lack nuclei in their mature forms) can also express HLA class I antigens (Rosenfield *et al.*, 1967), sometimes referred to as Bg (Bennet-Goodspeed) antigens on RBCs. The numbers of molecules expressed per platelet have been estimated to be in the range of 14,000 to 82,000 (Kao *et al.,* 1986), being far fewer on RBCs, with a range of 40 to 550 per cell (Giles *et al.,* 1990). In contrast, the number of HLA class I molecules on T lymphocytes is about 100,000 per cell and on B lymphocytes, there are about

260,000 molecules per cell (Everett *et al.,* 1987; Mollison *et al.,* 1997). In view of the low number of HLA class I molecules on most RBCs, there has been speculation whether they are integral membrane components or they are acquired on the membrane by adsorption from plasma, which contains both membrane-shed and secreted forms (Krangel, 1987). It has been reported that RBCs do not synthesise HLA per se, but HLA class I molecules are produced by their nucleated precursor cells (Rivera & Scornick, 1986). Three principal antigens – Bg^a, Bg^b and Bg^c – have been defined and correlated with HLA class I antigens B7, B17 and A28 respectively (Morton *et al.*, 1969). Unwanted positive results in cross-matching due to HLA are common because antibodies to HLA-A28 and HLA-B7 are frequently present in sera and anti-Bg^a has been found in more than 10% of multiply transfused patients and can be the cause of HTRs (Nordhagen & Aas, 1978; Panzer *et al.,* 1987; Mollison *et al.,* 1997).

2.5 Pre-transfusion compatibility testing

The goal of pre-transfusion compatibility testing is to provide the patient with a beneficial and safe transfusion (Shulman *et al.,* 2001; Lieb & Aldridge, 2005). The transfused blood components should have acceptable survival *in vivo*. Pre-transfusion testing, including the antiglobulin phase, is very important because adverse effects of accelerated RBC destruction can be severe. Even recipient's RBCs, albeit less frequently, sometimes undergo accelerated destruction (bystander or autoantibody mediated). Most HTRs result from errors in patient or sample identity; and in some cases blood group alloantibodies to private antigens are not detected by standard serological techniques using RBC panels. When testing samples from antenatal patients and patients transfused within the last 3 months, a fresh sample is obtained for compatibility testing if more than *3 days* have elapsed since the original sample was collected. If performed properly, pre-transfusion testing will ensure that a patient is issued the designated blood components, it will verify that the blood is ABO compatible and will detect the most clinically significant unexpected antibodies.

2.6 Prevalence of post-transfusion RBC alloimmunization

2.6.1 RBC alloimmunization in sickle cell disease patients

In heavily transfused SCD subjects, the RBC alloimmunization rate may approach 30% (Orlina *et al*., 1978). Acute or delayed HTRs may occur if an alloimmunized patient is exposed to the same foreign antigen during subsequent transfusion. In the Cooperative Study of Sickle Cell

Disease, Rosse *et al.* (1990) reported an overall rate of RBC alloimmunization of 18.6% in 1,814 multiply transfused SCD patients. They identified a positive linear correlation between the number of SCD patients sensitized and the number of RBC exposures. Seventeen percent of the alloimmunized patients demonstrated four or more antibodies with a predominance of anti-C, anti-E and anti-K. In the same study, children less than 10 years old had a lower rate of alloimmunization than those in older age groups. In SCD, nulliparous women are more likely to become sensitized to RBC antigens than multiparous females (Reisner *et al*., 1987). The suggested mechanisms underlying the increased incidence of alloimmunization in SCD patients include an altered immune response, increased frequency of certain HLA antigens, or lack of phenotypic compatibility between donor and recipient (Ambruso *et al.,* 1987; Cox *et al.,* 1988). Alarif *et al*. (1986) found a significant association between RBC alloimmunization and HLA-B35 among SCD patients. Caccese *et al.* (1987) demonstrated an increased functional activity in monocytes from patients with SCD reflecting an ongoing inflammatory state, when compared with monocytes derived from normal individuals.

When the distribution of antigens is different in the donor and recipient populations, greater alloimmunization may be expected. In a retrospective study, Vichinsky *et al.* (1990) found that racial and ethnic blood group antigen profiles between donor and recipient groups contribute to RBC alloimmunization in SCD. The frequencies of Duffy and Rh blood group system antigens are known to be distinctly different in Blacks and Caucasians. Despite the fact that 68% of Blacks lack the Fy^a and Fy^b and 99.9% of Caucasians have one or both antigens (Daniels, 2002), very few of the Fy(a-b-) Blacks form Duffy antibodies no matter how often they are transfused with Fy(a+) and/or Fy(b+) blood. This is because individuals of the Fy(a-b-) phenotype do not recognize the Duffy antigen as ‘foreign’ due to the presence of the Duffy glycoprotein on their tissue cells (Issitt & Anstee, 1998). In Brazil, where less heterogeneity between the donor and recipient groups exists, RBC alloimmunization rates in SCD are still substantial, suggesting that other mechanisms may be operative (Moriera *et al.*, 1996).

Following alloimmunization, antibodies to the Rh and Kell system antigens are most often detected, followed by antibodies to antigens of the Duffy and Kidd systems, while transfusion-induced antibodies to other RBC antigens are rarely found (Schroeder, 1999). To prevent the occurrence of alloimmunization, Davies *et al.* (1986) recommended extended RBC phenotyping of all SCD patients at the beginning of their transfusion therapy. This helps in deciding what

blood should be transfused and also aids in the identification of any antibodies that might develop. Thereafter, blood for transfusion should be matched for at least C, E and K antigens (Murphy, 2001). The stroke prevention trial (Vichinsky *et al.,* 2001) demonstrated that when SCD patients were given WBC-reduced RBCs that were matched for C, E and K antigens, the alloimmunization rate dropped from 3% to 0.5% per unit and HTRs dropped by 90%.

There is growing evidence that alloimmunization may lead to the production of autoantibodies and vice versa (Aygun *et al.,* 2002). Castellino *et al.,* (1999) reported the frequency of autoantibody formation as approximately 7.6% in a review of a large series of multiply transfused children with SCD. They also reported a strong association between autoantibody formation and the presence of RBC alloantibodies. The etiology behind the formation of these autoantibodies is poorly understood and not much information exists to suggest ways in which to lower the incidence of autoantibody formation. However, Ahrens *et al.,* (2007) found that blood transfusion appears to play a role in the majority of cases of autoantibodies associated with RBC alloimmunization. Clinically, it is important to recognize that post-transfusion haemolysis in which both autologous and transfused RBCs are destroyed may occur in patients with SCD.

2.6.2 RBC alloimmunization in other multiply transfused patients

In a retrospective study undertaken by Blumberg *et al.* (1983) on patients with disorders that often require multiple transfusions, the rate of alloimmunization (i.e. the proportion of patients with new antibodies) to RBC antigens was 11% in the aplastic anaemia, and 16% in the chronic myeloid leukaemia (CML) disease groups. In the same study, other groups of multitransfused patients had similar rates of alloantibody formation e.g. patients with renal failure (14%) and those with gastrointestinal bleeding (11%). Patients receiving chemotherapy for CML did not seem to be suppressed in terms of their ability to produce blood group alloantibodies when the D antigen was respected in the selection of the donor units. In contrast, none of the 99 patients with chronic lymphocytic leukemia (CLL) followed up for over a 10-year period produced blood group antibodies. Lymphoid leukemia patients are generally characterized by a lack of immunologic response and alloimmunization to RBC antigens following multiple transfusions, in this setting, is uncommon (Han *et al.,* 1981; Fluit *et al.,* 1990). There is often hypogammaglobulinaemia in lymphoid leukemia, which may be attributed to impaired functionality of B cells that are unable to upregulate HLA class II and costimulatory molecules

(Marshall-Clark, 2000) as is also observed in multiply transfused infants during the first few months of life (Ludvigsen *et al.*, 1987; Strauss *et al.*, 1999). Similarly absence of alloimmunization has been reported in some RhD negative AIDS patients receiving RhD positive RBC transfusions (Boctor *et al.,* 2003). This may be attributable to the decrease in $CD4^+$ T lymphocytes in AIDS. However, the hypergammaglobulinaemia associated with a positive DAT (Levine & Liebman, 1995) and the persistent immune activation (Eggena *et al.,* 2005) in the course of HIV infection may explain the post-transfusion alloimmunization that is occasionally reported. Seyfried and Walewska (1990) found that the highest rate of immune response to RBC antigens occurred in multitransfused patients (defined as 3 or more blood transfusions) with autoimmune haemolytic anaemia (28%), liver cirrhosis (31.5%) and the myelodysplastic syndromes (40.9%). In a retrospective study, Fluit *et al.* (1990) found that 22 out of 186 (11.8%) multitransfused patients with haematological disorders developed antibodies over a 3-month period, after receiving at least six RBC transfusions. Anti-E and anti-K were the antibodies most frequently found; they were detected in 12 and 15 patients respectively. In patients with transfusion-dependent thalassaemia, the rate of allo-immunization to RBC antigens was found to range from 5 to 37%, with a lower prevalence in children starting transfusions before an age of two years (Spanos *et al.,* 1990; Coles *et al.*, 1981; Wang *et al.,* 2006). A female preponderance of RBC alloimmunization upon transfusions is controversial (Raki 1999; Blumberg *et al.* 1984; Redman *et al.* 1996). In a recent review of literature, Verduin *et al* (2012) observed a higher RBC alloimmunization rate in transfused females with SCD only and not in other diseases that require multiple blood transfusions.

As discussed in section 2.3.2, HLA class II antigens present blood group peptides to CD4 T cells. Some peptides may be more optimally presented by the antigen-presenting groove formed by particular class II molecules. Reviron *et al.* (2005) and Chu *et al.* (2009) reported associations of anti-Jk^a alloimmunization with particular HLA-BRB1 alleles and anti-Mi^a alloimmunization with HLA-DRB1*0901 allele respectively, suggesting a role for MHC restriction in some cases of RBC antigen presentation (Picard *et al.,* 2009; Hoppe *et al.,* 2009).

2.7 Haemolytic transfusion reactions

Haemolytic transfusion reactions are one of the recognized consequences of post-transfusion RBC alloimmunization. An acute HTR is defined as the haemolysis of donor RBCs, within 24

hours of transfusion, by preformed alloantibodies in the recipient. Clerical errors (mislabelling of blood or misidentification of patients) account for 80% of acute HTRs, confirmed by national haemovigilance schemes which have been operational for several years in Europe and North America (Goodman *et al.,* 2003; SHOT, 2009). Symptoms and signs of acute HTRs are non-specific and include fever, chills, rigors, chest/back/abdominal pain, pain at the infusion site, nausea, vomiting, dyspnoea, hypotension, haemoglobinuria, oliguria/anuria, and disseminated intravascular coagulation (DIC). Most frequently, the offending antibodies are high titer IgM anti-A and/or anti-B although complement-fixing IgG antibodies in the recipient may be responsible as well. Immune-mediated haemolytic reactions can also rarely occur because of RBC antibodies in the plasma of the transfused product, be it in RBCs, fresh frozen plasma (FFP) or platelets. Cases of HTRs after transfusion of group O plasma containing products such as platelets, with high titer anti-A or anti-B to non-group O patients have been reported (Larsson *et al.,* 2000; Lozano & Cid, 2003; Josephson *et al.,* 2004).

Delayed HTRs are more common but usually less severe than acute haemolysis. Delayed reactions occur when a patient previously sensitized by pregnancy or blood transfusion receives "incompatible RBCs" because the low titers of circulating alloantibodies (typically against Rh and Kidd system antigens) escape detection by pre-transfusion testing. However, there is a rapid anamnestic response after transfusion of antigen-positive RBCs, leading to haemolysis. Delayed HTR often go unrecognized because they occur several days (usually within 5 - 10 days) after transfusion, which often means after hospital discharge. Delayed serologic transfusion reactions (DSTRs) are reactions identified serologically but not clinically. Delayed HTRs and DSTRs occur in approximately 1 in 1500 transfusions, with DSTRs being detected at rates two to fourfold higher than delayed HTRs (Ness *et al.,* 1990; Pineda *et al.,* 1999; Hendrickson & Hillyer, 2009). Obtaining a transfusion history and selecting offending antigen-negative RBCs for transfusion of patients with a history of clinically significant RBC alloantibodies is critical in decreasing the risk of delayed HTRs or DSTRs (Hendrickson & Hillyer, 2009). Patients with SCD or other major haemoglobinopathy syndromes who are chronically transfused are at greatest risk of alloantibody formation to RBC antigens and consequent HTRs. About 25% of the clinically significant RBC alloantibodies become undetectable over time, potentially confounding future transfusions and placing the patient at risk of an anamnestic antibody production and severe delayed HTRs (Rosse *et al.,* 1990; Schonewille *et al.,* 2000).

Approximately 40% of SCD patients who are alloimmunized have or will experience a delayed HTR (Knowles, 2001). Importantly, delayed HTRs can mimic various complications of SCD and should be suspected when patients present with appropriate symptoms (e.g. pain, fever, accelerated haemolysis) after a recent transfusion (Diamond *et al.,* 1980).

Another complication of RBC alloimmunization is the hyperhaemolysis syndrome which has a reported incidence of 4 - 11% (Aygun *et al.,* 2002; Talano *et al.,* 2003). In patients with SCD, clinical findings in the hyperhaemolysis syndrome occur approximately 1 week after the RBC transfusion and include the onset of increased haemolysis associated with pain and profound anaemia. The haemoglobin level often drops to below pre-transfusion levels. In many reported adult cases, the DAT remains negative and no new alloantibody is detected as the cause for these transfusion reactions (Talano *et al.,* 2003). Continuation of blood transfusion may be lethal, as this can further exacerbate haemolysis (Friedman *et al.,* 1993). It has been suggested that transfusion be withheld in severe haemolytic episodes, until hemolysis has faded spontaneously or after treatment with corticosteroids, high dose intravenous immunoglobulin or rituximab, a monoclonal antibody against B cells. The exact pathophysiologic mechanism of this syndrome is not well understood. A bystander haemolytic mechanism and transfusion suppression of erythropoiesis have been proposed (Petz *et al.,* 1997; King *et al.,* 1997). There is a broad clinical spectrum of autoantibody formation in association with red blood cell transfusions and reactions range from asymptomatic serologic detection to severe, life-threatening haemolysis (Sosler *et al.* 1989; Zumberg *et al.,* 2001; Garratty (2004).

2.8 Haemolytic disease of the fetus and newborn

Haemolytic disease of the newborn was first described in 1609 in a set of twins by a French midwife called Louise Bourgeois: the first twin was oedematous and stillborn, and the second was deeply jaundiced and subsequently died of what is now called kernicterus (Bowman, 1988). Over the centuries, this clinical picture was recognized and reported as two separate conditions. Diamond *et al.* (1932) realized that congenital anaemia, icterus gravis and hydrops, were manifestations of the same disease, which they named *erythroblastosis fetalis*. The identification of the cause of the haemolysis had to await the discovery of the Rh system (Landsteiner & Wiener, 1940) and the determination soon thereafter that HDFN occurred in an RhD-positive

fetus carried by an RhD-negative woman who had been immunized by the transplacental passage of RhD-positive RBCs during a prior pregnancy (Levine *et al.,* 1941).

Alloimmunization to the D surface antigen is the commonest cause of HDFN, which, before the introduction of anti-D immunoprophylaxis affected 1% of all newborns and was responsible for the death of one baby in every 2200 births (Kumar & Regan, 2005). By the 1970s, routine antenatal care in well-resourced countries included screening of all expectant mothers to select Rh-D negative cases and giving preventive treatment with anti-D after birth of a Rh-D positive child. This led to a dramatic decrease in the incidence of HDFN, particularly severe cases that were responsible for stillbirths and neonatal deaths (Mollison *et al.,* 1997). Despite the widespread use of this prophylaxis, a significant number of women still become alloimmunized for a variety of reasons, including no administration or insufficient dosage of RhIG in case of unrecognized miscarriage, leakage of fetal RBCs into the maternal circulation late in pregnancy, large fetomaternal hemorrhage (FMH) or exposure to traumatic deliveries including Caesarean sections, manual removal of the placenta, stillbirths and intrauterine deaths, blunt abdominal trauma during the third trimester, twin pregnancies (at delivery), external cephalic version, chorionic villous sampling, antepartum haemorrhage, ectopic pregnancy and an unexplained hydrops fetalis (Sebring & Polesky, 1990; Bowman, 1997). FMH involves smaller amounts: in 3% of the women, fetal RBCs are detectable in the maternal circulation during the first trimester of pregnancy; in 12% during the second trimester; in 46% during the third trimester; and in 64% of the women after delivery, usually in amounts less than 20 ml of fetal blood (Bowman *et al*., 1986). It is not uncommon for there to be silent leaks of RBCs from the fetus into the mother (with no pain or bleeding), especially in the third trimester. The maternal IgG antibodies traverse the placenta to the fetal circulation during gestation and cause RBC destruction with complications before birth, or anaemia and hyperbilirubinaemia after birth, or both. In its most severe form, HDFN produces hydrops fetalis, which is characterized by total body oedema, hepatosplenomegaly and heart failure, and can lead to intrauterine death. HDFN may also follow blood transfusion with antigen positive blood that is incompatible with the mother. Virtually all alloantibodies reactive by the IAT have been implicated in HDFN in different populations. The prevalence of D-negativity varies in different ethnic groups with 15% of Caucasians, 8% of Blacks and 1% of Asians being D-negative (Reid & Lomas-Francis, 2004). The D antigen accounts for about 50% of cases of maternal alloimmunization; the remainder is mainly due to

incompatibility to K, c, C/G, E, and Fy^a antigens and to low incidence antigens in the Rh, MNS, and Diego blood group systems (Heddle *et al.,* 1993). Anti-D formation is more frequent in D positive individuals of African descent than in Europeans, which is probably a result of the high frequency of aberrant *RHD* alleles belonging to the three African D clusters i.e. DIVa, weak D type 4 and DAU in some African populations (Touinssi *et al.,* 2009). Also, because the number of copies of the D antigen on each RBC is higher in the R_2 haplotype (range: 14,000 to 16,000) than in the R_1 haplotype (range: 9,000 to 14,600), fetuses whose RBCs are R_2 have more severe anemia than their R_1 counterparts (Mollison *et al.,* 1997). Ulm *et al.* (1999) reported that male fetuses were 13 times more likely to develop hydrops than female fetuses, and perinatal mortality was 3 times higher in male fetuses. However, this was not confirmed by other inestigators (Ramsey & Sherman, 1999).

From the perspective of prevention an initial step is to estimate fetal risk by establishing paternal *RHD* zygosity. After paternal testing has revealed the possibility of a heterozygous state for *RHD*, fetal testing is indicated. Fetal D antigen determination through noninvasive DNA testing from maternal plasma is now routine practice in some countries (Lo *et al.,* 1998; Daniels *et al.,* 2004). If the maternal race is black, then the presence of a maternal pseudogene or $Ccde^s$ gene should be considered in the scheme of fetal testing (Faas *et al.,* 1997; Singleton *et al.,* 2000). The presence of one of these genes in the fetus can lead to a false positive molecular diagnosis yet the fetus would be found to be D-negative by serology after birth, leading to unnecessary fetal interventions such as antenatal RhIG administration. Whenever an IAT-reactive antibody is detected during pregnancy, a cord blood sample at birth should be tested by DAT and if positive, the haemoglobin (Hb) and bilirubin levels monitored to initiate treatment (BCSH Guidelines, 1996). Monitoring maternal anti-D during pregnancy is very important to predict the severity of antenatal HDFN. In most centres, a critical titre for anti-D between 8 and 32 is usually used (Moise, 2005; Moise, 2008). Because the titre is not very reliable predicting fetal hemolysis, functional assays such as the monocyte monolayer assay (MMA) or antibody-dependent cellular cytotoxicity (ADCC) are performed in reference laboratories. Once sensitization has occurred the fetus should be monitored by preferentially non-invasive echo-doppler techniques to estimate the degree of fetal anemia. The following therapeutic options are open: controlled early delivery or intrauterine transfusion of which the latter has the best prognosis but requires a specialized centre (Urbaniak & Greiss, 2000; van Kamp *et al.,* 2004).

Interestingly, a fetus that is ABO incompatible with the maternal anti-A/B is less likely to have HDFN due to anti-D, presumably due to rapid removal of the ABO-incompatible RBCs by the naturally occurring anti-A/B. Although maternal-fetal ABO incompatibility is common, in general haemolysis is mild and the clinical course is relatively benign needing only phototherapy (Grundbacher, 1980; Drabik-Clary *et al.,* 2006). In cases of ABO incompatibility between the mother and the fetus, group O mothers are more likely to become sensitized (Ozolek *et al.,* 1994). This process occurs to a much less extent in group A or B neonates who are born to heterospecific A or B mothers, because in this situation, the respective anti-A or anti-B immunoglobulin is predominantly IgM and therefore unable to cross the placenta (Kaplan *et al.,* 2009).

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