Red blood cell alloimmunization after blood transfusion

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RED BLOOD CELL ALLOIMMUNIZATION

AFTER BLOOD TRANSFUSION

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GENERAL INTRODUCTION

1.1 Introduction

Since the proposal on the circulation of blood in man by the English physician William Harvey[1] in 1628 and the first published report on human-to-human blood transfusion by James Blundell[2], an obstetrician at the United hospitals of Guy's and St. Thomas', in 1818, blood transfusion has nowadays become a relatively simple and live-saving part of daily medical practice.
The discovery of the AB0 blood group system, by the Austrian pathologist Karl Landsteiner[3] together with his colleques von Decastello and Sturli[4], and the introduction of the AB0 blood grouping test for selected donors by Reuben Ottenberg[5] in 1911, greatly reduced the fatalities associated with blood transfusion in the early days of transfusion therapy. In 1921, Unger[6] reported intra-AB0-group transfusion reactions and recommended additional tests to exclude the possibility of a recipient's serum agglutinating the donor's red cells, now known as irregular alloantibodies.
After the introduction of the indirect antiglobulin test by Coombs[7] in 1945, which added a new dimension to the safety of blood transfusion, there was a rapid increase in the identification of alloantibodies that caused transfusion reactions or hemolytic disease of the newborn. This has led to the discovery of 245 blood group antigens classified in 29 blood group systems and 38 high or low frequency antigens not yet fulfilling the requirements for classification into a system[8].
Alloimmunization occurs when an incompatible antigen introduced in an immuno-competent host evokes an immune response. The way the immune system reacts depends on several factors. The immune response to carbohydrate antigens, is usually thymus *independent*. Multivalent antigens directly stimulate B cells to synthesize antibodies without the aid of helper T cells resulting in the majority of cases in the production of IgM antibodies. Individuals lacking a particular carbohydrate blood group antigen on their red cells can have 'naturally occurring' IgM antibodies, which are most probably stimulated by cross-reacting antigens present in the environment, such as on gut bacteria. The most important carbohydrate antigens for blood transfusion practice are the A- and B-antigens. Normal individuals who lack either the A or B antigen make IgM B- or A-antibodies, respectively. Since IgM antibodies are complement-binding, these antibodies can cause immediate and severe intravascular hemolysis after transfusion of incompatible red cells, which can lead to serious or fatal complications.
Numerous other blood group antigens reside on membrane proteins and comprise polymorphic determinants dependent primarily on amino acid sequence. These protein antigens can stimulate a thymus-*dependent* immune response, and the resulting IgG antibodies can cause extravascular clearance of antigen-positive

cells. These IgG antibodies may also cross the placenta, resulting in hemolytic disease of the newborn.
The most important irregular red blood cell alloantibodies in daily transfusion practice, in terms of frequency of occurrence, are directed towards the RH (anti-D, -C, -E, -c and -e), KEL (anti-K), FY (anti-Fy^a and -Fy^b), JK (anti-Jk^a and -Jk^b) and the MNS (anti-M, -S and -s) blood group systems. Of these, the D-antigen is the most immunogenic, resulting in more than 80% of immunocompetent D-negative persons becoming alloimmunized after a transfusion of D-positive erytrocytes[9,10]. This has resulted in prophylactic matching of red cell transfusions for the D-status. Such routine is not common to prevent other RBC antibodies, for which we rely on serologic screening before transfusion.
Retrospective studies in the general population reported antibody frequencies after transfusion of less than 1 to 3 percent. However, in multitransfused patients alloimmunizaton occurs in up to 70% of patients. Whether the recipient's immune system will react depends on genetic and acquired patient related factors, dose and route of administration and the immunogenicity of the foreign antigen.
Studies on the alloimmunization frequency in several patient cohorts and factors influencing these results are subject of this thesis.

1.2 Outline of the thesis

Since the discovery of the indirect antiglobulin test, several techniques have been applied in the transfusion laboratories to detect irregular RBC antibodies. In the Netherlands, up till the 1990's, it was common policy, in pre-transfusion testing, to detect all blood group antibodies present in patients, irrespective of their clinical importance. When it came clear that antibodies reacting at lower temperatures but not at 37 ºC are of no clinical significance and only generate useless extra investigations, routine pre-transfusion testing at low temperature was abbreviated. Furthermore, it has been shown that the antiglobulin phase crossmatch as part of compatibility testing in patients without RBC alloantibodies is of limited value.
Nowadays, before a transfusion event, patients are routinely tested for their AB0 and D blood groups and for the presence of irregular IgG alloantibodies using panels of red cells with homozygous expression of the most relevant blood group antigens. In case of an alloantibody, red blood cells lacking the corresponding antigen, are transfused after an antiglobulin crossmatch. When the patient has no irregular alloantibodies, the type and screen strategy together with an immediate-spin or computer crossmatch, is being applied in an increasing number of transfusion laboratories in the Netherlands.
However, despite the understanding of red cell antigens and their clinical significance in transfusion medicine, fatalities due to alloimmunization still occur.

The characterization of genes and the molecular basis of antigens and phenotypes has introduced DNA-based molecular methods for the detection of blood group antigens. The development of microassay technology will soon make it possible to analyze many blood group alleles on a single synthetic chip. Application of molecular genotyping to transfusion medicine practice will enable selection of donor units that are antigen matched for recipients at multiple blood group loci, potentially diminishing alloimmunization. The introduction of such a policy should be based on information regarding patient groups to whom it should be applied, for which RBC antigens it should be applied and the costs compared to the current policy.
The aim of this thesis was primarily to investigate whether we should change the current policy to improve transfusion safety. For this purpose the risk of red cell alloimmunization and identification of (patient related) factors associated with alloimmunization were investigated.

The aim of the studies, described in detail in the following chapters, is summarized in short.

Chapter 2
Review of the literature regarding blood groups and red cell alloimmunization

Chapter 3
Multicenter retrospective study on the influence of prestorage filter leucodepletion on the development of clinically significant red blood cell alloimmunization against antigens in the RH, KEL, FY, JK and MNS blood group systems. Comparisons were made between the transfused patient cohorts during two periods, two years before and two years after universal leucodepletion. To control for changes not related to leucoreduction, antibody incidence was compared to antibody prevalence.

Chapter 4
A 23-year retrospective study on the safety of the type and screen policy with regard to the presence of unexpected antibodies directed against low incidence antigens.

Chapter 5
A 10-year retrospective study on RBC alloimmunization in patients with oncohematologic diseases. Comparisons for myeloproliferative and lympho-proliferative diseases were made. The main goal of this study was to investigate, whether these patients should receive RBC transfusions matched for other antigens than ABO and D.

Chapter 6
The development of additional antibodies in non-multitransfused alloimmunized patients was the subject of this 20-year retrospective multicenter study. The aim of the study was to investigate to which extend these patients are prone to form additional antibodies after repeat transfusion and if extended matching should be applied in these patients.

Chapter 7
An 11-year retrospective single center national study on additional RBC alloimmunization in women, whose fetusses were treated with intra-uterine transfusion for hemolytic disease. In addition, a number of risk factors for the occurrence of additional antibodies were defined.

Chapter 8
The aim of this 20-year retrospective study was to achieve information on the risks of current pre-transfusion testing, with regard to the persistence of clinically significant RBC alloantibodies.

Chapter 9
A 5-year retrospective multicenter study analyzing factors influencing the rate and specificity of RBC alloimmunization. Special emphasis was taken on the time interval between transfusion event and antibody detection.

Chapter 10
General discussion concerning studies of the thesis and proposals for further studies.

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REVIEW OF THE LITERATURE

ON RED CELL ALLOIMMUNIZATION

Contents

2.1 History of red cell alloimmunization

Red blood cell alloimmunization results from the genetic red blood cell antigen disparity between donor and recipient or from mother and fetus.
The first reports on alloimmunization date from the 17th century describing hydropic stillborns. This disease, today known as hemolytic disease of the fetus or newborn (HDFN), is caused by immune IgG antibodies from the mother directed against the red blood cells of the fetus. These antibodies are transferred into the fetal circulation where they coat the fetal red blood cells causing extravascular hemolysis. In 1939, Levine and Stetson published a case report of HDFN and reported that the mother's serum agglutinated the red blood cells of her husband and also 80 percent of group O-donors[1]. The name for the antibody involved, anti-Rhesus, came from work of Landsteiner and Wiener who immunized rabbits and guinea pigs with blood from Macacus Rhesus monkeys[2]. In 1940, Wiener and Peters reported on the first hemolytic transfusion reactions due to anti-Rhesus[3]. Years later, this antibody turned out to be different from the human anti-Rh and it was renamed anti-LW, after Landsteiner and Wiener.
Antibodies with different RH specificities (e.g. C, c, E and e)[4-7] were discovered soon after and already in 1944 Sir Ronald Fisher proposed the current RH blood group system nomenclature[8].
After the development of the antiglobulin test by Coombs[9] in 1945, many other blood group antigens were recognized in the next years. Antibodies to the S and s-antigens, belonging to the MNS blood group system, of which the M and N antigens had already been discovered in 1927 by Landsteiner and Levine[10,11], were first reported in respectively 1947 and 1951[12,13].
Often the name of a blood group system was derived from the first patient described. Coombs identified the K-antigen in a patient (Mrs Kell) whose child had HDFN[14] and anti-k was found in 1949 by Levine and coworkers[15]. The FY system was named after Joseph Duffy, a hemophilac, who had become alloimmunized after several blood transfusions, and the JK system taking the initials of a baby named John Kidd, who suffered from HDFN[16-19].
Untill today, the aforementioned blood group systems, together with the ABO-system, are still the most important in transfusion medicine and pre-transfusion antibody screening is primarily focussed on detecting antibodies against these blood group antigens.

2.2 Red cell compatibility testing

The basis for a succesfull blood transfusion is the simple recognition of red cell clumping as a result of antigen-antibody reactions. The description of red cell

agglutination and its development as a tool in elucidating blood groups took place in the last 30 years of the 19th century. In 1869, Adolf Creite, was the first who reported that, after mixing serum from one species with red blood cells from another species, serum proteins had the property of both dissolving (lysis) and bringing about clustering (agglutination) of red cells[20]. Landois who extended Creite's in vitro experiments described agglutination as 'cells develop the ability to stick to neighboring cells and form larger or smaller clumps'[21].
In 1901, Landsteiner made a great breakthrough by elucidating the mechanism underlying intraspecies agglutination, through his discovery of the ABO-blood group system, clarifying the frequently observed hemolytic reactions after transfusion[22].
Minot advocated pretransfusion ABO determination of the patient and blood donors, to select compatible donors in advance, rather than performing crossmatches between the patient and random donors. This led to the first 'walking' blood bank[23]. Although Ottenberg[24,25] is credited for being the first to advocate and practice cross-matching besides ABO blood grouping as routine pretransfusion testing, Hektoen had proposed an early form of cross-match a year before[26]. These first cross-match procedures required 10-15 ml blood and at least a 2 hour incubation. Rous and colleques and Lee improved the technique by reducing the amount of blood needed and shortening the incubation time to less than 15 minutes[27,28].
The first recognition of non-ABO antibodies (probably against RH system antigens) was made by Unger in 1921, who stated that 'preliminary to transfusion, the blood of every patient should be grouped and then tested directly against that of the prospective donor'[29]. This statement was further strenghtened by reports on hemolytic transfusion reactions, especially in previously transfused patients[30-32].
Levine observed that these irregular antibodies were detected better after a 30 minutes incubation at 37 ºC and Diamond and coworkers enhanced agglutination reactions further by the addition of bovine albumin to the testsuspension[33,34].
A milestone in transfusion medicine and compatibility testing was the re-discovery of the antiglobulin test by Coombs, Mourant and Race in 1945[9,35-37]. After the 'Coombs' test became routine in compatibility testing[38] thousands of antibodies were detected.
To further increase transfusion safety, sensitive cross-matching protocols were developed, including direct antiglobulin tests, autocontrols and minor crossmatches (i.e. donor serum and recipient cells). Enzyme treated red blood cells[39] and additives such as bovine albumin[34], low ionic strenght media[40], polybrene[41], and polyethylene-glycol (PEG)[42] were used to enhance agglutination and to further shorten incubation times. By these sensitive techniques many new bloodgroups were discovered. Yearly, new blood group antigens are identified by

unusual serologic findings[43-49]. Subsequently a number of pretransfusion tests that did not add to transfusion safety were abandonded[50,51].
Currently, routine pretransfusion tests focus primarily on potential clinical significant antibodies that only react in the indirect antiglobulin phase after incubation at 37 °C.

2.3 Blood group diversity and function

Antigens are defined by antibodies, which can be immune or 'naturally' occuring human antibodies, as well as deliberately stimulated antibodies in animals. Blood group antigens are cell surface molecules and reside on a variety of structures e.g. proteins, polysaccharides, glycoproteins, glycolipids and lipoproteins. The membrane proteins are subdivided into structural (integral membrane or transmembrane proteins) or functional structures (structural integrity, transporter, enzymes, receptors).
Blood groups antigens are the product of genes. Evolutionary pressure from various environmental pathogens is thought to be responsible for the generation of genetic variants, resulting in survival advantage. A wellknown example are individuals whose phenotype is Fy(a-b-) and lack gp-Fy on their erythrocytes, preventing the invasion of malaria Plasmodium vivax parasite[52].
The genes encoding the blood group proteins have been mapped to different chromosomes throughout the genome. Many antigens are related by arising from mutations of the same 'parent' molecule and together form blood groups systems. Within these systems, antigens excist either as different epitopes on the same molecule or as the products of allelic genes. In case of polysaccharide blood group antigens, genes code for enzymes that cause the production of specific red cell membrane carbohydrates[53-55].
The development of DNA sequenching and amplification techniques (polymerase chain reaction) has created the possibility for molecular characterization of the genes encoding blood group antigens[56,57]. The most common mechanism responsible for diversity in blood groups arises from single nucleotide polymorphisms (SNPs). These SNPs can be silent or affect the translated gene product (missense or non-sence mutations). It is estimated that two thirds of all blood group antigens are defined by missense SNPs in blood group genes[58]. The SNPs cannot only alter the antigen expressed by a certain blood group molecule but also modify the number of copies expressed on the red cell membrane, e.g. weakened expression of the D-antigen.
There is increasing knowledge of the functional aspects of the molecules that express RBC antigens and the potential pathophysiological significance of these structures[59-61]. Because there are important interactions between proteins at the

cell surface and the cytoskeleton, gene mutations resulting in complete lack of antigen-bearing molecules result in red cell abnormalities as well as in other organ dysfuctions (e.g. RH-null, Leach, McCloed), but in general the antigenic composition does not affect intrinsic red cell function.
In the clinical transfusion practise, applications of genomic typing assays include donor typing for RBC, HLA and platelet antigens, fetal RBC phenotype prediction to determine the risk of hemolytic disease, genotyping of multiple transfused patients and in situations where the RBC phenotype cannot be accurately determined by serological techniques[62-66].
Today, almost all of the genes underlying expression of the human blood group systems have been cloned and the polymorphisms responsible for the phenotypes encountered in different individuals and populations are increasingly being clarified.

2.4 Immunogenicity

The nature of the immune response to blood group antigens depends on several factors, including the dose and route of administration, genetic host factors and the immunogenicity of the antigen. Immunogenicity is the ability to stimulate a specific immune response, estimated as effector cells or antibody production. Compared to the immune response against micro-organisms blood group antigens are generally poor immunogens. After exposure by transfusion only 1-3 percent of recipients respond with antibodies against a red cell antigen. Antigen factors such as chemical and physical form, number of antigen sides, degradability and whether a response is T-cell dependent influence the host's immune response[67].
The most immunogenic blood group antigens are A and B, because the natural antibodies to these antigens occur in virtually all individuals lacking the corresponding antigen. With regard to immune alloantibodies, the D-antigen appears to be the most immunogenic of all blood group antigens. Studies deliberately exposing healthy D-negative volunteers to D-positive blood, to obtain IgG anti-D for immunoprophylaxis purposis, showed that appoximately eighty percent of D-negative individuals will produce serological detectable anti-D[68-71]. Studies on D-immunization after D-positive RBC transfusions in D-negative patients reported comparable[72] or lower immunization rates[73-79]. In a number of these studies the low rate of D immunization is associated with a depressed immune system[73-76].
It is presumed that the explanation for the strong immunogenicity of the D-antigen is related to the genetic basis and organization of the RH system. DNA analysis revealed that the corresponding *RHD* gene is deleted in D-negative individuals and that no alternative allelic form exists at the same locus in

Caucasians. Therefore, a D-negative recipient of D-positive cells will recognize an entirely foreign antigen, with several distinct epitopes, whereas in case of most other blood group disparities a single or a few amino acid differences are seen as foreign. Even the weak D type red blood cells with very low antigen density have been reported to be capable of anti-D immunization[80-82].

The exact immunogenicity of other antigens is unknown, as few studies exist deliberately exposing antigen-negative persons to antigen-positive RBC[83]. With regard to K-immunogenicity, Schabel et al found anti-K immunization 3-months after K-incompatible transfusions in 11 out of 116 K-negative patients (9.5%)[84].

Giblett[85] estimated the relative immunogenicity for a number of RBC antigens compared to K-immunization by relating the observed frequency with which a specific antibody is found in the population to the estimated frequency of an immunizing event by transfusion, using the formula:

$$\frac{\text{Number of antibodies of interest x Probability of exposure to K antigen}}{\text{Number of K antibodies x Probability of exposure to antigen of interest}}$$

Based on her calculations, the K-antigen was 2.5 times more potent than c-antigen, 3 times more potent than E-antigen, 21 times more potent than Fy^a-antigen and 71 times more potent than Jk^a-antigen in inducing antibody formation. Combined data from 3 studies performed between 1974 and 1995 show comparable relative immunogenicity results for E-antigen, but c-antigen was 3 times (range, 1.2-4.2) less immunogenic, while Fy^a- and Jk^a-antigens were respectively 3 (range, 2.3-3.5) and 8 times (range, 5.7-12.7) more immunogenic than reported by Giblett[86-88].

Factors such as the sensitivity of antibody detection techniques, which has improved considerably over these study years, and the (patient) population under study may have had great impact on the occurrence of certain antibody specificities and therefore on its calculated immunogenicity.

Genetic recipient factors may determine as to whether a person will response to a foreign RBC antigen or not. The adaptive immune system reacts only to foreign antigens if CD4 T-lymphocytes are activated upon interaction with peptide fragments presented by class II major histocompatibility complex (MHC) molecules on antigen presenting cells. The binding groove formed by the various MHC class II genes has a variable affinity for different peptides, with consequences for peptide presentation to T-cells. Recent studies showed that, within a particular ethnic group, the intrinsic immunogenicity of a given red cell antigen is, amongst other factors, related to the presence of particular HLA-DRB1* molecules, capable of effective binding and presentation of blood group derived peptides to CD4 T lymphocytes[89-92].

2.5 Alloimmune response to Red Blood Cell antigens

The immune system consists of two closely connected defense layers, the innate and the adaptive or specific immune system. The first is evolutionary older and consists of barriers such as skin and mucosal surfaces and soluble factors in which broad pattern recognition leads to phagocytosis, complement activation and extracellular killing.
The adaptive immune system is responsible for specific antibodies to red blood cell antigens. The first step in this response requires the recognition of foreign antigen. The production of naturally occuring IgM antibodies, such as anti-A, anti-B and anti-M, is primarily a T-cell independent response. Antibody production to these antigens is stimulated by B-cell receptor (sIg) binding to bacterial polysaccharide molecules, which are cross-reactive with the repetitive carbohydrate structures of human red blood cell antigens[93-95]. B-cells are activated, by cross-linking of their antigen receptors, to differentiate into IgM antibody secreting cells. A T-cell independent response usually does not induce a reponse maturation leading to immunoglobulin class switching from IgM to IgG, a process typical for T-cell dependent responses[96].
The main mechanism for alloimmunization involves the presentation of the donor antigen peptides by APCs to the T-cell receptor (TCR) on recipient CD4 T cells (a T-cell dependent response). Presentation of alloantigens may involve two distinct routes, the direct and indirect pathway of allorecognition. In direct recognition, the foreign (donor) HLA class II antigens expressed on donor APCs are directly recognized by recipient CD4 T-cells[97,98]. This occurs mainly for foreign HLA antigens[99,100]. Leucoreduction of blood products, removing donor APCs, has greatly reduced the occurrence of HLA immunization, particular in case of platelet transfusions, where fresh viable APCs enhance immunogenicity[101,102]. Because mature RBC lack HLA class II antigens, direct antigen presentation will not occur.
Red blood cells have an approximate lifespan of 120 days[103], after which the senescent red blood cells and during aging formed microvesicles are phagocytized by splenic and hepatic macrophages. This process takes place either through the phosphatidylserine receptor binding to externalized phosphatidylserine on apoptotic cells or by FcγR recognition of IgG (auto)antibodies bound on senescent cells[104,105]. We assume that when no alloantibodies are present allogeneic RBC are removed by similar mechanisms.
After phagocytosis, the RBC antigens are proteolysed into small peptide fragments in lysosomes and short linear segments of 12-28 amino acids are associated with newly formed HLA class II molecules in postlysosomal vesicles. Genetic HLA class II restriction determines which peptides are tightly bound in

the HLA class II groove[90,92,106-108]. The peptide/HLA complex is transported to the plasma membrane of the macrophage where they are presented to TCR and immunoglobulin receptors on B-cells[109,110]. B cells by themselves can also take up native antigen through their membrane immunoglobulin, process this and present the peptides to activated antigen specific CD4 T-cells. Antigen specificity of T and B cell receptors is obtained by recombination of variable (V), diversity (D) and joining (J) gene segments, N-region nucleotide addition and somatic hypermutation, thereby creating the possibility to recognize an almost unlimited array of different aminoacid sequences[96,111,112].

The TCR variable antigen recognition unit of CD4 T-cells recognizes specific foreign amino acid sequences from processed exogenous antigens presented in the context of the self HLA class-II molecules.

Whether T-cell recognition of foreign peptides leads to an effective immune response depends on additional signals, generated by co-stimulatory cell surface structures.

The first step after engagement of the TCR/MHC class-II/foreign peptide complex is the activation of CD28 on the CD4 T-cell and CD80 or CD86 on the APC[113]. If these costimulatory signals are not activated during initial antigen exposure, the T cell cascade is down regulated, eventually leading to functional inactivation (anergy)[114].

When, in case of non-self peptides, the CD4 accessory molecules on the T-cells bind to their ligands on the APC, the strenght and specificity of the interaction is increased and additional signals (IL-2) for T-cell activation are provided. Activated T-cells rapidly expand resulting in a 100-fold increase. This activation also induces the expression of the inhibitory cytotoxic T-lymphocyte antigen (CTLA)-4 on the expanding T-cell clone, which competes with CD28 for binding with CD80 and CD86, balancing the expansion of the antigen-specific T-cell clone by limiting IL-2 production[115,116]. A second mechanism controlling homeostasis of immune response involves direct cell-cell contact. Activated T-cells express cell-surface Fas (CD95) and engagement of Fas by Fas-ligand bearing cells triggers apoptosis in the Fas-expressing cells by activated enzymes from the caspase cascade which degradates DNA[117,118]. Finally, if antibodies are produced the immune response is cooled down by immunecomplexes activating inhibitory signals.

Activated T-cells produce IL-2 (T-cell growth factor) leading to proliferation and differentiation into effector T_h cells, memory T_h cells, and suppressor T_h cells. Effector T_h cells can differentiate into two major subtypes of effector cells, T_h1 and T_h2 cells, defined by the specific cytokines they produce. The original black and white concept of the T_h1/T_h2 paradigma is later reconsidered as a model in which there are no discrete subsets but rather a continuum of different combinations of cytokine secretion reinforcing each other's actions[119-121].

Activated T_h cells express CD154 (CD40L) which interacts with CD40 on B cells and this CD40/CD154 interaction drives the B-cells into cell cycle[122]. Besides, T_h2 cytokines also induce B-cell activation and division (IL-4) and promote the differentiation into antibody-forming plasmacells (IL-6, IL-10). Upon activation, B cells undergo repeated cell divisions and differentiation to form a clone of short-lived antibody secreting plasma cells. These plasma cells initially secrete low-affinity IgM antibodies, but in the course of the response, switch to the production of high affinity IgG antibodies by means of gene-segment rearrangement of the constant part of the Ig molecule and somatic mutation of the rearranged variable gene.
The primary antibody response is generally relatively slow, it may take several weeks to months before antibody reaches a detectable level and will therefore, in most cases, not affect the RBC survival of the immunizing transfusion.
Despite the antigenic differences between donor and recipient RBC, the likelihood of alloimmunization is, depending on the antigen, in the order of 1-8 percent in the general recipient population. Besides genetic factors, several unknown or ill defined factors may influence the immune response. It is observed that intravenous injection of high concentrations of antigens that are close to self-antigens and persist for a long period of time may delete T-cell responses and more recent studies showed that phagocytosis of apoptotic cells inhibits the production of pro-inflammatory cytokines (the 'danger' signal) and thereby T-cell activation[123-125]. In this respect it is interesting whether longer stored RBC, with a higher number of apoptotic cells, elicit different immune reponses than fresh RBC. It may be clear that more studies are needed to elucidate all factors involved in the immune response towards red blood cell transfusions.
When the antigen has disappeared activated cells are eliminated through apoptotic death, but a small fraction of cells differentiate into memory B and T cells, which are long-lived and provide long-term protection against the antigen concerned. Compared to naïve cells, these memory cells, with an increased antigen sensitivity, require far less antigen and stringent activation requirements, resulting in a more rapid, greater and effective formation of high affinity IgG antibodies upon antigen rechallenge.

Antibody mediated red blood cell destruction

There are tree main pathways of RBC destruction by antibodies. In most cases, an ABO incompatible transfusion elicits a complement mediated acute intravascular hemolytic transfusion reaction (AHTR). The C_H2 domain of IgM antibodies complexed with the RBC antigens binds to C_1q (complement recognition unit) triggering the complement cascade through the classical pathway. After subsequent activation of the complement activation unit (C2-C4-C3) the membrane attack complex (C5-C9) is formed. This complex penetrates the lipid bilayer of the red blood cell membrane making the cell permeable resulting in

osmotic cell lysis. An AHTR is characterized by the release of vasoactive amines (e.g. histamine) and other mediators/cytokines, (e.g. IL-1, TNF-alpha and IL-6 and IL-8) which cause vasodilation, hypotension, and contraction of bronchial and intestinal smooth muscle. The clinical symptoms, although variable in individual patients, are sudden onset of fever, chills, facial flushing, chest or low back pain, hypotension, dyspnea, hemoglobinuria, renal damage, disseminated intravascular coagulation, shock and even death[126].

In case of an incompatible RBC transfusion in an immunized patient, immediate destruction may occur if antibodies are still present but have not been detected during antibody tests, or a delayed transfusion reaction, typically after 5-14 days, which occurs after a secondary immune response.

The IgG antibodies, bind specifically to the foreign RBC antigens and target them for extravascular phagocytosis and lysis predominantly via the interaction of the IgG constant domain with Fcγ receptors on splenic macrophages. Most IgG RBC antibodies do not activate complement efficiently enough to activate the MAC causing RBC lysis. However, some (e.g. Kidd-antibodies, although in part IgM[127]) can, completely activate the complement cascade, and may cause intravascular hemolysis, while others, such as some K and Duffy antibodies, can activate complement only to the C3b stage. The latter are predominantly destroyed in the liver after binding with the CR1 receptor on Kupffer cells.

Although hemolysis by IgG antibodies may occur and be accompanied by the complete array of symptoms described for acute hemolytic transfusion reactions and even involve destruction of the patients' own RBCs[128-132], referred to hyperhemolysis, but in many cases there are no clinical symptoms. Factors such as antigen density, number of bound antibodies, antibody IgG subclasses and the RES capacity affect the rate of destruction[133-135]. Ness and colleques introduced the term 'serologic transfusion reaction' for post-transfusion cases of a positive direct antiglobulin test without signs of hemolysis, which were found about 2-5 times more often than hemolytic transfusion reactions[136-138].

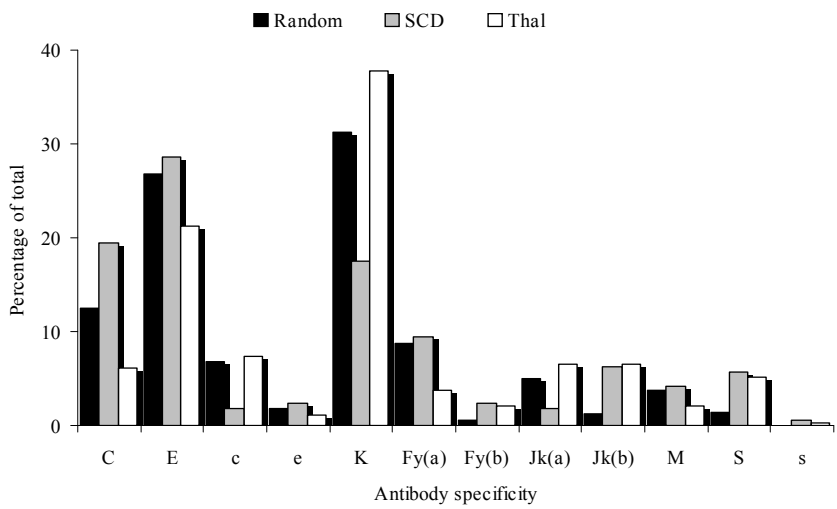
2.6 Red blood cell antibodies in disease

Observational studies in random patients, who most often receive incidental transfusions, and pregnant women, estimated the antibody prevalence between less than 1 to 3 percent[86-88,139-147], but prospective systematic studies and studies in multitransfused patients reported on an up to over 70 percent alloimmunization incidence[148].

Three large retrospective studies on alloimmunization in random hospital transfusion recipients, covering the period 1975-1995 and a total of 10,226

antibodies, showed that antibodies to RH and K blood group antigens comprise almost 80 percent of clinically significant non-D antibodies (fig 1)[87,88,147].
Transfusion dependent diseases are characterized by a high alloimmunization frequency and is highest in sickle cell patients. Combined data from 18 studies on 4005 sickle cell patients[148-165] and 11 studies regarding 3394 thalassemia patients[128,130,166-174] showed an overall alloimmunization risk of 22 (range 3-76) respectively 13 (range 5-28) percent. A total of 1606 RBC antibodies were reported in 675 sickle cell patients[148,150-156,162-165] and 834 antibodies in 446 thalassemia patients[128,130,150,167-173]. Multiple antibody specificities were present in 46 respectively 35 percent of patients. The immunization rate, expressed as the number of antibodies per 100 transfusions, varied between 1.7 and 4.0.

Fig. 1. Clinical significant non-D antibody specificities[1] in random patients and in patients with sickle cell disease (SCD) and thalassemia patients (Thal).



[1] For comparison only clinically significant non-D antibodies that are routinely screened upon are taken into account. These antibodies comprise 55% of antibodies in random patients and 78% of antibodies in hemoglobinopathy patients.

Most other diseases requiring transfusions exhibit lower immunization risks (table 1). Differences in immunization risk and antibody specificity for various diseases are dependent on a number of factors. The genetic disparity between patient and donor RBC phenotypes is considered to be the main reason for the high immunization risk in patients with sickle cell disease. Especially C, Fy^a, Fy^b, Jk^b and S RBC antigens are significantly less frequent ($p<0.001$) in the predominantly black sickle cell patients than in the predominantly white

donors[128,154,162,175] and antibodies against these antigens are more frequently found than in most other patients (fig 1). Studies in sickle cell patients performed in Jamaica[148] and Brasil[156,162], with a closer racial matching of donor and recipients, showed a three times lower immunization risk compared to European and American studies (9% versus 27%). This led to the policy to prophylactically match donor RBCs for RH and K antigens in patients with hemoglobinopathies. Studies, although not in a randomized controlled design, evaluating this policy found a reduction in the number of immunized patients and lower immunization rates[128,154,160,168,169,176].

A dysfunctioning immune system, either hyper- or hyposensitive can result in an enhanced respectively a reduced antibody production. Patients with myelodysplastic syndromes are fully immunecompetent, associated with a high incidence of autoimmune phenomena[177], and also in a high immune response against allogeneic RBC antigens[178-182]. On the other hand, patients with lymphoid leukemia[178,179], AIDS[183,184] and hematopoietic stem cell transplantation[185-189] show a highly reduced RBC alloimmune response probably related to the disease's pathophysiology or the intensive immuno-suppressive therapy. However, the immuno-suppressive therapy in myeloid leukemia[178,179] and organ transplant[76,190-193] and the impaired immune response in end-stage renal disease[143,178,180,183,194-198] does not prevent alloimmunization against allogeneic RBC antigens and is comparable to surgical patients[199-201].

Table 1. Alloimmunization risk in various diseases

Disease	References	Number of patients per study (range; total)	Immunization risk (median; range)
SCD[1]	148-162	34-1044; 3409	30.0; 9.9-46.8
Children	161-165	42-245; 596	18.5; 7.8-29.5
Thalassemia	128,130,150,166-173	39-1434; 3424	9.7; 5.0-28.4
Hematologic			
MDS[2] and CMPD[3]	178-182	16-112; 231	23.2; 12.5-58.6
Myeloid leukemia	178,179	35-209; 244	7.5; 5.7-8.6
Lymphoid leukemia	178,179	13-193; 206	0.3; 0.0-0.5
Renal failure	143,178,180,183,194-198	81-405; 1296	5.9; 1.1-14.0
Transplantation			
Organ	76,190-193	35-1132; 3007	6.2; 2.7-9.0
HSC[4]	185-189	117-217; 885	2.3; 1.3-9.1
AIDS	183,184	72-81; 153	2.6; 1.4-3.7
Surgery	199-201	374-530; 1356	5.3; 2.1-8.0

[1] Sickle cell disease; [2] Myelodysplastic syndrome; [3] Chronic myeloproliferative disease
[4] Hematopoietic Stem Cell

The formation of red cell antibodies may be influenced by the patients' age at which the transfusions are given or when chronic transfusion therapy is started. Four studies on RBC alloimmunization performed in (pre-term) neonates who received multiple transfusions during the first 3-4 months of life did not encounter any RBC antibodies[202-205]. Also, in hemoglobinopathy patients it has been shown that alloimmunization risk was significantly lower in patients who started transfusion therapy at a very young age (<3 years) compared to those who started later in life[153,168,169], although Ameen et al[130] reported an immunization frequency of 30% in 190 thalassemia patients who all started transfusion therapy before the age of 1 year. An immature immune system and some form of acquired immune tolerance to allogeneic RBC antigens is held responsible for the reduced alloimmunization risk.

A number of studies reported on an increasing number of alloimmunized patients dependent on the number of RBC units transfused[143,148,153,156,158,164,176], although others do not confirm this association[148,152,165,171,196,198,201]. The conflicting results are explained by the number of transfusions, the interval between transfusions-events, the frequency of antibody testing (single versus serial) and the specificity of the antibodies (e.g. common clinically significant versus non-significant and low-incidence) in the different studies. Besides, for most studies all transfusions administered to the patients were considered untill antibody detection. After transfusion with an incompatible antigen, a primary immune response needs time to result in a serological detectable antibody and additional transfusions can be given during this period. As a consequence the number of transfusions needed to elicit an antibody response can be overestimated. Based on the frequency of common RBC antigens in the caucasian population, most patients will theoretically encounter alloantigens during the first 3-4 transfusions. Blumberg et al showed that the rate of antibody formation per transfusion actually decreases with increasing numbers of transfusions (e.g. 7.9 per 1000 transfusions when less than 15 units were transfused compared to 2.5 when more than 44 units were transfused)[178]. This is in agreement with others who reported that the majority of alloimmunized patients have made the antibodies early during the transfusion course and probably after the first few encounters with the foreign antigen[147,150,160,162,163,166,195].

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3

ALLOIMMUNIZATION TO RED BLOOD CELL ANTIGENS AFTER UNIVERSAL LEUCODEPLETION. A REGIONAL MULTICENTRE RETROSPECTIVE STUDY

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Henk Schonewille, Anneke Brand. Alloimmunization to red blood cell antigens after universal leucodepletion. A regional multicenter study. Brit. J. Haematol. 2005;129:151-156

SUMMARY

Leucodepletion has shown to reduce human leucocyte antigen immunization, but studies on the effect of leucodepletion on red cell alloimmunization reported discordant results. We conducted a retrospective multicentre study to determine whether prestorage filter leucodepletion alters the development of clinically significant red blood cell alloimmunization against the RH, KEL, FY, JK and MNS blood group systems. Two periods were investigated, 2 years before and 2 years after universal leucodepletion. Comparisons were made between the transfused patient cohorts. To control for changes not related to leucoreduction, we compared antibody incidence with antibody prevalence in the two study periods. Newly detected antibodies (n = 4770) were found in 4115 patients from 19 participating hospitals. Of these, 857 antibodies in 659 patients were because of transfusions given in the study periods. The immunization risk was 0.13% for both periods. No differences were found regarding incidence of new antibodies, nor for patients regarding age, sex, previous antibodies, multiple antibodies, additional antibodies, number of transfusions, transfusions episodes, and days from transfusion to date of immunization.

In conclusion, compared to buffy-coat leucoreduction, universal prestorage filter leucodepletion did not alter the development of clinically significant red blood cell alloimmunization.

INTRODUCTION

Contaminating leucocytes in allogeneic blood transfusion are presumed to play an important role in immunomodulatory effects in the recipient. One hypothesis is that these contaminating leucocytes in the blood products downregulate a T-helper cell type 1 (Th1) immune response and drive the recipient toward a T-helper cell type 2 (Th2) response. Such skewing towards type 2 immunity may enhance alloantibody formation (Blumberg & Heal, 2000).
Leucodepletion has been shown to reduce human leucocyte antigen (HLA) alloimmunization (Vamvakas, 1998). Leucodepletion also removes the donor antigen presenting cells (APCs) abrogating the direct pathway of alloimmunization by donor-recipient T cell interaction. Besides, donor leucocytes readily express activation and costimulatory molecules upon recognition of recipient antigens (Fast, 2000). Whether the removal of leucocytes from cellular blood products ameliorates the antibody response by the indirect classical pathway is unknown.
Red blood cell (RBC) alloimmunization, a result of indirect immunization, is a common unwanted transfusion effect that occurs in up to 40% of patients depending on patient related factors, number of transfusion events and the type and timing of studies performed (Shirey & King, 2000).
In the Netherlands, standard RBC products have been leucoreduced [white blood cells (WBC) $<1 \times 10^9$] by buffy-coat removal since many years. The indication for filter-method leucodepletion (WBC $<1 \times 10^6$) was originally restricted to special patient-groups, primarily to prevent HLA-immunization to minimize refractoriness to platelet transfusions.
In November 2001, universal leucodepletion, by means of pre-storage filtration, of all RBC and platelet products, was implemented in the Netherlands as a precautionary measure to reduce the possible transmission of variant Creutzfeld Jacob disease. All transfusions have been leucodepleted since January 2002.
Recently, the effect of leucodepletion on RBC alloimmunization was addressed in a number of studies (Strauss *et al*, 1999; Singer *et al*, 2000; Blumburg *et al*, 2003; van de Watering *et al*, 2003), with discordant conclusions. We investigated the effect of leucodepletion on RBC alloimmunization in a large non-selected patient cohort in a region covering 25% ($\approx 4.2 \times 10^6$ inhabitants) of the Netherlands.
We conducted a 4-year retrospective regional multicentre study to investigate the incidence of newly developed RBC antibodies upon transfusion before and after the implementation of universal leucoreduction. To control for possible changes not related to leucoreduction, we compared the antibody incidence with the antibody prevalence in patients admitted to these hospitals in the same interval, but who did not receive transfusions in the two study periods.

METHODS

Hospitals

Hospital transfusion laboratories (n = 27) in the Dutch Sanquin Bloodbank Southwest region were asked to participate in this retrospective study regarding RBC alloimmunization before and after universal leucodepletion. The study periods were from January 1999 to December 2000 and from January 2002 to December 2003. Hospitals were asked to provide details of their transfusion policy and the antibody detection test methods employed over the years. Yearly, total numbers of antibody screens and RBC transfusions were used to normalize the number of antibodies found for the periods under study. However, data on the total number of patients with antibody screens and complete data on the number of transfused patients were not available.

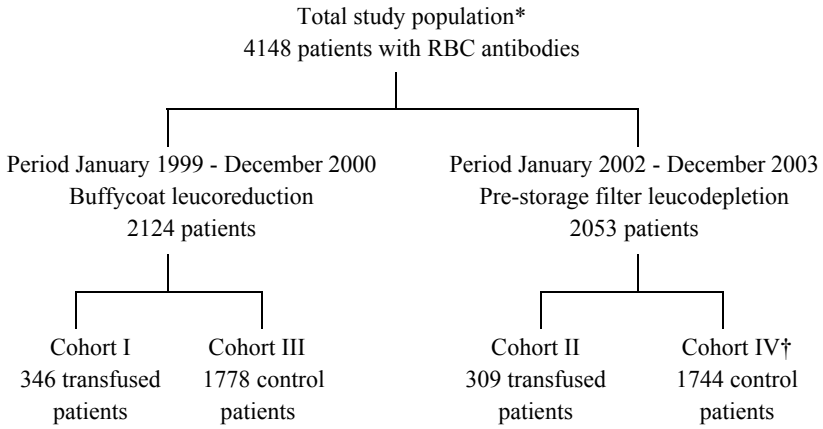
Antibodies

We retrospectively analyzed the records of all patients with newly detected RBC antibodies, during the 4-year period, by searching the participating hospital computer databases. Data collected included sex, age and the transfusion history. No detailed information on pregnancies, transfusions and antibodies detected in other hospitals was available.

Only the clinically significant RBC alloantibodies of the RH, KEL, FY, JK and MNS blood group systems were included. Autoantibodies and antibodies to low and high frequency antigens were excluded. Anti-D was excluded because D-immunization is not related to RBC transfusions in the majority of cases. Comparisons were made between two periods of two years each, 1999-2000 vs. 2002-2003, 2 years before and 2 years after the implementation of universal leucodepletion. We compared new antibodies that were detected in patients transfused during the study intervals (antibody incidence) with antibodies in patients admitted to these hospitals in the same period but who had not received transfusions during the study periods (antibody prevalence) (Fig. 1). This control group consisted of all patients for whom a blood type and screen was performed.

New antibodies were defined as antibodies detected for the first time in a patient, additional antibodies were defined as new antibody specificities, in a patient with (historical) antibodies.

Fig. 1 Study flow diagram of alloimmunized patients



* The total study population, i.e. all patients with antibodies, was divided into four cohorts:
Cohort I, patients who formed antibodies upon transfusions in the period 1999-2000 (antibody incidence)
Cohort II, patients who formed antibodies upon transfusions in the period 2002-2003 (antibody incidence)
Cohort III, patients presenting with antibodies without transfusions in the period 1999-2000 (antibody prevalence)
Cohort IV, patients presenting with antibodies without transfusions in the period 2002-2003 (antibody prevalence)
† Including 29 patients who formed antibodies in the period 1999-2001 and who presented with additional antibody specificity in the period 2002-2003

Statistics

The statistical software packages, EXCEL 5.0 (Microsoft, Redmond, CA, USA); and Statistical Package for the Social Sciences (SPSS) 11.0, (SPSS Inc. Chicago, IL, USA) were used for data management and analysis respectively. The chi-square or Fisher exact test were used for comparisons of proportions in the two periods. Medians were compared by the Mann-Whitney test. Groups were assumed to differ significantly when the probability level was less than 0.05.

RESULTS

Nineteen of 27 hospitals in this Southwest region participated in the study, which enclosed 9909 (70%) of the regional hospital beds and 80% of transfusions (n=494,505) in the 4-year period. Antibody detection methods did not change during the study years and either a polyethylene glycol (PEG) enhancement tube test or a low ionic strength solution (LISS) enhancement column technique was

used, both tests of comparable sensitivity to detect RBC antibodies. Transfusion policy, especially regarding the prevention of alloantibodies (e.g. c, E and K matching) did not change but antibody screens increased by almost 4% during the periods, while RBC transfusions decreased by 11%, indicating a more widely applied type and screen policy, associated with a restrictive transfusion policy (Table I).

Table I. Hospital production data in the study periods.

	Study period	
	1999-2000	2002-2003
Number of antibody screens	332,210	344,503
Number of units transfused	261,879	232,626
Ratio screens/transfusion	1.3	1.5

Table II. Antibody frequency and specificity for transfused and control patients

	Transfused cohort I and II n = 655			Control cohort III and IV n = 3522			
Antibody specificity	1999-2000	2002-2003	% of all antibodies	1999-2000	2002-2003	% of all antibodies	P-value*
E†	168	133	35.1	504	519	26.1	<0.0001
K	99	92	22.3	556	473	26.3	0.017
C‡	29	27	6.5	204	189	10.0	0.0018
Fy^a	16	21	4.3	198	214	10.5	<0.0001
M	11	6	2.0	187	215	10.3	<0.0001
c	32	46	9.1	151	125	7.1	0.046
Jk^a	55	47	11.9	84	72	4.0	<0.0001
S	8	8	1.9	63	60	3.1	0.057
Jk^b	11	19	3.5	10	20	0.8	<0.0001
e	6	10	1.9	11	16	0.7	0.0019
Fy^b	3	6	1.1	15	16	0.8	0.59
s	2	1	0.4	5	5	0.3	0.91
k	1	0	0.1	1	0	<0.1	0.80
All	441	416		1989	1924		0.77§

* Antibody specificity cohort I + II versus cohort III + IV
† Including anti-D+E (n=44), ‡ Including anti-D+C (n=275)
§ P-value cohort I/I+III versus cohort II/II+IV

In the four study years, antibodies (n = 4770, Table II) were detected in 4148 patients (female to male ratio 3.1, mean age 59, range 1-105 years). Of these, 124 patients already possessed historical (non-D) antibodies before entering the study period (Tables III and IV). This included 29 cases that had made antibodies upon

transfusion in the period 1999-2001 (cohort I) and who were identified with additional antibodies in the period 2002-2003 (Fig. 1, cohort IV).
Of the total 4148 patients with antibodies, 399 (9.6%) patients had multiple antibody specificities at the initial antibody screening test. This distribution was similar for the two study periods without differences between the four cohorts (p=0.41). A total of 224 patients (5.4%, including the 124 patients with historical antibodies) made 239 additional antibodies during subsequent antibody screening tests.

Table III. Demographics, transfusion and alloimmunization data for transfused patients (cohorts I and II)

	Cohort I 1999-2000	Cohort II 2002-2003	p-value
Number of immunized patients	346	309	n.a.
Female-male ratio	2.0	1.7	0.25
Mean age at immunization	66	65	0.27
Transfusion history:			
Median number of units transfused	4	4	0.79
Median number of transfusion events	1	1	0.47
Alloimmunization:			
New antibodies	441	416	n.a.
Patients with historical antibodies	7	7	1.0
Patients who presented with multiple antibodies	48	50	0.38
Patients who formed additional antibodies	19	24	0.35
Median interval after transfusion to immunization (days)	43	38	0.36
Patients with antibodies within 14 days after transfusion	84	74	1.0
New alloimmunization rate (%)*:			
Patients immunized/antibody screens	0.10	0.09	0.061
Patients immunized/ units transfused	0.13	0.13	0.98
Antibodies/antibody screens	0.13	0.12	0.18
Antibodies/units transfused	0.17	0.18	0.40

* Number of antibody screens and transfusions, see table 1
n.a.: not applicable

The antibody specificity distribution between the transfused patients (cohorts I and II) and control patients, that presented with antibodies, but were not transfused during the study periods (cohorts III and IV) differed significantly for both study periods (Table II, $p<0.0001$). The incidence of the antibody specificities E, e, Jk^a and Jk^b was significantly higher in the transfused group

compared to the frequency in the control group, whereas anti-C, -c, -K, -Fy[a] and -M were increased in the control group.
No differences in antibody incidence or prevalence between the two study periods were observed. Similarly, patients age, sex, multiple antibodies, historical antibodies and additional antibodies were comparable (Tables III and IV). Regarding the transfused cohorts, number of units transfused, transfusions events and interval from transfusion to immunization were comparable.
Alloimmunization within 14 days after transfusion, presumably reflecting a secondary immune response, was similar for both study periods (Table III).
The alloimmunization rate for transfused patients normalized to the number of antibody screens or to the number of transfusions showed no significant difference between the periods 1999-2000 and 2002-2003 (Table III). In cohorts III and IV the alloimmunization rate, expressed as antibodies identified per antibody screen, showed a significant decrease (Table IV).

Table IV. Demographics and alloimmunization data for control patients (cohorts III and IV)

	Cohort III 1999-2000	Cohort IV 2002-2003	p-value
Number of immunized patients	1778	1744	n.a.
Female-male ratio	3.4	3.4	0.98
Mean age at immunization	60	59	0.25
Alloimmunization:			
New antibodies	1989	1924	n.a.
Patients with historical antibodies	49	61	0.24
Patients who presented with multiple antibodies	173	182	0.43
Patients who formed additional antibodies	84	97	0.25
New alloimmunization rate (%)[*]:			
Patients immunized/antibody screens	0.54	0.51	0.10
Antibodies/antibody screens	0.60	0.56	0.029

[*] Number of antibody screens, see table 1
n.a.: not applicable

DISCUSSION

In a 4 year retrospective multicentre 'before-and-after' study we investigated the incidence of clinically significant antibodies that developed against RH, KEL, FY, JK and MNS antigens and found that the immunization rate, expressed as immunized patients and antibodies per transfusion event (0.13% and 0.17%, respectively), was not favourably affected by universal leucodepletion. Our study concerned non-selected random transfused patients with RBC antibodies. The

effect of universal leucodepletion in different patients groups (i.e. chronically multi-transfused patients) was not studied.

We conducted our study the last 2 years before and the first two years following the implementation of universal leucodepletion. We excluded changes in laboratory and transfusion practices that could affect the results, except for an increase in blood typing and screening and a 11% reduction in the use of RBCs. However, in terms of preventing alloimmunization, we would have expected a decreased number of immunized patients as a result from this transfusion policy.

Other causes of bias or uncontrolled confounders that could have affected the results can never be excluded entirely but are unlikely given our closely related study periods.

In a recent study by Blumberg *et al* (2003), RBC alloimmunization was found to be reduced in relation to an increased use of leucodepleted red blood cell products in a group of 410 AML patients and in 217 random transfusion recipients. This effect was only significant in the AML patients (8.2% vs 2.8%, p=0.016). In the random patient group, antibodies known to be not necessarily induced by blood transfusion (e.g. Lewis, P1, Lutheran, HTLA, and Bg) were decreased, while alloimmunization to RH, KEL, FY and JK antigens only showed a non significant decrease over the years. Singer *et al* (2000) studied alloimmunization in 64 multi-transfused thalassemia patients and reported a non-significant RBC alloimmunization decrease that could be related to the parallel change to the use of leucodepleted RBC blood products. It was, however, more likely that a change in transfusion policy, in terms of preventive of RH and K antigen matching, contributed to the decreased immunization. Strauss et al (1999) compared alloimmunization in 47 preterm infants after transfusion of fresh unmodified versus leucodepleted RBC in a randomized controlled blinded trial and found no RBC antibodies in either study arm. van de Watering *et al* (2003) did not find a reduction of RBC alloimmunization by leucodepletion in a randomized controlled trial study in 374 cardiac surgery patients.

The number of transfusion events could have played a role in the differences found regarding the effect of leucodepletion on RBC alloimmunization. In the study by Blumberg *et al* (2003) patients were transfused by a mean of 17 RBC units, probably administered during several events, while patients in the study of van de Watering *et al* (2003) and in our study received a median of only 4 units (mean seven units), often as a single transfusion event. RBC alloimmunization is more likely after multiple transfusion events than after a single transfusion event (Issitt & Anstee, 1998).

Another aspect that could influence the differences in study outcomes is the time interval between transfusion and the antibody screening. Recent studies (Ramsey & Larson, 1988; Schonewille *et al*, 2000), observed that antibodies could become undetectable over time. The mean number of weeks between the transfusion date and new alloimmunization varied, between 260 (non-WBC reduced) and 340

weeks (100% WBC reduction), in the groups studied by Blumberg *et al* (2003). Theoretically, this could have resulted in 7% decline in detectable antibodies (calculated from Schonewille *et al*, 2000). In our study, the median interval between transfusion and antibody detection in the two study periods in the transfused groups (cohort I and II) differed by only 5 days, and therefore unlikely that the antibodies had become undetectable.

From prospective studies on alloimmunization in recently transfused patients (Redman *et al*, 1996), the antibody specificity distributions were in agreement with our transfused patients. The different antibody specificity distribution in recently transfused patients (cohorts I and II) compared with the control patients who had received transfusions in the past (cohorts III and IV) (Table 2) is probably dependent on two factors: the time needed for an antibody to develop to detectable levels and the period the antibody level remains high enough to be detectable. For instance, Kidd antibodies are frequently seen soon after transfusion but are often only detectable for a short period of time (Heddle *et al*, 1995, Schonewille *et al* 2000). In terms of a role of leucocytes in RBC products creating more cytokine activation and a Th2 skewing of the immune response, one would have expected rather a difference in immediate antibody response, such as antibodies against JK antigens, which was not the case.

In conclusion, this pre- and post intervention, retrospective multicentre study in a large non-selected patient cohort, found no differences in the development of clinically significant red blood cell alloimmunization by prestorage filter leucodepletion compared with buffy-coat leucoreduced RBC transfusions.

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4

THE IMPORTANCE OF ANTIBODIES AGAINST LOW-INCIDENCE RBC ANTIGENS IN COMPLETE AND ABBREVIATED CROSS-MATCHING

Henk Schonewille, Annette M van Zijl, Pierre W Wijermans. The importance of antibodies against low-incidence RBC antigens in complete and abbreviated cross-matching. Transfusion 2003;43:939-944

ABSTRACT

BACKGROUND: It is common practice to perform an antiglobulin cross-match only when unexpected RBC alloantibodies are present, to detect antibodies against additional RBC antigens. In this study, the incidence of unexpected antibodies to low-incidence antigens (Ab-LIA) over a period of 23 years was investigated.

STUDY DESIGN AND METHODS: Records of RBC antibodies and the accompanying transfusion history from 1978 through 2000 was retrospectively examined. Complete cross-matches were performed for all RBC transfusions before 1991. As of 1991, the type-and-screen policy was applied. To study the incidence of anti-Wr^a, a prospective study was conducted on sera from 462 patients sent to the transfusion laboratory and 486 blood donors.

RESULTS: The records of 1795 patients containing 2257 RBC antibodies were examined. In 89 patients, a total of 94 Ab-LIAs was found. Anti-Wr^a was the most frequently encountered Ab-LIA. Thirty-nine patients had Ab-LIA in combination with other antibodies, 20 of which were autoantibodies. Eighty percent of these Ab-LIA were found at the first positive antibody screening test. Fifty-one solitary Ab-LIA were found in 50 patients, 37 during antibody screening tests, and 14 after positive complete cross-matches conducted before 1991. After an RBC antibody was detected, 664 patients received a total of 7792 RBC transfusions. Since the introduction of the type-and-screen policy, only one anti-Wr^a has been discovered during complete cross-matching. No transfusion reactions due to Ab-LIA were reported during the study period. In the prospective study, 12.3 percent of patients and 4.3 percent of blood donors had anti-Wr^a.

CONCLUSIONS: Although Ab-LIAs are found coincidentally in the sera of only 2 to 3 percent of patients with other RBC antibodies, they are formed often. Because we found no difference in serologic incompatibility, due to Ab-LIAs, between patients with and without other blood group antibodies, we conclude that blood can be transfused safely to patients without performing a complete cross-match.

INTRODUCTION

Antibodies to low-incidence antigens (Ab-LIAs) are discovered coincidentally during routine RBC antibody (screening and/or identification) investigations, after unexpected positive cross-matches or after an incompatible transfusion resulting in a hemolytic transfusion reaction. A number of studies[1-14] mention the presence of Ab-LIAs. The incidence of Ab-LIAs in these studies varied between 0.3 and 10 percent.

It has become common practice to perform an antiglobulin cross-match only when the transfusion recipient has unexpected alloantibodies (UA), to detect additional RBC incompatibility.[15] The chance of an incompatible blood transfusion due to Ab-LIA, based on the incidence of some LIAs, has been estimated to be 1 in 1600 to 1 in 10,000 transfusions.[16] In studies[11,14,17,18] on the risk of incompatible transfusions after immediate spin cross-match, the incidence of serologic or delayed hemolytic transfusion reactions was found to be more than 1 in 1800 or 1 in 100,000 transfusions, respectively.

Many sera contain Ab-LIAs from the IgM or IgG immunoglobulin class, which can be naturally occurring or immune antibodies.[16,19-21] In autoimmune hemolytic anemia, Ab-LIAs are found in up to 30 percent of the patients.[16]

Although Contreras et al.[19] studied Ab-LIAs in hyperimmunized donors, we know of no systemic investigation of Ab-LIAs in patients with unexpected RBC alloantibodies.

We retrospectively investigated the incidence of coincidentally discovered Ab-LIAs in a patient population with unexpected alloantibodies over a period of 23 years. In addition, we also prospectively studied the presence of anti-Wr^a in hospital patients, pregnant women, and blood donors to estimate the incidence of the most frequently encountered Ab-LIAs when tested.

MATERIALS AND METHODS

We examined retrospectively all the records on RBC antibodies in the transfusion laboratory computer database of Leyenburg Hospital (a 600-bed, multidisciplinary teaching hospital) during a 23-year period (i.e., 1978–2000).

The study includes all RBC antibodies found. Antigens with a frequency of less than 10 percent of the population and routinely not present on antibody screening-test cells were considered to be low-incidence antigens (LIAs). Information collected included patient's age and sex, dates and results of tests for RBC antibodies, and transfusion history, but no detailed information on pregnancies, transfusions, and antibodies detected in other hospitals was available.

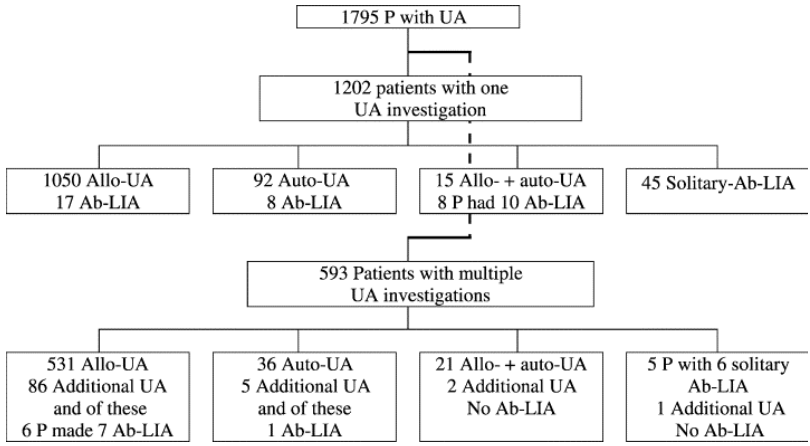
Blood samples from successive patients submitted to the blood transfusion laboratory between January 1978 and December 2000 were screened for RBC antibodies as previously described.[22] In short, a two- (1978–1986) or three-cell set (1987–2000) of reagent RBCs for antibody detection was used. The antibody-detection RBCs were not selected to have LIAs. The technique for antibody detection consisted of a saline, albumin, and antiglobulin tube test (1978–1990); later a LISS gel test (DiaMed AG, Murten, Switzerland) (1991–2000) was used. Antibody identification was accomplished with commercial panels of cells obtained by means of similar methods or additional techniques (e.g., PEG, enzyme) whenever needed. If the specificity could not be determined, the blood sample was sent to a reference laboratory for further analysis.
Cross-matching, including an IAT, was performed routinely from 1978 through 1990; as of 1991, the "type and screen" policy (immediate centrifugation using saline-suspended donor RBCs without antiglobulin test) was adopted for all patients, including hematologic patients, until antibody formation was detected. When an antibody was identified, RBC components that were negative for the corresponding antigen were transfused after complete cross-matching, including an indirect antiglobulin phase.
We also conducted a prospective study on successive hospital patients (n=108), pregnant women (n=193), and blood donors (n=486) to investigate the incidence of anti-Wr^a in a population without unexpected alloantibodies. In addition, we also tested frozen serum samples from 161 patients with UA for the presence of anti-Wr^a.

Statistics

Statistical software packages (Excel 5.0, Microsoft, Redmond, CA; NCSS 6.0, Jerry L. Hintze PhD, Kaysville, Utah) were used for data management and analysis, respectively. The Chi-square test was used to compare the incidence of Ab-LIAs between groups. Groups were assumed to differ significantly when the probability level was less than 0.05.

RESULTS

Fig. 1. Flow sheet of the patients with antibodies.



P=patients

Figure 1 presents a flow sheet of the patients with antibodies. The records of 1795 patients contained RBC antibodies (female-to-male ratio 3.2). Median patient age at the time of first antibody detection was 63 years (mean, 60; range, 15–97).
A total of 3418 antibody specificity tests was performed (median, 1; mean 1.9; range, 1–42). For 1202 patients (67%) one antibody specificity test and for 593 patients (33%) more than one antibody specificity test were performed. We identified 2257 RBC allo- and/or autoantibodies; the blood group system specificities are listed in Table 1.

Table 1. Antibody blood group specificity and frequeny for 1795 patients with 2257 antibodies

Blood group system	Number	Percentage of total	Blood group system	Number	Percentage of total
RH	955	42.3	LIA	94	4.2
KEL	443	19.6	JK	64	2.8
FY	165	7.3	HTLA/HLA	48	2.1
MNS	121	5.4	Other sytems	30	1.3
LE	110	4.9	Unidentified	27	1.2
			Autoantibodies	200	8.9

In total, 1428 patients had one antibody, 303 had two, 41 had three, and 23 patients had more than three antibodies.
Fifty-one solitary Ab-LIAs were found in 50 patients, 14 were discovered by positive complete cross-matches conducted before 1991, and 37 were discovered during antibody screening tests. Five patients had two Ab-LIAs.
The characteristics of the Ab-LIAs are listed in Tables 2 and 3. Thirty-nine patients had 43 Ab-LIAs in combination with other antibodies. The incidence of patients with Ab-LIAs in combination with other RBC antibodies is 2.2 percent. In 38 patients, these antibodies were found coincidentally during antibody specificity tests. In 20 (53%) cases, autoantibodies accompanied the antibodies.

Table 2. Type and frequency of antibodies against LIAs

Antibody type	Frequency (N) First + additional*	Solitary	In combination with other antibodies
Wr^a	24 + 2	8	18
Lu^a	23 + 1	16	8
C^w	18 + 3	11	10
Unidentified	12 + 0	11	1
Kp^a	7 + 0	3	4
V	2 + 0	2	NA
Co^b	0 + 1	NA	1
Vr	0 + 1	NA	1
Total	86 + 8	51	43

* First, the AB-LIA is found (together with the UA) during the first antibody detection test; additionaly, the Ab-LIA is found after subsequent testing in patients with known UA.

After initial antibody demonstration, 94 patients formed 121 antibodies in 106 additional antibody-forming episodes. There were no differences ($p>0.05$) in the number of patients who formed additional antibodies between patients with one (n=79/482) and those with more than one antibody (n=11/111) at initial screening. In seven patients, these additional antibodies were Ab-LIAs.
Thirty-five (81%) Ab-LIAs were found during the first antibody identification test, and eight were found after subsequent testing. In a patient with anti-c, -E, -K, and -Lu^a, an additional anti-C^w was found. A 75-year-old transfusion-dependent (80 U in 30 months) female patient with a chronic myelomonocytic leukemia and anti-K made anti-Lu^a and anti-Wr^a, as well as anti-c and -E, autoantibodies and HLA antibodies.

Table 3. Characteristics of Ab-LIAs

Ab-LIA	Accompanying antibodies	Number of patients	Test-type at first discovery*
Wr^a	None	7	CCM
Wr^a	E	2	AI
Wr^a	K	1	CCM
Wr^a	AA	6	DAT+/AI
Wr^a	K + AA	1	DAT+/AI
Wr^a	Auto-e + AA	1	DAT+/AI
Wr^a	Auto-D + AA	1	DAT+/AI
Wr^a	E + c + AA	1	DAT+/AI
Wr^a	E + auto-C + AA	1	DAT+/AI
Wr^a	E + auto-e + auto-C + AA	1	DAT+/AI
Lu^a	None	15	AS
Lu^a	E, K	1, 2	AI
Lu^a	E + c	1	AI
Lu^a	C + e + Fya	1	AA
Lu^a	C + Lea + AA	1	DAT+/AI
Cw	None	11	AS
Cw	E, c, K, S	3, 1, 1, 1	AI
Cw	E + K + AA	1	DAT+/AI
Cw	Auto-e + auto-c + AA	1	DAT+/AI/Eluate
Kp^a	None	2	CCM
Kp^a	None	1	AS
Kp^a	Lea, K + E	1, 1	AI
Kp^a	K + AA	1	DAT+/AI
V	None	2	CCM
Co^b	E + c	1	AI
Vr	E + K + S + Jkb + AA	1	DAT+/AI
$Wr^a + Lu^a$	None	1	CCM
$Wr^a + C^w$	C + AA	1	AI
$Lu^a + C^w$	C + e + K	1	AI
$Wr^a + Lu^a$	E + c + K + AA + HLA	1	DAT+/AI
$Wr^a + Kp^a$	K + S + Jkb + auto-e + AA	1	DAT+/AI
Unidentified	None	10	AS
Unidentified	None	1	CCM
Unidentified	Fya	1	AI

* CCM = complete cross-match; AA = autoantibody; AI = antibody identification; AS=antibody screen.

An anti-C^w was found in the eluate of the RBCs of a patient with auto-e, auto-c, and unidentified autoantibodies after receiving 28 e- and c-matched transfusions in 11 transfusion episodes. This patient did not suffer from a hemolytic transfusion reaction at the clinical level.
The specificity of the Ab-LIAs could not be identified in 12 cases.
After an RBC antibody was detected, 664 patients received a total of 7792 RBC transfusions (median, 5; mean, 11.7, range, 1–326) in 2803 transfusion episodes (median, 2; mean, 4.2; range, 1–101). The number of complete cross-matches for all transfused hospital patients during the study period was estimated to be 117,000 (1978–1990, 110,800; 1991–2000, 6200) and the number of transfusions 204,000. During the whole study period, no transfusion reactions were reported to have been caused by Ab-LIAs.

Ab-LIAs detected by "complete cross-match" versus "abbreviated cross-match"

From 1978 to 1990, 22 Ab-LIAs were found - nine Ab-LIAs during 1896 antibody identification tests and 13 during 110,800 complete cross-matches. After the type-and-screen policy was applied (from 1991), only one Ab-LIA was found during 6200 cross-matches in patients with unexpected alloantibodies, and 70 Ab-LIAs were found during 1522 antibody identification tests.

Table 4. Type of investigation during which Wr^a antibodies were found

	Antibody identification		Anti-Wr^a found during complete cross-match
	Anti-Wr^a in patients without UA	Anti-Wr^a in patients with UA	
1978-1990	NA	3/902	8/110.800*
1991-2000	NA	14/893	1/6200†
Prospective	46/787	32/161	NA

* Number of complete cross-matches for all patients
† Number of complete cross-matches only for patient with UA
NA, not applicable

Wr^a antibodies

Wr^a antibodies were the most frequently encountered Ab-LIA (Table 4). Out of 19 nonsolitary Wr^a antibodies, 15 were discovered together with unidentified autoantibodies. One anti-Wr^a was discovered after a positive cross-match in a patient with anti-K.
The prospective study on the presence of anti-Wr^a revealed the presence of this antibody in 46 of 787 persons (5.8%) without RBC antibodies (21/486 [4.3%] blood donors, 14/193 [7.3%] pregnant women, 11/108 [10.2%] hospital patients) and 32 of 161 (19.9%) patients with alloantibodies.

DISCUSSION

In this study we found that in the total cohort of patients with RBC antibodies, 5.0 percent had Ab-LIAs detected by screening with RBCs not selected to have Ab-LIAs or by cross-matching, and 2.2 percent of these Ab-LIAs were accompanied by other RBC antibodies.

Twelve Ab-LIAs were of unknown specificity. Further investigations of the specificity of an Ab-LIA are often not undertaken because of the high costs involved and the limited clinical relevance.

We found no Ab-LIAs together with D-antibodies (n=334) retrospectively, but prospectively four anti-Wr^a were discovered in 25 patients with anti-D ($p<0.00001$). Cleghorn,[23] who compared recently delivered women without anti-D to those who made anti-D during pregnancy, found that the presence of anti-Wr^a increased from 1 in 50 to 1 in 14 women. Based on these findings and the fact that anti-D is one of the most frequently encountered antibodies in our study, we should have encountered combinations of anti-D with Ab-LIAs in seven (95% CI, 2–12) patients.

We found five patients with multiple Ab-LIAs, and anti-Wr^a was present in four cases. Anti-Wr^a was also identified prospectively in 19.9 percent of patients with allo-UA. This value is in agreement with Contreras et al.,[19] who found that 12 percent of plasmas from donors hyperimmunized against Rh antibodies contained antibodies to Wr^a antigens ($p=0.2$). Because they also found that 57 percent of hyperimmunized donors had multiple Ab-LIAs, they postulated that the production of antibodies to RBCs in general stimulates the production of Ab-LIAs. Because we only tested for the presence of anti-Wr^a and found our results are comparable to those of Contreras et al.,[19] it is conceivable that many more different Ab-LIAs are actually present in our population of patients as well as healthy individuals.

In our prospective study, we found anti-Wr^a in 7.3 percent of pregnant women and 10.2 percent of hospital patients without other RBC antibodies. These incidences are in agreement with those of Wallis et al.[24] who found anti-Wr^a in 7.9 percent of their hospital patients. They noted that the anti-Wr^a incidence among patients decreased with age, but they could not find an association with sex, AB0-D blood group, type of illness, or medical or surgical speciality. Issitt and Anstee[16] state that probably as the result of an unbalanced immune system together with RBC destruction, Wr^a antibodies are frequently formed in patients with autoimmune hemolytic anemia and in pregnant and recently delivered women. On the other hand, one could hypothesize that hospitalization in itself or other stress situations could alter the immune system and therefore enhance anti-Wr^a production. Cleghorn,[23] in this regard, suggested that most human sera

contain many Ab-LIAs and that some nonspecific boost is needed to "activate" them. The mechanisms of the antigen presentation involved are still unclear, but RBC transfusions with the corresponding antigen are not always involved. Another explanation could be that during activation of the immune system, certain proteins are formed that cross-react with the Wr^a-antigen on RBCs.

As far as blood donors are concerned, we found a much higher incidence of anti-Wr^a than reported in a number of other studies[23-25] (4.3 vs. 0.4–1.3%, $p<0.00001$). In accordance with our results, Contreras et al.[19] found anti-Wr^a in 1 of 50 donors hyperimmunized with microbial antigens (Herpes-Zoster, HBsAg, or Tetanus Toxoid) and no other RBC antigens. Further investigations are needed to explain these discrepancies.

Forty-eight percent of Ab-LIAs are found simultaneously the first time that other RBC antibodies are detected. Only for 7 out of 593 (1.2%) patients who underwent multiple antibody-specificity investigations were Ab-LIAs found after the initial testing. In view of the facts that once a patient has made an RBC antibody the chance of additional antibodies is three to four times[6,13] greater and sera from patients with RBC antibodies are tested against a variety of test RBCs during repeated antibody investigations and cross-matches and therefore there is a greater chance of encountering LIAs, we should even coincidentally have encountered more Ab-LIAs. It could mean that the risk of additional Ab-LIA formation in patients with RBC antibodies is low. This is in contrast with our prospective findings concerning anti-Wr^a in patients with UA. Also, Dunsford[26] found that 14 of 48 sera with anti-E also contained anti-Wr^a, in agreement with our results, where 8 of 36 anti-E sera and 3 of 8 sera containing multiple antibodies including anti-E also contained anti-Wr^a. It is not known if the above findings are specific for the combination of an UA and anti-Wr^a. Anti-C^w, however, was only present in 10 patients with other UA, despite the fact that most RBC identification panels comprise C^w-positive cells.

In view of the fact that 14 Ab-LIAs were found after a positive indirect antiglobulin phase cross-match, the risk of serologic incompatibility in this study is 1 in 8300 transfusions. Before 1991, 13 serologic Ab-LIA incompatibilities were found in 110,800 complete cross-matches in all patients versus 1 in 6200 complete cross-matches in patients with UA from 1991 through 2000 ($p>0.10$). This means that the risk of an serologic incompatible transfusion due to an Ab-LIA is comparable in patients with and without UA. The low incidence of positive complete cross-matches and incompatible transfusions (1 in 204,000) due to Ab-LIA is in agreement with others.[14,17,18,27,28] Because up to 30 percent of RBC antibodies can become undetectable over time,[22,29,30] patients are likely to receive RBC transfusions on a type-and-screen basis when transfused in another

hospital, where the antibody history is unknown. This is also the case for patients with Ab-LIAs.

Taken together, it is likely that many units carrying a LIA have been transfused to patients with the corresponding antibody. No transfusion reactions due to Ab-LIAs have been reported to our laboratory. Because many transfusion reactions probably have gone unrecognized and therefore only severe reactions are routinely reported to the laboratory, this could reflect the mildness of the possible transfusion reactions in most cases of Ab-LIA incompatibility. The consequences of incompatibility based on Ab-LIAs in terms of in vivo RBC survival are unknown. For patients with normal hematopoiesis who receive a single transfusion for surgical reasons, this incompatibility probably has no serious harmful effects, but in patients with a dysfunctioning marrow who are transfusion dependent, the maximum efficiency per transfusion must be obtained. Serologic incompatibility can be an indicator of shortened in vivo RBC survival, resulting in additional transfusions.

In conclusion, some Ab-LIAs (e.g., anti-Wr^a) are often formed but are seldom detected because LIA is missing from detection RBCs and/or cross-matched RBCs. Prospective studies can give insight into the incidence and consequences of the most common Ab-LIAs in different populations. The results of such studies can have an impact on the transfusion policy applied for selected groups of patients. Because the risk of a transfusion reaction due to an Ab-LIA is very low, transfusion without complete cross-matching can be considered safe.

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5

ALLOIMMUNIZATION AFTER BLOOD TRANSFUSION IN PATIENTS WITH HEMATOLOGIC AND ONCOLOGIC DISEASES

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ABSTRACT

BACKGROUND: Because of intensive marrow depression and improved survival, patients with hematologic and oncologic malignancies are dependent on transfusion for a longer period. It has been advocated that these patients should receive blood that is matched for blood group antigens other than ABO and D. A retrospective study was performed on the rate of alloimmunization against red cell antigens in 564 patients with malignant hematologic diseases over a period of 10 years.

STUDY DESIGN AND METHODS: Records of transfusion and immuno-hematologic studies of all patients (n =1066) with malignant myeloproliferative and lymphoproliferative diseases diagnosed between 1987 and 1996 at one hospital were collected from the hospital computer blood bank files. Transfusions were correlatedwith antibody formation. Factors affecting this correlation were analyzed.

RESULTS: Seventy-one antibodies were found in 51 patients. The overall immunization rate was 9.0 percent. Fifty percent of antibodies were formed after 13 units had been transfused. Once a patient had formed an antibody, the probability of additional antibodies increased 3.3-fold. Anti-c, anti-E, and anti-K composed the majority of antibodies found. Four patients formed RH system antibodies after incompatible platelet transfusions. Patients who underwent intensive chemotherapy formed antibodies at a much lower rate than other patients. More than40 percent of antibodies became undetectable after the first detection. No difficulty was encountered in finding compatible blood for these patients.

CONCLUSIONS: Antibody formation in hematologic malignancies is comparable to that in other diseases requiring multiple blood transfusions. Extensive antigen matching before transfusion of patients with hematologic and oncologic malignancies is not necessary and leads to increased costs.

INTRODUCTION

Transfusion support is vital to the management of patients with hematologic disorders and malignancies. Many such patients require blood transfusion during the course of their illness, and almost all patients who undergo chemotherapy or radiotherapy become anemic. It is well known that alloimmunization to red cell (RBC) antigens resulting from the genetic disparities between donor and recipient is one of the risks of blood transfusion. The risk depends on the recipient's exposure to the foreign antigen and its immunogenicity. Immunization may also be influenced by the number and frequency of the transfusions as well as the recipient's sex, age, and underlying disease[1-19]. Clinically significant RBC alloantibodies develop in more than 30 percent of patients receiving multiple transfusions and can pose major problems in the case of long-term transfusion therapy. Several authors found that RBC alloimmunization mainly occurs after the first few transfusions [11,12]. RBC antibodies, which can become undetectable over time [20,21], can cause delayed transfusion reactions after incompatible blood transfusions. Several authors[6,10-14,22] advocated that transfusions given to patients who are likely to become transfusion-dependent over a longer period of time should be matched for antigens other than ABO and D (e.g., C, E, c, and K) in an attempt to prevent alloimmunization. However, cost-effective considerations as well as the question of whether this would truly add to the patient's safety make this policy a matter of debate[1,4,17,23].

Patients undergoing chemotherapy, especially those with leukemia, exhibit a lesser antibody response than do other patients. Impairment of the immune status as a result of the malignant process[4,12,24,25] or transient immunosuppression due to intensive chemotherapy could enable patients to develop a tolerance for or unresponsiveness to incompatible transfusions[2,24-30].

We retrospectively analyzed RBC alloantibodies detected and identified in serum from patients with hematologic and oncologic malignancies submitted to our laboratory.

MATERIALS AND METHODS

Laboratory testing

Blood samples from hematologic and oncologic patients submitted to the blood transfusion laboratory of the Leyenburg Hospital (The Hague, the Netherlands) between January 1987 and December 1996 were screened for RBC alloantibodies by use of a selected three-cell set of reagent RBCs for antibody detection. The technique for antibody detection involved the use of 100 µL of serum and 50 µL of 5-percent RBCs (serum-to-cell ratio, 40:1), a saline, albumin, and antiglobulin

tube test (1987 through 1990); later (1991 through 1996), 25 µL of serum and 50 µL of 0.8-percent RBCs (serum-to-cell ratio, 63:1) were used in a low ionic-strength solution gel test (DiaMed AG, Cressier Switzerland).
Antibody identification was accomplished with commercial panels of cells tested by similar methods or additional techniques (e.g., polyethylene glycol, enzyme) whenever needed. If the specificity could not be determined clearly, the blood sample was sent to a reference laboratory for further analysis.
The results of both antibody screening and antibody identification were valid for 72 hours (a transfusion episode); then, in case of transfusion, a fresh blood sample was tested.
Crossmatching, including an indirect antiglobulin test, was performed routinely from 1987 to 1990; as of 1991, the type and screen policy was used for all patients until antibody formation. Subsequently, complete crossmatching including an indirect antiglobulin phase was performed.

Patients

The identification of all patients (n=1066) with a malignant myeloproliferative (n=399) and lymphoproliferative (n=667) disease diagnosed between 1987 and 1996 at the Leyenburg Hospital was obtained by searching the computer database. Patients who never received a transfusion (n=369) and those who did not undergo at least one antibody screening after the first blood transfusion (n=133) were excluded. This left 564 evaluable patients.
Myeloproliferative diseases consisted of acute myeloid leukemia (AML, French-American-British [FAB] classifications M0-M7)[31], myelodysplastic syndromes (FAB: RA [refractory anemia], RARS [RA with sideroblasts], RAEB [RA with excess blasts], chronic myelomonocytic leukemia, and RAEB-T [RA with excess blasts in transformation])[32], and myeloproliferative syndromes (MYPRO) (chronic myelogenous leukemia, polycythemia vera, essential thrombocythemia, and myelofribrosis). Lymphoproliferative diseases consisted of Hodgkin's lymphoma and non-Hodgkin's lymphoma (NHL), myeloma, and acute lymphocytic leukemia (ALL) and chronic lymphocytic leukemia (CLL).

Methods

Records of transfusions and immunohematologic studies on the patients were collected from the hospital's computer blood bank files. The data included all transfusions and tests performed for each patient during all previous admissions to this hospital. Information collected included age, sex, transfusion history (date, quantity, and type of blood component), and results of tests for RBC antibodies. No detailed information on pregnancies and transfusions in other hospitals was collected. We excluded 1) antibodies that were present before the study began, 2) antibodies against low frequency antigens that were coincidentally found, 3) non-RBC-stimulated antibodies, and 4) autoantibodies. Only results obtained from

antibody screening after transfusion were considered. When an antibody had been detected, we recorded its specificity, the number of previous RBC transfusions, and the number of transfusion episodes since the first transfusion.
Patients received RBC-containing transfusions compatible with their ABO and D phenotype. These included packed RBCs, white cell-reduced RBCs, washed RBCs, and whole blood. Patients who received packed RBCs and had a febrile nonhemolytic transfusion reactions twice, subsequently received white cell-reduced blood components.
Platelet components were prepared by combining the platelets from the buffy coats of five to eight donors in one platelet pack. The transfused platelet components were ABO and D compatible whenever possible. After an antibody had been identified, RBC components that were negative for the corresponding antigen were transfused.

Statistics

Statistical software packages (Excel 5.0, Microsoft, Redmond, CA; NCSS 6.0, Jerry L. Hintle, Kaysville, UT) were used for data management and analysis, respectively. The Kaplan-Meier product limit estimator was used to calculate the rate of antibody formation. Product-limit antibody formation plots were derived from the formula 1 - [antibody formation (t)]. The log rank test (nonparametric) was used to compare antibody formation. The chi-square test was used to compare the incidence of antibodies in persons with different diseases. The Kruskal-Wallis test (nonparametric ANOVA) was used to compare medians. When multiple comparisons were performed, the Bonferroni test value was used to test for significant differences. In other cases, groups were assumed to differ significantly when the probability level was less than 0.05.

RESULTS

Patient data are shown in Table 1. The median age at diagnosis was 69 years (range, 16-96), and the male-to-female ratio was 1.2.

Table 1. Patient number and age (years) according to disease

Disease	Patients		Evaluable		Median age	Range
	Total	Transfused	Number	Percentage		
NHL	343	212	175	83	69	16-90
MDS*	160	137	105	77	74	30-94
AML	102	96	87	91	67	18-89
Myeloma	117	94	86	86	70	34-91
MYPRO	137	64	44	69	69	28-96
CLL	115	43	31	72	71	41-85
ALL	25	23	21	91	42	18-81
Hodgkin lymphoma	67	28	15	54	38	20-80
Total	1066	697	564	81	69	16-96

*Myelodysplastic syndrome

The 564 patients received 15,287 units of RBCs in 6182 transfusion episodes. The total number of units transfused per patient ranged from 1 to 338, with a median of 16 units. Men received a median of 19 units, and women received a median of 15 units ($p>0.05$). Gender and age had no influence on the rate of antibody formation. The median number of units transfused, the number of transfusion episodes, and the months of transfusion support are shown in Table 2. Patients with myeloproliferative diseases received almost three times as many units of blood ($p<0.0001$) in more than twice as many episodes ($p<0.0001$) over more than twice as long a period ($p=0.0202$) as patients with lymphoproliferative diseases. The median number of units transfused was 15 for the nonimmunized group and 47 for the immunized group ($p<0.0001$).

Patient sera were screened for antibodies a median of 10 times (range, 2-93). The screening revealed that 1) Ten (1.8%) patients had a total of 12 RBC antibodies upon entering the study, and one of them formed additional antibodies. The latter antibodies were included in the study; 2) Seventeen antibodies against low-frequency antigens were coincidentally found (9 anti-Wr^a, 5 anti-Lu^a, 2 anti-C^w, and 1 anti-Kp^a); 3) There were 3 non-RBC-stimulated antibodies (2 anti-M and 1 anti-Le^b); and 4) There were 16 autoantibodies (13 aspecific autoantibodies and 3 auto-anti-e).

Table 2. RBC transfusion data by disease

Disease	Transfusion					
	Number		Episodes		Months of support	
	Median	Range	Median	Range	Median	Range
AML	31	3-126	12	1-52	6	1-35
MDS	30	2-229	12	1-89	13	1-94
MYPRO	24	2-183	11	1-67	11	1-88
Total*	30	2-229	12	1-89	9	1-94
ALL	23	2-98	9	1-43	4	1-87
Myeloma	15	2-66	7	1-30	7	1-60
CLL	15	2-152	6	1-67	4	1-84
NHL	8	1-338	4	1-84	3	1-116
Hodgkin lymphoma	7	2-22	3	1-10	2	1-32
Total†	11	1-338	5	1-81	4	1-116
Overall total	16	1-338	7	1-89	5	1-116

*All myeloprofiferative diseases
† All lymphoproliferative diseases

A total of 71 antibodies were formed by 51 patients (9.0% of the total group; 28 men and 23 women). Fifty percent of the first antibodies were detected after 13 units had been transfused (Fig. 1). Antibody formation first occurred after a median of 12 units had been transfused in six episodes.

Table 3. Incidence and specificity of RBC antibodies

Single antibody	Number of patients	Two antibodies	Number of patients	Multiple antibodies	Number of patients
D	3	D+E	1	E+c+AA*	1
E	7	C+e	1	E+K+Fya	1
c	1	C+E	1	E+c+K	1
K	10	C+K	1		
Jk[a]	1	E+c	5		
AA	12	E+K	1		
		E+Fy[a]	1		
		E+AA	2		
		K+AA	1		

* Aspecific alloantibody

The risk of alloimmunization, defined as the total number of alloantibodies divided by the total number of units transfused, was 0.5 percent. When related to the number of transfusion episodes, the risk was 1.15 percent.

Fig 1. Percentage of first antibodies formed in relation to the number of units transfused. Fifty percent of antibodies were formed after 13 transfusions

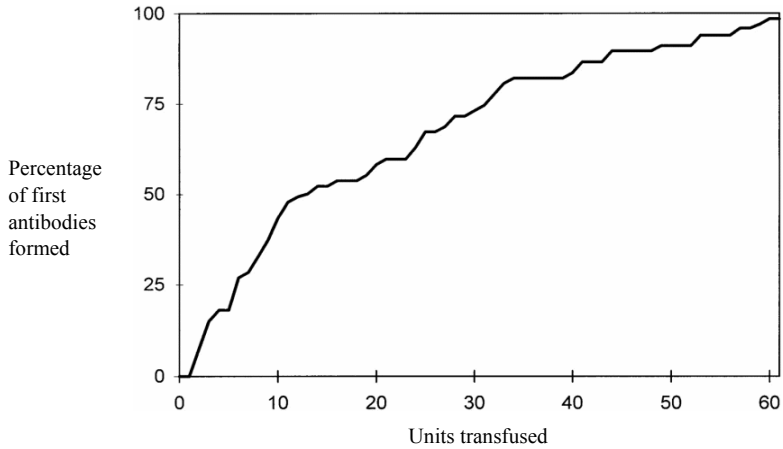
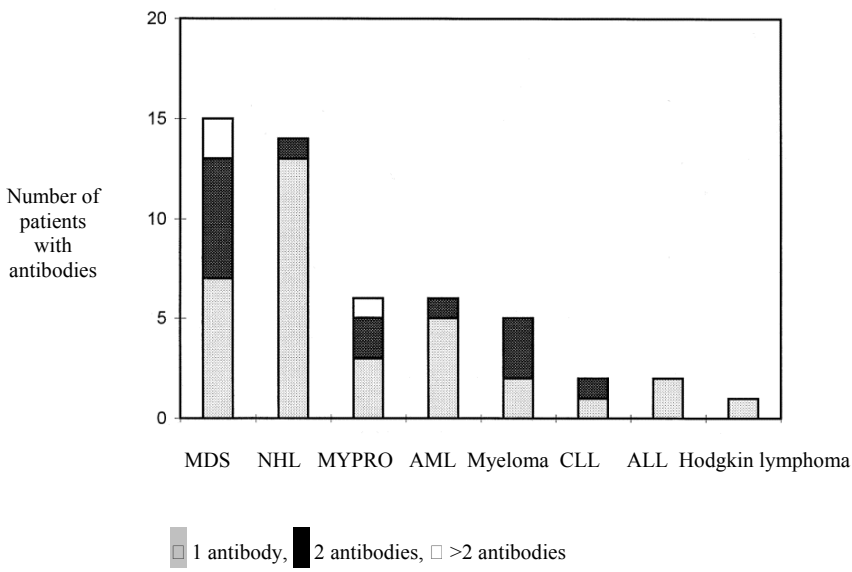


Fig 2. Number of patients with antibodies in relation to hematologic disease



The incidence of antibody formation and the antibody specificity are presented in Table 3. The distribution of alloantibody specificity did not differ significantly among patients with different diseases. Thirty-four patients formed one antibody,

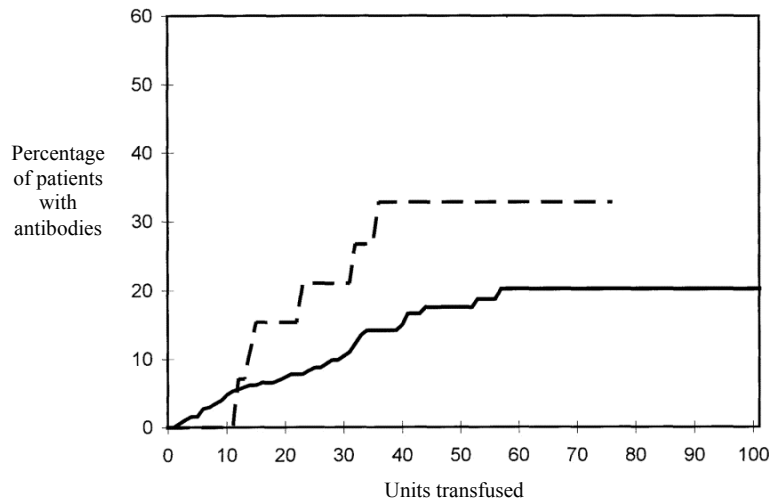
14 patients two antibodies, and 3 patients three antibodies. Figure 2 depicts the results by disease.

These results indicate that, once a person is immunized, the risk of producing additional antibodies increases 3.3-fold.

A total of 58 analyses of antibody specificity were documented: 46 for one antibody, 11 for two antibodies, and 1 for three. There was no difference between responders (those who formed 1 antibody) and "super-responders" (those who formed more than one antibody at one time) in terms of the number of units transfused before antibody formation occurred (median number of units, 15 and 9, respectively; $p>0.05$).

A Kaplan-Meier estimate of the cumulative probability function for all diseases illustrates the relationship between the first and additional antibody formation and the number of transfusions given (Fig. 3).

Fig. 3. First versus additional antibody formation in relation to number of units transfused.



—, Transfusions before first antibody; - - - -, transfusions between first and additional antibodies. $p>0.05$.

A logrank test revealed no significant difference ($p>0.05$) between the rates of antibody formation. Second antibody formation occurred after the transfusion of 19 additional units (Table 4).

Twenty-one patients received more than 100 units of blood; 14 did not become immunized - in one case, not even after receipt of 229 units. Investigation of alloantibody formation after the last transfusion was performed after a median of 14 days (range, 4 days-9 years). No acute or delayed serologic transfusion

reactions were reported. Febrile nonhemolytic reactions and allergic urticarial reactions were seen incidentally.

Table 4. Number of RBC transfusions preceding first and second antibody formation episodes.

	Patients		RBC number		Transfusion episodes	
Disease	Number	Frequency	Median	Range	Median	Range
Before first antibody						
MDS	15	14.3	12	4-60	5	2-24
MYPRO	6	13.6	19	2-57	7	1-22
ALL	2	9.5	43	41-44	20	19-21
NHL	14	8.0	18	3-40	7	1-19
Hodgkin lymphoma	1	6.7	10		5	
Myeloma	5	5.8	6	2-11	2	1-6
AML	5	5.7	17	10-28	8	6-10
CLL	1	3.2	53		21	
Total	49	3.2-14.3	12	2-60	6	1-24
Before second antibody						
MDS	4	26.7	14	12-23	6	4-10
MYPRO	1	16.7	32		9	
NHL	1	7.1	35		9	
Total	6	7.1-26.7	19	12-35	8	4-10

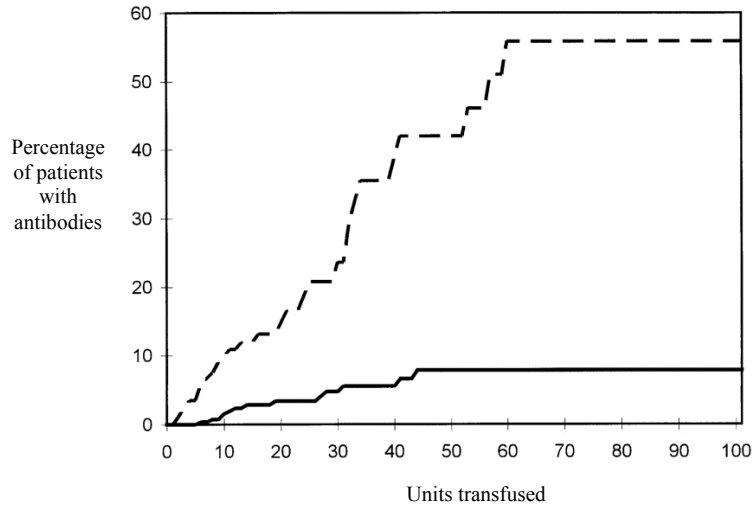
Antibodies and platelet transfusions

Three-hundred four patients received a total of 4263 platelet transfusions (median, 10; range, 1-80). Seventeen patients in this group formed a total of 21 RBC alloantibodies. The risk of alloimmunization to RBC antigens was lower for patients who received platelet transfusions (0.21%) than for patients who did not (0.97%). Moreover, RBC alloantibody formation in patients who received platelet transfusions during the course of their disease was slower than that in patients who did not need platelet transfusions (Fig. 4, $p<0.0001$).

Thirty-three patients received a total of 244 D-incompatible platelet transfusions (median, 4; range, 1-31). Three patients, two with AML with maturation (AML-M2) and one with T-cell prolymphocytic leukemia, developed anti-D after 7, 7, and 11 D-incompatible platelet transfusions, respectively.

Anti-E was detected after two platelet transfusions in a D-positive male patient with MDS-RA who had screened negative for antibodies on two previous occasions and had not received RBC transfusions.

Fig 4. RBC antibody formation in patients with and without platelet transfusions.



—, Patients who received platelet transfusions; - - -, patients who did not receive platelet transfusions. P<0.0001

Individual antibodies

Anti-E was found in 20 patients after a median of 13 units had been transfused, anti-c in 8 patients after the transfusion of 6 units, and anti-K in 15 patients after the transfusion of 17 units. Antibodies from the RH and KEL systems together represented 73 percent of antibodies detected. The most frequently encountered combination was anti-E and anti-c (7 times). Anti-E occurred 14 times in various combinations.

Persistence of antibodies

We found that, in 26 cases (51%), 33 RBC antibodies (46%) did not always persist after recognition and were not detected on at least two consecutive occasions over time. The frequency of nondetection for each antibody is shown in Table 5.

Table 5. Antibody detectability

Antibody specificity	Time in months to become undetectable				
	<1	1-3	3-6	6-12	>12
D					
E		5		2	2
e		1			
C		1	1		
c		1	1	2	2
K	4	2	1	1	
Jk^a					1
AA*	1	2			1

* Aspecific alloantibody

Case reports with lymphoplasmacytoid B-cell NHL

A patient (IgG immunocytoma) who received a total of 338 units of blood formed anti-C after receiving 24 units and anti-K after 60 units; no more antibodies were formed during the transfusion of the next 278 units of blood. The most prolific antibody responder, a patient with essential thrombocythemia complicated with an inexplicable anemia, produced four antibodies (anti-E, anti-c, anti-K, and anti-Lu^a) after receiving only 3 transfusions; no further antibodies were formed during the next 53 transfusions.

One patient, who entered the study with anti-Lu^a, formed anti-D and anti-E after an accidental transfusion of 4 units of D-incompatible white cell-reduced RBCs.

A patient with therapy-resistant CLL complicated with pure RBC aplasia and an autoimmune hemolytic anemia (aspecific autoantibodies) formed anti-E after 53 white cell-reduced RBC transfusions.

DISCUSSION

In several studies, the question has arisen as to whether patients who are transfusion dependent over a longer period, such as those with sickle cell disease or thalassemia, should receive blood transfusions that are matched for antigens other than ABO and D, in an attempt to prevent the formation of RBC alloantibodies[1,4,6,10-14,17,22,23]. Because of intensified treatment and improved survival, patients with hematologic malignancies are also in need of transfusion support over a period of several years; therefore, the same question could apply for that patient population.

This retrospective study was designed to discover the prevalence of RBC alloimmunization due to transfusion in 564 patients with hematologic and oncologic malignancies. We found 71 RBC antibodies in 51 patients, for an

overall alloimmunization rate of 9.0 percent. Patients received a median of 16 units of RBCs. Fifty percent of the antibodies occurred after 13 transfusions, which indicates that the majority of antibodies are formed early during the course of transfusions. Several authors[2,4,12,17,18] reported comparable results for the risk and the number of transfusions. There are also studies that found higher[1,6,11,22] and lower[16,19,30,33-35] immunization rates. Factors such as the patient population under study, the transfusion policy, the time and frequency of testing, the sensitivity of the test methods, and the technical expertise of the transfusion laboratory staff play an important role in the difference in results obtained. Ramsey and coworkers[20,21], who studied the persistence of RBC alloantibodies, reported that clinically significant alloantibodies may become undetectable in time; although this was not investigated systematically in the present study, our findings were similar. On the one hand, this may lead to underestimation of the number of antibodies formed; on the other hand, the consequence may be that the patient receives incompatible RBCs and experiences a secondary immune response, which compromises the benefit of the transfusion. This implies that information on previously detected RBC alloantibodies should always be available.

Age and gender were of no influence on the rate of antibody formation. Seyfried and Walewska[9] found that the probability of alloimmunization is a quadratic function of age. We could not reproduce these findings. The overall immunization risk was 0.5 percent per RBC unit transfused. Redman et al.[18], who also performed serial posttransfusion testing for alloantibodies, obtained a much higher (5.9%) alloimmunization risk per unit transfused for a population of 452 immunocompetent patients who had undergone elective surgery. They found 59 RBC antibodies after the transfusion of 1002 units; the proportion of immunized patients (10.4%) did not differ significantly from the values in our and other [2,3,7,9,13,17] studies. When we consider the fact that many antibodies are formed early during the course of transfusions, one would expect that, after the initial increase, the immunization risk per unit would eventually decrease as the number of units transfused increased. In our study, the immunization risk was 0.45 percent after 2 units had been transfused; it increased to 0.65 percent after the transfusion of 11 units and stabilized at 0.46 percent after about 50 units had been transfused (Fig. 1).

Seventeen patients formed more than one antibody. The risk of additional antibody formation therefore increased 3.3 times. Thus, we agree with others[2,7,12,18], who found increased risk factors between 2.7 and 4.1.

The most frequently encountered combination was anti-E and anti-c: 35 percent of the patients with anti-E also developed anti-c. Shirey et al.[41] found that the overall rate of immunization against c in R1R1 patients with anti-E was 37 percent.

For 16 of 71 antibodies, the specificity could not be identified; this is in agreement with the study of Redman et al.[18], who found 9 antibodies of

unrecognized specificity in a total of 51 positive screening tests. In four cases, aspecific antibodies were found in combination with specific antibodies.
We had one patient with CLL who presented with anti-E and aspecific antibodies after 53 transfusions. While several studies[2,4,9,12] indicated that lymphocytic leukemia may be characterized by a lack of immunologic response, we were not able to demonstrate a significant difference between CLL patients and patients from other disease groups, as far as the risk of antibody formation is concerned.
As in other studies[12,13,18], antibodies against RH system antigens, especially -E and -c, and Kell system antibodies made up most of the antibodies found. On the basis of genetic frequency, 402 patients in our study were at risk of forming anti-E, 105 patients anti-c, and 510 patients anti-K. When we consider the relative potency of the antigens - that is, the actual frequency with which alloantibodies are encountered - compared with the calculated frequency of the opportunity for immunization (E, 1.7%; c, 2.0%; and K, 5.0%)[42] we can expect a median of 16 transfusions to find 28 patients with anti-E, 23 patients with anti-c, and 35 patients with anti-K. Anti-E was observed in 20 patients, anti-c in 8 patients, and anti-K in 15 patients, which suggests a relative potency of 1.2 percent, 0.7 percent, and 2.1 percent, respectively. For anti-c and anti-K, these relative potencies are significantly lower ($p = 0.007$ and $p = 0.004$, respectively) than those found by Giblett[42].
Four patients formed RBC antibodies after transfusion of platelets: three formed anti-D and one formed anti-E once. Patients who received D-incompatible platelet transfusions were not treated with anti-D immunoglobulin. Because platelets do not bear RH system antigens[52], immunization is enhanced through RBC contamination of the platelet components. Woodrow and Donahue[53] found that as little as 0.1 mL of D-positive blood can immunize a D-negative recipient. In our study, only three patients formed anti-D after 244 incompatible platelet transfusions, which indicates that, for patients treated for hematologic and oncologic malignancies, the capacity to form anti-D after incompatible platelet transfusions is very low. Although the anti-E could have occurred naturally,[37] that seems unlikely, because it was not detected on two previous occasions.
Patients who received intensive chemotherapy and therefore required platelet transfusions produced antibodies against RBC antigens at a much lower rate than patients who did not need platelet support (Fig. 4).
During the period under study, no (delayed) hemolytic transfusion reactions were reported. According to others[16,25,28,43,44], we should have encountered three such reactions (range, 0.4-8.1). This difference might be explained by low awareness of this possibility, the fact that many reactions are clinically asymptomatic, or the possibility that the reaction was masked by the severity of the underlying disease.

Several authors found that patients with hematologic malignancies and those who undergo intensive chemotherapy have a low capacity for forming antibodies against foreign antigens[9,19,24,26,27,30,43] and a lower frequency of transfusion reactions[25,28] (most probably due to their altered immune status), which results in a tolerance for or an unresponsiveness to blood group antigens. Other authors[45-49] discovered that transfusion itself induces immunosuppression, although the mechanisms involved are not yet completely understood[50,51]. The low rate of response to foreign antigens seen in our study could be due to a combination of all three factors mentioned above.

Except for the one patient with anti-e in combination with anti-C, we never had any difficulty finding compatible blood for our patients with antibodies. In the majority of cases (85%), the theoretical probability of finding compatible blood exceeded 20 percent.

In patients with a hematologic and an oncologic malignancy, RBC antibody formation is comparable to that for other diseases requiring multiple transfusions. Patients with a hematologic malignancy who receive intensive treatment form RBC antibodies at a lower rate than those who are not treated intensively.

In this cohort of patients, we encountered a low incidence of severe delayed hemolytic transfusion reactions and no real problems in finding compatible donors. Therefore, "prophylactic" matching for antigens other than ABO and D, as recommended by several authors, seems unnecessary in this clinical setting. In addition, extensive matching leads to increased costs at a time when we should seek ways to reduce operational costs while maintaining a high standard of patient care.

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6

ADDITIONAL RBC ALLOANTIBODIES AFTER BLOOD TRANSFUSIONS IN A NONHEMATOLOGICAL ALLOIMMUNIZED PATIENT COHORT: IS IT TIME TO TAKE PRECAUTIONARY MEASURES?

Schonewille H, van de Watering LMG, Brand A. Additional RBC alloantibodies after blood transfusions in a nonhematological alloimmunized patient cohort: is it time to take precautionary measures? Transfusion 2006;46:630-35

SUMMARY

BACKGROUND: Red blood cell alloimmunization is common in transfused patients. Most studies report on the rate of alloimmunization in chronically transfused patients, which can be as high as 60%. Less is known on the incidence of clinically relevant antibodies in accidentally transfused patients. Because the probability of repeat transfusion increases with longer life expectancy, it was wondered to which extend non-chronically transfused alloimmunized patients are prone to form additional antibodies after repeat transfusion events

STUDY DESIGN AND METHODS: A 20-year retrospective multicenter study analysing additional alloantibody formation, against the RH, KEL, FY, JK and MNS blood group systems.

RESULTS: After additional transfusions, 21.4 percent of 653 patients produced additional antibodies, resulting in 157 new antibody specificities. At the end of the study 33.4 percent of patients had multiple antibodies. Eighty of 140 patients (57%) who formed additional antibodies did so after 1 transfusion episode of a median of 2 units RBCs. Based on the antigen profile of 316 patients, 83 percent of antibodies could have been prevented by extended matching for the C, E, c, K, Fy^a and Jk^a antigens. Considering the current available donors in our region, 1-10% potential donors would be available for 39 percent of patients and greater than 10 percent of potential donors for 61 percent of patients.

CONCLUSION: It has been shown that nonhemato-oncologic alloimmunized patients are high antibody responders, with a more than 20 times increased risk to form antibodies compared to first-time alloimmunization risk. If extended matching for C, c, E, K, Fy^a and Jk^a antigens in the future is considered, this group should be taken into account.

INTRODUCTION

Alloimmunization is common in transfused patients. The risk of immunization increases with the number of transfusion events, although in most polytransfused patients antibodies are formed early in the course of treatment[1,2]. Most studies have been performed in chronically transfused patients, for example, hemoglobinopathies[3-10], hematologic malignancies[1,2,11-14], organ transplant recipients[15-17] and patients with renal failure[11,18,19]. Alloimmunization in these groups has a reported incidence up to 60 percent, with an up to fourfold increased risk of multiple antibodies compared to the risk of single antibodies[1,2,15,21]. In other transfused populations alloimmunization was described between less than 1 and 10 percent, depending on the study design (e.g. retrospective resp. prospective)[20, 21,30].

Alloimmunization may cause several problems ranging from inconvenience due to delay in obtaining compatible blood to (delayed) hemolytic transfusion reactions. The development of alloantibodies was recently found to be associated with the development of RBC autoantibodies. Such autoantibodies can lead to shortened life span of the recipients own RBCs and may cause clinical hemolysis[7, 22-25].

Because of longer life expectancy of the population and an increased probability of repeat surgery or diseases requiring blood transfusions, the clinical relevance of red blood cell antibodies in non-chronically transfused patients increases.

We wondered to which extend non-chronically transfused patients who became alloimmunized are prone to form additional antibodies after one or more repeat transfusion events. To address this question, we retrospectively studied alloantibody formation over a 20-year period in a large non-hematological transfused alloimmunized patient cohort with special emphasis on additional antibody-forming episodes.

MATERIALS AND METHODS

We examined retrospectively all the records on warm-reacting RBC antibodies in the transfusion laboratory computer databases of HAGA Hospital, location Leyenburg (a 600-bed multidisciplinary teaching hospital in the Hague, the Netherlands) and Leiden University Medical Centre, (a 900 bed top-clinical care hospital, Leiden, the Netherlands) during a 20 year period, from 1983 through 2002. We included only patients with antibodies against the RH, KEL, FY, JK and MNS blood group systems who experienced at least one subsequent transfusion event after alloimmunization. We excluded patients with a hemato-

oncologic disease, patients with warm reacting autoantibodies, patients with antibodies against low and high-frequency antigens and patients receiving preventive extended matched (e.g. c, E and K) RBC transfusions. Information collected was patient's age; sex; RBC typing for C, E, c, e, K, Fy^a, Fy^b, Jk^a, Jk^b, M and S antigens; dates and results of RBC alloantibody screenings and transfusion history. The results of antibody investigation were valid for 72 hours (a transfusion episode). Detailed information on pregnancies, transfusions, and antibodies detected in other hospitals was not available.
Primary antibodies were defined as the first time that antibodies were identified in patients in each hospital during the study period, and additional antibodies were defined as new antibody specificities detected after subsequent transfusions. After an antibody was identified, patients received RBC transfusions that were negative for the antigen corresponding to the implicated alloantibody, after complete cross-matching, including an indirect antiglobulin test. Preventive antigen-matching (i.e. c, E and K) was not routinely performed in alloimmunized patients.

Statistical analysis

Statistical software packages (Excel 5.0, Microsoft , Redmond, WA; SPSS 11.0, SPSS 11.0, SPSS Inc., Chicago, IL) were used for data management and analysis respectively. The chi-square or Fisher exact test was used for comparisons of proportions in two groups. Student's t test, Welch test, or Bonferroni test was used when (multiple) comparisons between groups were performed. After univariate analysis, multivariate analysis was used to determine factors that were independently associated with additional antibody production.
The Kaplan-Meier product limit estimator was used to calculate the rate of additional antibody formation.
Groups were assumed to differ significantly when the probability level was less than 0.05.

RESULTS

Patient characteristics

During the study period, 1043 patients belonging to the selected patients' categories presented with irregular antibodies. Of these 653 patients returned for a subsequent transfusion request.

Because statistical analysis revealed no differences in additional antibody formation regarding sex (female-to-male ratio 2.1 [1.8 if anti-D was excluded]; except for anti-D frequency at inclusion, which was higher in female compared to male patients, $p<0.001$) and hospitals, with respect to all tested parameters, data was not stratified for these factors during analyses. Patient age (median, 63 years; range, 1-96) was not associated with additional antibody formation.

The median study period per patient, from inclusion to date of last antibody screening, was 22 months (range, 2 days to 20 years). Additional antibody screening tests after first antibodies were performed a median of three times (range, 1-37 times).

Table 1. Transfusion characteristics in 653 alloimmunized patients

	Patients presenting with alloantibodies	Patients who developed new antibodies
Number of patients	653	140
Mean number of units transfused	9.0	5.9
(total, median, range)	(5850, 5, 1-120)	(809, 4, 1-41)
Mean number of transfusion episodes	2.8	1.9
(total, median, range)	(1894, 2, 1-35)	(265, 1, 1-11)
Number of transfusion episodes		
1	277	80
2	110	31
3	83	17
4-6	116	9
>6	67	3
Mean number of transfusion months	10.3	7.2
(median, range)	(0.3, 0.03-175)	(0.03, 0.03-118)

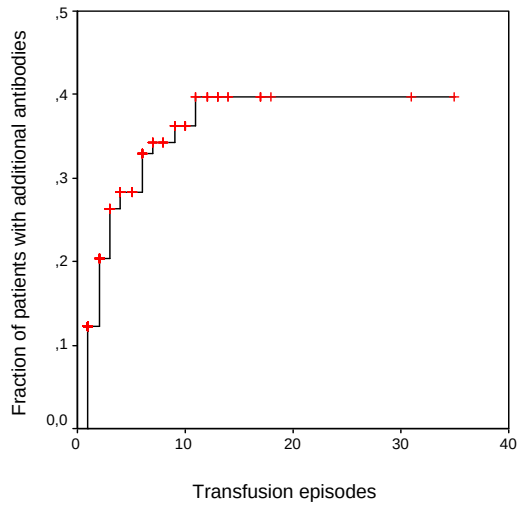
Transfusion characteristics

The 653 patients received a median of 5 units of RBC in a median of 2 transfusion episodes over a period of a median of 10 months (transfusion months). Almost 60 percent of patients experienced less than three transfusion episodes (table 1).

Eighty of the 140 patients (57%) who formed additional antibodies did so after one additional transfusion episode of a median of 2 units of RBC (range, 1-10

units), 31 patients (22%) developed additional antibodies after 2 additional transfusion episodes of a median of 5 units (range, 3-14 units), 17 patients (12%) did so after 3 episodes of a median 8 units (range, 6-18 units) and 12 patients (9%) after 4-11 transfusion episodes of a median of 19 units (range, 7-41 units; Table 1 and Fig. 1, Kaplan-Meier estimate).
Multivariate analysis (variables; numbers of: RBC units transfused, transfusion episodes, transfusion months, and screening tests) revealed that additional antibody formation was independently associated with the number of transfusion episodes (inversely, $p<0.001$), indicating additional alloimmunization early during transfusion therapy, and the number of antibody investigations ($p<0.001$), reflecting longer patient follow-up.

Fig. 1. Kaplan Meier estimate of the rate of additional antibody formation after transfusion in alloimmunized patients



Red blood cell antibodies

At presentation, 653 patients had antibodies against 772 RBC antigens, 555 patients had an antibody against a single RBC antigen and 98 patients (15.0%) had multiple antibodies. After additional transfusion episodes, 140 patients (21.4%) developed additional antibodies, detected after a median interval of 12.5 months (range, 3 days to 240 months), resulting in 157 new antibody specificities. Of these 140 patients, 79 patients received additional transfusions and 10 patients (12.7%) experienced a third antibody forming episode (140/653 vs 10/79, $p=0.076$) making 11 additional antibody specificities. The odds ratio (OR) for

additional antibody formation compared to primary antibody formation, which in a previous study was 0.9%[31], is 29.1 (95% confidence interval [CI], 22.7-37.2; p<0.0001).

Table 2. Number and antibody specificity in 653 alloimmunized patients

Antibody Specificity	Primary Antibodies		Additional Antibodies		p Value*
	Number	Percent of total	Number	Percent of total	
D	97	12.6	2	1.2	<0.0001
C	51	6.6	16	9.5	0.19
E	228	29.5	34	20.2	0.017
c	38	4.9	27	16.1	<0.0001
e	8	1.0	1	0.6	1.0
K	178	23.1	39	23.2	1.0
Fy^a	67	8.7	14	8.3	1.0
Fy^b	2	0.3	2	1.2	0.15
Jk^a	46	6.0	12	7.1	0.6
Jk^b	16	2.1	7	4.2	0.16
M	23	3.0	1	0.6	0.10
S	17	2.2	11	6.5	0.009
s	1	0.1	2	1.1	0.084
Total	772		168		

* p Value primary antibody specificity versus additional antibody specificity as fraction of total first antibodies and additional antibodies

The antibody specificity between first and additional antibodies differed with regard to anti-E, which was formed more often during the first antibody forming episode whereas anti-c and anti-S were more often formed after subsequent transfusion episodes (Table 2). The number and specificity of antibodies at presentation were not correlated with the incidence of additional antibody formation. D-negative patients with non-D antibodies formed additional non-RH antibodies as equally often as D-negative patients with anti-D.

The red blood cell antigen profile was known in 316 patients (47.8%). In these patients, the number of antigens to which antibodies could be formed was not associated with the incidence of additional antibody formation or with the number of additional antibodies formed.

We calculated the additional immunization risk per antigen per unit transfused in patients who lacked the corresponding antigen, based on the number of a specific additional antibody formed divided by the *estimated* number of antigen positive transfusions. The results showed that the immunization risk varied considerably for specific antigens (Table 3).

At the end of the study 33.8 percent of 653 patients had multiple antibodies. The most frequently encountered antibody combinations, with or without other antibody specificities, were DC (n=28), cE (n=22), EK (n=17) and KFy^a (n=11), representing 57 percent of all antibody combinations.

Laboratory test problems associated with multiple alloantibodies

A patient with multiple alloantibodies places the transfusion laboratory staff at great problems identifying the specificity of the individual antibodies and excluding the presence of other clinically significant antibodies. At presentation we found 31 different antibody combinations in 98 patients; at the end of follow-up 64 different antibody combinations were present in 221 patients. With standard antibody specificity techniques (e.g., low-ionic-strenght saline enhanced and enzyme-test), a routine 3-cell screening test panel and a 11-cell identification test panel (DiaMed AG, Murten, Switserland), we calculated the number of antibodies that could not be excluded, based on the absence of a negative reaction with at least one test RBC with homozygous expression for the corresponding antigen. At presentation, identification of the alloantibodies and exclusion of possibly underlying antibodies was possible in 453 patients, but for 200 patients, one to six clinically relevant antibodies could not be excluded. At the end of the study, for 240 patients one to 8 antibodies could not be excluded.

Preventive extensive antigen matching in alloimmunized patients

For 316 patients the C, E, c, e, K, Fy^a, Fy^b, Jk^a, Jk^b, M and S phenotype was known. We performed the theoretical exercise for these patients regarding extended matching. To prevent additional antibodies (except for anti-e and anti-M) for 95 percent of patients less than 1 percent (minimum, 0.22%) of potential Caucasian donors per RBC unit were to be expected for 15 percent of patients, 1 to 10 percent of donors are expected to be compatible for 64 percent of patients and more than 10 percent compatible donors for 21 percent of patients. If extended antigen matching was limited to the C, E, c, K, Fy^a and Jk^a antigens, preventing 83 percent of additional antibodies, 1 to 10 percent of potential donors would be available for 39 percent of patients and more than 10 percent of donors for 61 percent of patients.

Table 3. Additional immunization risk per antigen per antigen-positive unit transfused in 316 RBC antigen-phenotyped alloimmunized patients

Antigen	Number of patients*	Number of additional antibodies	Number of RBC units transfused	Number of antigen- positive RBC units †	Immunization risk (%) ‡
K	211	19	2340	211	9.0
C	80	7	762	192	3.6
E	182	17	1952	566	3.0
c	61	12	646	503	2.4
e	4	1	55	53	1.9
Jk^a	64	10	763	587	1.7
Fy^a	89	10	1094	722	1.4
Jk^b	56	5	575	420	1.2
S	156	6	1829	1006	0.6
Fy^b	56	2	710	589	0.3
M	70	1	817	637	0.2

* Number of patients with a specific RBC antigen to which an additional antibody could be formed.
† The number of antigen-positive RBC units was calculated as total units transfused in patients with absent antigen multiplied by the change on specific mismatched RBC antigens. The chance of mismatched antigens was based on the antigen frequency in general Caucasian population (i.e., c = 0.78, e = 0.96, K = 0.09, Fy^a = 0.66, Fy^b = 0.83, Jk^a = 0.77, Jk^b = 0.73, M = 0.78, S = 0.55; for C and E, the patient's Rh(D) factor was taken into account $D^{pos}C^{pos}$ = 0.80, $D^{neg}C^{pos}$ = 0.05, $D^{pos}E^{pos}$ = 0.33, $D^{neg}E^{pos}$ = 0.06)
‡ Immunization risk: number of additional antibodies per number of corresponding antigen positive units transfused.

DISCUSSION

In this 20-year retrospective study in two hospitals, we investigated the formation of additional RBC alloimmunization in 653 patients who initially presented with RBC antibodies and who had returned for a subsequent transfusion event. We found that 21.4 percent of patients formed additional antibodies after repeat transfusion episodes and 12.7 percent of these patients experienced a third antibody forming episode. Most additional antibodies were formed after one additional transfusion episode of a median of 2 units of RBCs. At the end of the study 33.8 percent of patients had multiple antibodies.

In agreement with previous studies regarding primary antibody formation in multitransfused patients[1,2], most additional antibodies in alloimmunized patients are also formed after a few additional transfusions. Fifteen patients experienced more than 11 transfusion episodes and none formed additional antibodies (Fig. 1).

Most studies report on the presence of multiple RBC antibodies in chronically transfused patients, with an up to fourfold increased risk of multiple antibodies compared to the general risk of antibody formation[1,2,4,9,15,26-28].
The calculations used are based on the formula:

$$\frac{\text{Number of patients with multiple antibodies / Number of patients with a single antibody}}{\text{Number of patients with antibodies / Number of transfused patients}}$$

The results from these calculations do not give any indications regarding the risk of additional antibody forming episodes in alloimmunized patients after subsequent transfusion episodes.
From a number of informative studies[14,20,28,29], some including polytransfused patient groups, we could calculate that the incidence of additional antibodies after transfusion in alloimmunized patients is between 9 and 22 percent. Only few (7-14) alloimmunized patients in these studies, however, were retransfused.
In retrospective studies, primary antibody formation in non-chronically transfused patients has a reported incidence of approximately 1 percent[30]. We previously analysed RBC alloimmunization in transfused patients during a 5-year period and found 0.9 percent alloimmunized patients, corresponding with an immunization risk of 0.2 percent per unit transfused[31]. The current study shows additional RBC immunization in 140 of 653 alloimmunized patients after subsequent transfusion (OR, 29.1; 95% CI, 22.7-37.2; $p<0.0001$).
The formation of a first antibody apparently identifies high responders against subsequent alloantigenic challenges. The fact that the chance for subsequent antibody- forming episodes is so much higher than in hemato-oncologic patients[1,2] may be explained by immunocompromised conditions in these patients.
To our knowledge this is the first study calculating RBC alloimmunization risks for specific antigens in a RBC antigen-phenotyped population. Although factors influencing antibody specificity are not taken into account, such as time interval between transfusion and antibody screening, the results, in terms of antigen ranking, are in agreement with a previous study calculating antigenicity for the most important antigens[31]. Because antibody investigations in the present study were performed for a transfusion indication, the immunization risk per antigen may be even higher, considering that antibodies may have disappeared[33,34].
Although alloantibodies are considered not harmful, they place the patients at increased risk for complications such as transfusion delay, (delayed) hemolytic or serologic transfusion reactions, and even autoimmunization, compromising their clinical care. The last complication has recently been shown not to be restricted to chronically transfused patients[25].

Extended antigen matching (e.g. c, E and K), preventing the formation of the majority of RBC antibodies in chronically transfused patients, has been advocated for selected patients populations. Some reports applying this policy showed a significant drop in alloimmunization rate and delayed hemolytic transfusion reactions[7,34]. Discussion remains, however, regarding to which patient populations this should be applied[7,35].

Such selection is of importance considering the high costs involved and donor feasibility. The costs are primarily based on extended donor and patient antigen typing. No data are available addressing costs involved with complex laboratory tests to identify antibody specificities in case of multiple antibodies and delayed and prolonged medical care, associated with transfusion delay, hemolytic transfusion reactions, additional transfusions, and autoimmunization.

Donor genotyping for the most common clinically relevant blood group antigens, by means of automated DNA techniques, will soon be available. This will, most likely, reduce costs as well as workload involved in donor and patient antigen typing, compared to current phenotyping techniques and increase the opportunity for the use of extended phenotypically compatible transfusions.

We calculated that donor feasibility could be problematic (<1% donors available) for the Blood bank in 15 percent of patients in case of extended matching included Jk^b and S antigens. If antigen matching is restricted to antigens with the highest immunization risk (i.e. C, E, c, K, Fy^a and Jk^a), however, finding compatible donors should be no problem in the Netherlands with yearly $6.2x10^5$ blood donations from $4.5x10^5$ donors.

In summary, we showed that nonhematooncological patients who present with an alloantibody have a high probability of forming additional antibodies upon a subsequent transfusion indication. If extended matching for C, E, c, K, Fy^a and Jk^a antigens in the future is considered, this patient group should be taken into account.

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HIGH ADDITIONAL MATERNAL RED CELL ALLOIMMUZATION AFTER RH- AND K-MATCHED INTRAUTERINE INTRAVASCULAR TRANSFUSIONS FOR HEMOLYTIC DISEASE OF THE FETUS

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ABSTRACT

OBJECTIVE: Intra-uterine transfusion (IUT) is a life saving therapy for the severely anemic fetus with hemolytic disease. However, maternal additional antibody formation is a complication of the procedure. In this study, we determined antibody formation after introduction of preventive D-, C-, c-, E- and e- and K-antigen matching of IUT donors.

STUDY DESIGN: A retrospective folluw-up study

RESULTS: During an 11-year period, 686 RH and K matched IUTs were performed in 233 pregnancies and in 95% (652/686) post-transfusion antibody testing was performed after a median interval of 21 days. Twenty-five percent (53/212) of the women formed 64 new antibodies and, compared to our previous study, this incidence was not decreased by the use of RH and K matched donors. After delivery 72% (153/212) of the women had multiple RBC antibodies. Additional antibodies were in 48% (31/64) directed against RH and K antigens, induced by the fetus or as natural antibodies. In 52% (33/64) the antibodies were directed against non-RH and K antigens and in 65% (11/17) of eligible cases the IUT donor and not the fetus expressed the corresponding antigen(s).

CONCLUSION: Despite RH and K matching, women treated with IUTs still show strikingly broad red cell alloimmunization. More extensive IUT donor red cell matching including FY, JK and S antigens to reduce the formation of new red cell antibodies should be explored.

INTRODUCTION

In hemolytic disease of the fetus and newborn (HDFN), maternal IgG antibodies against fetal red cell blood antigens cross the placental barrier causing hemolysis, with the risk for severe fetal anemia, hydrops fetalis and intrauterine death. In severe HDFN, intra-uterine transfusions (IUTs) are administered to the fetus to prevent or reverse hydrops fetalis and to prevent fetal death[1]. IUT suppresses the fetal (compensatory) erythropoiesis, and after one or more IUTs most of the fetal blood is of donor origin[2].

Despite D immunization prevention programs, anti-D is still the major cause of severe HDFN[3,4]. Severe HDFN due to other blood group incompatibilities is less common and usually due to antibodies against c and K, or rarely to E, Kidd, Duffy, MS or private antigens[2]. Apart from K-immunization clinical presentation and management is similar to HDFN caused by anti-D[5-10]. Anti-K immunization may cause a different clinical picture and severe disease early in pregnancy due to the destruction of K-positive erythrocyte progenitor cells[8,11-13].

Intra-uterine transfusion with red cells lacking the offending antigen is a life-saving procedure, however the treatment is not without risk. Fetal complications occur in 1 to 3% of the procedures[3,14]. Additional maternal red cell antibodies are often formed and can complicate subsequent pregnancies and future transfusions[15-19].

We previously analysed additional red cell immunization after IUTs in the period 1987 through 1992[17]. Since 1993, as for all high risk transfusion recipients, IUT donors were matched for the D-, C-, c-, E-, and e- and K-antigens.

Here, we present the follow-up regarding formation of IUT induced irregular antibodies from September 1994 through September 2005. This study was undertaken to evaluate the aforementioned transfusion policy.

METHODS

Patients

From the computer database of the Leiden Medical University Center (LUMC), we retrospectively analyzed the records of all women who had received IUTs, during an 11-year period since September 1994. The LUMC is the Dutch national reference center for intrauterine transfusion treatment.

Maternal data collected from the computer database included RBC antigen profiles, RBC antibodies, antibody titers, previous transfusion history, 'pregnancy number', date and number of each IUT and the route of IUT administration (trans- vs, paraplacental). We excluded from the analysis women who were not available for subsequent antibody testing after receiving only one IUT and

women who could not form additional antibodies because their RBCs possessed the corresponding antigens.
When available, the RBC antigen profiles of the fathers and IUT-donors were collected.
Fetal data collected were limited RBC antigen profile, sex, gestational age at initial IUT, and presence of hydrops.

Intra-uterine transfusions
Ultrasound-guided fetal blood sampling, and in case of anemia followed by IUT, was performed as described previously[3], whenever hydrops fetalis was present or fetal anemia was suspected on the basis of MCA Doppler (peak systolic velocity in the middle cerebral artery) and/or ultrasonographic findings (fetal liver length and spleen perimeter)[20-22].
Intra-uterine transfusions were prepared from fresh (<3 days old) leucodepleted (filtered pre-storage), irradiated (25 Gy) and CMV sero-negative RBCs. The supernatant was removed and adjusted to a hematocrit of 80% with 0.9% saline. The donors were D, C, c, E and e compatible with the mother, K-negative, and antigen-negative with maternal antibodies, including a complete crossmatch with maternal serum in the IAGT.

Red cell serology
Maternal, paternal and IUT donor RH, KEL, FY, JK and MSs antigen profiles were determined.
Fetal RH, KEL and JK antigens were determined form a cord blood specimen *prior* to the first transfusion. Duffy and MSs antigens were not determined, due to the very limited volume of fetal cells and the necessity to elute the bound IgG antibodies before testing for these antigens using an indirect antiglobulin (IAGT) test. In case where both maternal and paternal antigens were known, the presence of FY and MSs antigens on fetal RBC were deduced.
Maternal blood samples were tested against a screening panel one day prior to each (subsequent) IUT. In maternal sera we evaluated the development of additional clinically significant RBC antibodies e.g. anti-D, -C, -c, -E, -e, -K, -Fy^a, -Fy^b, -Jk^a, -Jk^b, -M, -S and -s.

Statistics
Statitical software packages (Excel 5.0, Microsoft, Redmont CA; SPSS 11.0, SPSS inc. Chicago, Illinois) were used for data management and analysis. Students T-test and paired T-test (Wilcoxon signed rank test) were used when comparisons between groups were performed. The chi-square or Fisher exact test were used for comparisons of proportions in two groups. We evaluated risk factors for additional antibody formation by univariate analysis. Significant

factors in the univariate analysis as well as factors reported in the literature as risk factors were applied to a multivariate binary logistic regression analysis to determine the variables independently associated with additional antibody formation. Included in the analysis were: transplacental access, total number of IUTs administered, D immunization, number of antibodies at presentation, number of additional antibodies possible, gestational age at first IUT, previous transfusion history, presence of hydrops, maternal age, maternal antibody titer at presentation, fetal gender, and pregnancy number.
Groups were assumed to differ significantly when the probability level was less than 0.05.

RESULTS

Patient characteristics

From September 1994 through September 2005, 241 women were treated with IUTs. Excluded were, 12 women receiving only one IUT and not available for subsequent antibody testing and 17 women not at risk for additional RBC immunization because their RBCs possessed the corresponding antigens. Available for analysis were 212 women (median age, 33 years; range, 19-44 years) who gave birth to 237 children (male to female ratio, 1.1) after 233 pregnancies. There were 4 twin pregnancies and 17 women had successive (2-4) pregnancies. Twenty-nine women, involving 34 pregnancies, had a history of blood tranfusions.
Eight women (3.8%) underwent IUT therapy during their first pregnancy. The antibodies involved were anti-D (n=3), anti-K (n-3) and anti-e and anti-c one each. More than 80% of IUT cases affected the second to fourth pregnancy.
The 212 women possessed 412 antibodies at presentation (Table I). There was no association between pregnancy number and the number of antibodies at presentation. Multiple antibodies, mainly combinations of anti-CD, were present in 139 pregnancies (59%).

Intra-uterine transfusions

In the 233 pregnancies 686 IUTs (median 3; range 1-8 IUTs) were performed. First IUTs were performed at a median gestational age of 28 weeks (range, 17.6-35.6 weeks). The median interval between first and second IUT was 15 days (range, 6-28 days) and the median interval between subsequent transfusions 28 days (range, 5-38 days).
In 135 pregnancies (58%) at least one IUT was administered through transplacental access. A transplacental route of IUT administration was associated with an increase in median D-antibody titer between first and second IUT (two-

thousand respectively four-thousand; p<0.001, Wilcoxon signed rank test), while it did not rise when the paraplacental route was employed (p=0.48).

Table I. Number of antibody specificities in 233 pregnancies with HDFN before the first, during and after the last IUT.

Antibody specificity	Antibodies detectable before the first IUT		Historical antibodies not detectable before the first IUT	Additional antibodies during IUT treatment[1]			Antibodies detectable after IUTs
	Causal antibody of HDFN	Additional antibodies		After IUT 1	After IUT 2	After IUT 3-6	
D	191	0	1	(1)			192
C	0	102	13	13 (2)	4 (2)	4	127
E	1	21	6	6 (1)	(1)	2 (1)	33
c	8	0	0	1			9
e	1	0	0				1
K	32	4	2	1			37
Fy^a	0	3	0	4		1	8
Fy^b	0	1	0	2	1		4
Jk^a	0	9	3	9	3		21
Jk^b	0	0	0	3	2	1	6
M	0	1	1	1	(1)	1	4
S	0	8	2	1 (1)	1	2 (1)	14
s	0	2	0	1			3
Total	233	151	28	42 (5)	11 (4)	11 (2)	459

[1] Number of non-detectable historical antibodies that re-appeared during IUT treatment between brackets

Additional antibodies during IUT treatment

After 652 (95%) of 686 IUTs a serologic screening was performed. In the remaining 34 cases antibody follow-up was not performed after the last IUT.

The median interval between two consecutive antibody tests was 20 days (range, 2-49 days). The median interval was 15 days after the first IUT, 28 days after subsequent IUTs and 18 days after the last IUT.

Twenty-eight women were known to have irregular antibodies in the past, but these were not detectable at presentation for the first IUT (historical antibodies). During IUT therapy, 11 of these 28 historical antibodies re-appeared. These were not classified as new antibodies (Table I). In 4 of 8 eligible cases the corresponding antigens were absent on the fetal RBCs. With regard to the 17 non-emerged antibodies, the corresponding antigens were present on fetal RBCs in 7 of 15 eligible cases.

Table II. Uni- and multivariate analysis of variables associated with the chance on additional antibody formation after IUTs

Variables included in the analysis	P-value univariate analysis	P-value multivariate analysis
Transplacental access	<0.001	<0.001
Total number of IUTs administered	0.003	0.015
D immunization	0.051	0.027[1]
Number of antibodies at presentation	0.065	0.34
Number of additional antibodies possible[2]	0.35	0.036
Gestational age at first IUT	0.30	0.38
Previous transfusion history	0.39	0.60
Presence of hydrops	0.38	0.34
Maternal age	0.46	0.78
Maternal antibody titer at presentation	0.67	0.97
Fetal gender	0.76	0.22
Pregnancy number	0.99	0.76

[1] Assuming that the formation of additional anti-C (n-21) was predominantly associated with D immunization as an independent variable for additional antibody formation, the analysis was also performed after the exclusion of C-antigen and C-antibodies. The results for D changed marginal, p=0.034.

[2] The number of additional antibodies possible for each pregnancy was calculated from the absence of corresponding RBC bloodgroup antigens on maternal RBCs.

Fifty-three women (25%) formed new additional antibodies (Table I) during 54 pregnancies. Five women formed additional antibodies on 2 separate occassions during IUT therapy. The mean immunization rate, expressed as new antibodies per IUT, was 18% after the first IUT and 5.5±1.2% after subsequent IUTs.

The percentage of women with multiple antibodies increased from 59% at admission to 72% after delivery and the number of women with non-Rh/K antibodies increased from 25 (12%) to 52 (25%).

Univariate analysis revealed that the chance of additional antibody formation after IUT was significantly associated with transplacental access (p<0.001) and the total number of IUTs (p<0.003), but not with other variables. When a multivariate analysis was employed, transplacental access (OR, 6.3; 95% CI, 2.5-15.7), D immunization (OR, 2.8; 95% CI, 1.1-6.8), number of additional antibodies possible (OR 1.5; 95% CI, 1.03-2.25) and the total number of IUTs (OR 1.4; 95% CI, 1.07-1.87) were independently associated with additional antibody production (Table II).

Etiology of new additional antibodies

The mothers' extended RBC antigen profile was known in all cases, the fathers' profile in 36% of cases. The fetal RH-antigens were determined in 227 (98%), K-antigen in 218 (94%) and JK-antigens in 200 (86%) cases. RH and K antigens

of the IUT donors were known in all units and in 61% of units for FY, 63% for JK and in 43% for MSs bloodgroups.

RH and K antibodies: In 95 pregnancies additional maternal antibodies could have been formed against 97 RH and 7 K incompatible antigens of the fetus. This actually occurred in 29 pregnancies (31%), with a total of 31 antibodies. In 27 of the 31 cases with additional RH and K system antibodies, the fetus possessed the antigen, but in 4 pregnancies, antibodies were formed although both the IUT donors and the children did not carry the corresponding antigens (Table III). Alternatively, 5 D-negative and 7 K-negative women did not form additional antibodies against the corresponding antigens, despite the antigens being present on the fetal cells. Two of these women responded with the formation of additional antibodies against other RBC antigens.

Table III. Etiology of additional maternal antibodies after IUT

Antibody specificity	Antigen presence on donor RBCs	Antigen presence on fetal RBCs	Number of cases	Causative RBCs
C	neg	pos	21	Fetal
E	neg	pos	5	Fetal
	neg	neg	3	None implicated
c	neg	pos	1	Fetal
K	neg	neg	1	None implicated
Fy^a	pos	neg	2	Donor
	pos	n.d.	1	Donor possible
	neg	n.d.	1	Fetus probable
	n.d.	n.d.	1	Inconclusive
Fy^b	pos	neg	2	Donor
	n.d.	n.d.	1	Inconclusive
Jk^a	pos	neg	4	Donor
	pos	pos	4	Both possible
	pos	n.d.	1	Donor possible
	n.d.	pos	3	Fetus possible
Jk^b	pos	neg	2	Donor
	pos	pos	2	Both possible
	n.d.	pos	2	Fetus possible
M	pos	n.d.	1	Donor possible
	n.d.	n.d.	1	Inconclusive
S	pos	neg	1	Donor
	pos	n.d.	1	Donor possible
	n.d.	n.d.	2	Inconclusive
s	n.d.	n.d.	1	Inconclusive

n.d., not determined

Non RH and K antibodies: After IUT therapy 33 additional antibodies were formed. In 17 cases both the fetal and donor RBC antigen profiles were known; in 11 of 17 cases (65%) the offending antigens were present in the donor and not in the fetus. The etiology could not be determined with certainty in 6 cases because both the donor and the fetus carried the antigens and in 16 cases because data regarding the corresponding antigens on fetal cells and/or donor cells were not available (Table III). In one of these cases, an additional anti-Fy^a was formed after the first IUT, despite the IUT donor lacked the Fy^a-antigen.

COMMENT

Since 1987 our hospital performs intra-uterine intravascular erythrocyte transfusions (IUT) for HDFN as the single national center in the Netherlands, with a population of 17 million and approximately $2x10^5$ births annually. In our previous study, concerning 280 IUTs in 91 pregnancies in the period 1987-1992, we reported that the formation of additional antibodies following one or more IUTs was 26% [17]. In 1993, we have adopted a new preventive program concerning RH and K antigen matching for recipients with a high risk for alloimmunization. In this retrospective study in patients receiving IUTs, we evaluated an 11-year period after introduction of this practice besides matching for the antigens involved in HDFN. Despite our new policy the percentage of new cases of alloimmunization remains 25% of women treated with IUT. RH and K matching of the IUT donor did not prevent the formation of antibodies against these antigens in 31% of pregnancies at risk.

In 233 pregnancies with 686 IUTs, 53 of the 212 (25%) women formed new additional RBC antibodies. Of these additional antibodies, 48% were directed against RH and K antigens. The number of women with non-RH and -K antibodies increased from 25 (12%) to 52 (25%), predominantly due to Kidd antibodies. In 11 out of 17 (65%) informative cases, the IUT donor and not the fetus carried the immunizing antigens.

After delivery, multiple antibodies were present in 72% of the women. However, since antibody screening after the last IUT was performed after a median of 18 days and not at all in 34 (15%) cases, the real figure is likely higher, considering that from the second IUT onwards, the immunization rate did not decline between subsequent IUTs.

Additional antibodies are formed by fetomaternal hemorrhage (FMH). FMH is common after transplacental puncture [23]. Although in our previous study we could not find a significant difference in additional antibody formation among women who had undergone transplacental puncture and among those who had not [17], the current study confirms the results from others that transplacental puncture is associated with enhanced antibody response [16,24].

Additional antibodies may have been induced by fetal or donor RBC antigens. The additional RH and K antibodies are obvious of fetal origin, or in incidental cases these antibodies occurred without an immunizing event as natural antibodies. The rise in anti-D titer, the re-appearance of historical antibodies as well as the formation of additional antibodies all occur predominantly after the first IUT, which has a three times higher immunization rate than subsequent IUTs (18.0 versus 5.5%). At one hand this can be explained by boostering of an immune response by fetal cells, and on the other hand by the fact that during the first IUT, there is actually exposure to RBCs from two donors, i.e. IUT and fetal RBCs. With subsequent IUTs fetal RBCs are virtually absent [2]. Although very small amounts of (fetal) cells can boost antibodies, it is questionable whether these cells can induce primary immunization, because these small amounts of incompatible, IgG sensitized cells, will almost immediately be removed by splenic macrophages [25].

Donor RBCs in this study were RH-compatible, K-negative and compatible with the maternal antibodies. These RBCs will survive normally and, despite the small dose, apparently are capable of initiating primary antibody production as was seen in 65% of eligible non-RH/K antibody cases.

In pregnancy there exists a shift of the Th1/Th2 balance towards a T-helper cell type 2 (Th2) favouring humoral immunity [26]. This may not only explain the fast new antibody formation, upon small amounts of FMH, but also the boost of historical antibodies in the absence of the corresponding antigen, or of natural antibodies (bystander effect) as seen in 4 cases in our study.

The development of additional antibodies potentially increases the risk for the mother and the current and future pregnancies and can complicate further transfusion therapy. Additionally, antibodies may become undetectable over time [27], posing the mother at risk for delayed hemolytic transfusion reactions in case of transfusions later in life.

Women who received IUTs represent the highest red cell multi-alloimmunized population, with at least 72% possessing antibodies against multiple red cell antigens.

In conclusion, we found that additional antibody formation during IUT therapy is common, associated with transplacental puncture increasing FMH and not reduced, despite RH and K antigen matching of IUT donors. Although in 17 of 33 immunizations against non-RH/K antigens the RBC antigen profiles of both donor and fetus were available, in 65% of FY, JK and S antibodies the fetus lacked the antigen.

We presume that donor antigens may play a major role even if the antigens are also expressed on fetal RBCs, because fetal RBCs are promptly removed by maternal antibodies and the virtual absence of fetal red blood cell production after 1-2 IUTs.

To reduce this high rate of alloimmunization, enhancing the risk for complications during pregnancies and maternal transfusions, we propose to study RBC immunization after IUTs, not only matched for RH and K, but also for JK, FY and S antigens, which are, following RH- and K-antigens, known to be the most immunogenic antigens. Prospective studies are needed to investigate the effects of employing this policy and will clarify the nature of additional RBC immunization during IUT treatment.

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8

RBC ANTIBODY PERSISTENCE

Henk Schonewille, Hans L. Haak and Annette M. van Zijl. RBC antibody persistence. Transfusion 2000;40:1127-1131

ABSTRACT

BACKGROUND: A person exposed to foreign blood group antigens may produce antibodies. The persistence of antibodies varies among people and among antibodies. A study was performed to investigate the persistence of clinically significant RBC alloantibodies over a period of 20 years.

STUDY DESIGN AND METHODS: A retrospective examination was performed of all records of RBC antibodies in the transfusion laboratory computer database from 1978 through 1997. Records of patients who underwent at least one antibody investigation after an antibody had been detected were studied. The study included all antibodies against the RH, KEL, FY, JK and MNS blood group systems. An antibody was regarded as not persistent if, after previous detection, the screening or panel studies became negative for the antibody under study. Anti-D due to RhIg administration was excluded.

RESULTS: An analysis was performed of 480 records consisting of 593 antibodies that fulfilled the criteria. Median antibody follow-up was 10 months (range, 1-240). In 137 patients, 153 (26%) antibodies became undetectable over the course of time. After initial negative screening investigations, 312 antibodies were formed. The antibodies that were still detectable had a median follow-up of 7 months (range, 1-193). A patient's age, sex, and antibody specificity were of no influence on the length of time that antibodies were detectable. Antibodies detected with a more sensitive screening technique were less persistent ($p=0.0002$). For 28 patients, detection of antibodies was highly irregular.

CONCLUSIONS: About 25 percent of all antibodies became undetectable over the course of time. The antibody screening technique used, rather than the antibody specificity, affected these results. To prevent delayed hemolytic transfusion reactions, precise antibody documentation is of great importance.

INTRODUCTION

A person exposed to foreign blood group antigens through transfusion, pregnancy, or tissue transplantation may produce alloantibodies. Pretransfusion RBC compatibility testing, including the serologic history, is performed to detect clinically significant alloantibodies that are or were present in the serum of potential transfusion recipients. The persistence of antibodies at a serologically detectable level varies among people and among antibodies[1-8]. Antibody immunoglobin type and quantity, and sensitivity of the screening method are the major factors that determine pretransfusion detection.

Once an antibody has been detected, good documentation and the patient's and doctor's awareness of the presence of the antibody are essential to avoid incompatible blood transfusions, especially when these patients are treated in various hospitals, as a result of hospital specialization. In the event of a previously identified clinically significant alloantibody, blood lacking the relevant antigens should be selected for transfusion. This should be done even if the current antibody detection test is negative, to avoid delayed hemolytic transfusion reactions (HTRs).

The frequency of delayed HTR varies considerably, depending on the criteria of diagnosis. The impact of delayed HTR on morbidity and mortality is not well-known and probably depends on the antibody involved. Because they are often mild, self-limiting reactions, many delayed HTRs resolve undetected; however some result in additional transfusions to alleviate anemia.

We investigated retrospectively the persistence of clinically significant, RBC alloantibodies over a period of 20 years.

MATERIALS AND METHODS

We examined retrospectively records on warm-reacting RBC antibodies in the transfusion laboratory computer database of Leyenburg Hospital (a 600-bed, multidisciplin-ary teaching hospital in The Hague, the Netherlands) during a 20-year period, from 1978 through 1997. Records of patients who underwent at least one antibody investigation after an initial antibody had been detected were studied.

The study included all antibodies against the RH, KEL, FY, JK, and MNS blood group systems. When anti-D was discovered in women of child-bearing age, we asked the treating physician if RhIg had been administered recently. If so, we excluded the anti-D from our study. Information collected included the patient's age and sex and the dates and results of tests for RBC antibodies. No detailed

information on pregnancies and antibodies detected in other hospitals was available. An antibody was regarded as not persistent if, after previous detection, the screening or panel studies became negative for that antibody on one or more subsequent occasions.

Blood samples from successive patients submitted to the blood transfusion laboratory from January 1978 through December 1997 were screened for RBC alloantibodies by use of a selected 2-cell (1978 through 1986) or 3-cell (1987 through 1997) set of reagent RBCs for antibody detection. The first technique for antibody detection consisted of 100 μL of serum and 50 μL of 5-percent RBCs (serum: cell ratio, 40:1) to perform an indirect antiglobulin (IAT) tube test including readings at saline and albumin 37°C phases (1978 through 1990); later, 25 μL of serum and 50 μL of 0.8-percent RBCs (serum: cell ratio, 63:1) were used in a LISS gel test (DiaMed AG, Bonaduz, Switzerland) (1991 through 1997).

New antibody identification or reconfirmation of previous antibodies was accomplished with commercial panels of RBCs obtained by means of similar methods or additional techniques (e.g., PEG, enzyme) when needed. If the specificity could not be determined clearly, the blood sample was sent to a reference laboratory for further analysis. Antibody titers were not investigated routinely.

We evaluated the reactive antibody screening for each new sample for which antibody testing was requested. The results of both antibody screening and antibody identification were valid for 72 hours; in case of transfusion, a fresh blood sample was then tested. Data on antibody identification were kept on file in writing as well as in the computer database.

Crossmatching, including an IAT, was performed routinely from 1978 to 1990; as of 1991, the type-and-screen policy was applied for all patients until antibody formation. After an antibody had been identified, RBC products that were negative for the corresponding antigen were transfused after complete crossmatching, including an IAT. Antibody survival studies were performed on antibodies that were formed after initial negative screening in our laboratory.

Statistics

Statistical software packages (Excel 5.0, Microsoft, Redmond, WA; NCSS 6.0, Jerry L. Hintze, PhD, Kaysville, UT) were used for data management and analysis, respectively. The Kaplan-Meier product limit estimator was used to calculate the rate at which antibodies became undetectable. We used Cox's proportional-hazard regression method to study the impact of factors on antibody detectability in time. The log rank test (nonparametric) was used to compare antibody persistence. The chi-square test was used to compare the incidence of

antibody undetectability between groups. Groups were assumed to differ significantly when the probability level was less than 0.05.

RESULTS

The records of 1291 patients with RBC antibodies were evaluated; 1066 records showed the patients had the antibodies listed above, and 480 of these records (female: male ratio, 2.7) fulfilled the criteria. Median patient age at the time of antibody detection was 66 years (mean, 62; range, 19-97). Patient sera were screened for antibodies a median of three times (mean, 3.9; range, 2-92).
We identified 593 antibodies; 89 percent were detected in more than one sample. One hundred fifty-three antibodies (26%) in 137 patients (29%) became undetectable over the course of time (Table 1). Of these, 55 percent were undetectable in more than one consecutive specimen. In the period 1978 through 1990, 23 percent of the antibodies became undetectable; in the period 1991 through 1997, 36 percent became undetectable (p=0.012). This increase was not related to a specific antibody.

Table 1. Antibody specificity and detectability over the course of time (all antibodies)

Antibody specificity	Absolute number	Percentage of all antibodies	Undetectable over the course ot time	Percentage of this antibody
K	166	28	48	29
E	136	23	52	38
D	118	20	15	13
Fy^a	55	9	7	13
C	49	8	9	18
c	28	5	9	32
Jk^a	17	3	6	35
S	10	2	1	10
Jk^b	6	1	4	67
e	5	1	1	20
Fy^b	2	<1	0	0
s	1	<1	1	100

Antibody follow-up from 1978 through 1990 lasted a median of 6 months (mean, 24; range, 1-143), and that from 1991 through 1997 lasted a median of 5 months (mean, 13; range, 1-84).
Three hundred twelve antibodies (53%) were formed after initial negative screening studies, whereas 281 antibodies were already present when the first screening was performed in our laboratory. Of the new antibodies, 108 (35%)

became undetectable. The antibodies that were still detectable had a median follow-up of 7 months (mean, 24; range, 1-193). The duration of detectability is presented according to antibody specificity in Table 2.

Table 2. Detectability over the course of time of antibodies acquired during study

Antibody specificity	Number	Time (months)			Percentage undetectable
		Mean	Median	Range	
e	3	39	39	1-76	33
D	17	58	34	1-193	47
S	10	45	23	1-182	10
c	22	13	8	1-60	32
E	91	20	7	1-182	42
K	108	25	6	1-188	34
Fy^a	28	24	6	1-162	14
C	16	18	6	1-73	25
Jk^b	5	9	3	1-26	30
Jk^a	12	10	1	1-62	33

A Kaplan-Meier estimate of the cumulative probability function for all RBC antibodies formed during the study illustrates the relationship between the type of antibody and the length of time it was detectable (Figs. 1 and 2). Cox's proportional-hazard regression function showed that age, sex, and antibody specificity had no influence on the length of time that antibodies were detectable. When the detectabilities of various antibody types were compared, the log rank test revealed no significant differences between the rates at which different antibodies became undetectable.

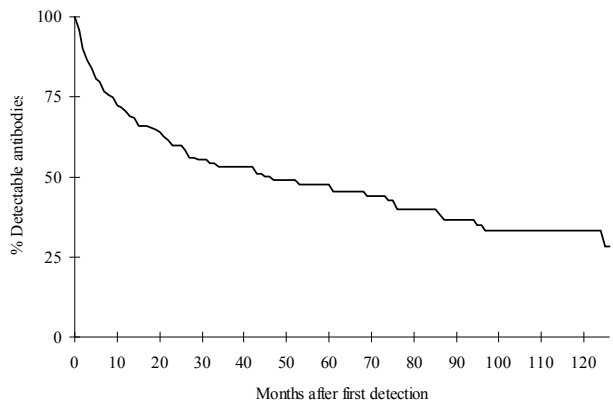


fig. 1. Antibody persistence (all antibodies, n=312)

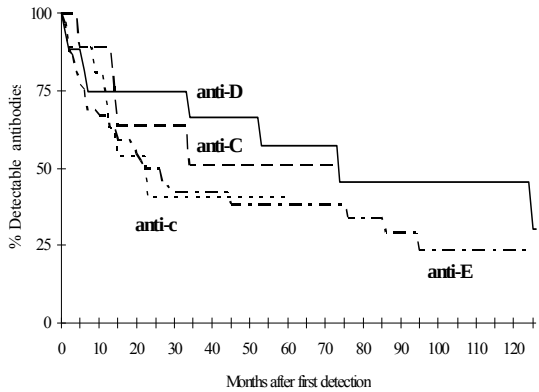
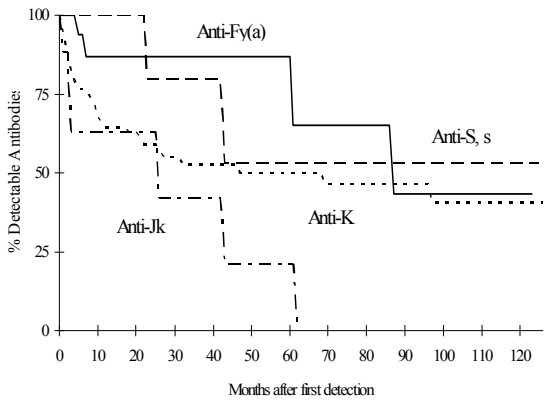


Fig. 2A [upper figure]) RH system antibody persistence: anti-D, n = 17; anti-C, n = 16; anti-E n = 91; anti-c, n = 22. Fig. 2B [figure below]) Non-RH system antibody persistence: anti-K, n = 108; anti-S, n = 10; anti-Jk^a and Jk^b*, n = 17; and anti-Fy^a, n= 28. *Because of small numbers of Jk^a and Jk^b antibodies (table 2), they were lumped together.



RBC antibodies detected by the tube albumin-IAT method persisted longer than those found by the column LISS-IAT technique (Fig. 3, p=0.0002). If we consider all D-antibodies (RhIg cases excluded), then those that were already present when the patients entered the study (n=101) seemed to persist longer than

newly formed ones (n=17) (Fig. 4, p=0.000036). This difference could not be demonstrated for other antibodies.

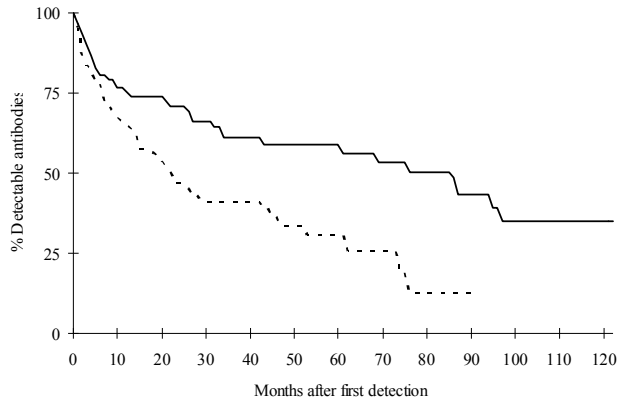


Fig 3. Antibody persistence versus detection technique: albumin, tube test , —— (n = 127) ; and LISS, column test, - - - - (n = 179). p=0.0002

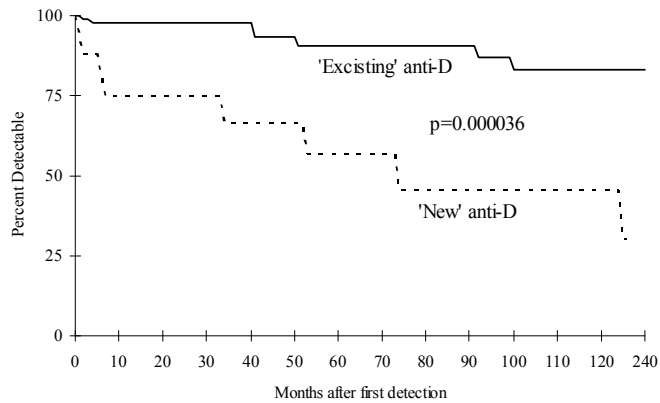


Fig. 4. D-antibody persistence: anti-D present at first examination, —— (n = 101); and anti-D developed during study, - - - - , (n = 17).

For 28 patients, antibody detection varied: 30 antibodies (14 anti-E, 11 anti-K, 2 anti-D, and 1 each anti-C, -c, and -S) sometimes could not be detected, but at other times, they were detected. Two patients exhibited a highly variable antibody response (Table 3). Patient R, a male born in 1947, and his sister HR, born in 1962, both had sickle cell anemia and had received numerous (exchange) transfusions from 1981 and 1982, respectively.

Table 3. Two patients with sickle anemia

Patient HR		Patient R	
Period*	Antibody	Period	Antibody
02/81-09/83	None	08/82-08/89	None
10/83-11/83	K	10/89	K
12/83-04/84	None	12/89-08/93	None
05/84-12/84	K	10/93-12/93	E
09/85-12/92	None	02/94	None
01/93	K	03/94	E
02/93	None	05/94	None
03/93	E	06/94	E
05/93-07/93	None	07/94-08/94	None
08/93-02/94	E	11/94-05/96	E
04/94	None		
05/94-08/94	E		
09/94	None		
10/94-11/94	E		
01/95-07/95	K		
09/95-01/96	E + K		
02/96-04/96	None		
06/96	K		
07/96-01/97	None		
02/97	K		
03/97-04-98	None		
07/98	K		

* Month/Year

DISCUSSION

Because of hospital specialization, patients are not always treated in the same hospital. Awareness of existing antibodies may not be reported from one hospital to another; in addition, because RBC alloantibodies can become undetectable over time[1-6], the risk of delayed HTRs due to the transfusion of incompatible blood increases.

This retrospective study was designed to discover the frequency and rate at which RBC alloantibodies become undetectable. We found that 26 percent of RBC alloantibodies were not discovered on one or more occasions after their first detection. New antibodies were still present after a median of 7 months. The data, although not statistically significant, varied considerably between antibodies. A patient's age, sex, and type of antibody had no impact on the rate at which antibodies became undetectable. Our results are comparable to those of others[3-6] as far as the overall undetectability of antibodies is concerned. There were differences in the nondetectability of anti-C (this study, 18%; Ramsey, 54%) and anti-Fy^a (this study, 13%; Rosse, 47%).

Antibodies discovered by means of the tube albumin IAT were more persistent than those found with the column LISS IAT. It is known that antibody uptake by reagent RBCs is much faster at low ionic strength than at normal ionic strength[9]. Thus, antibodies at low titers can also be detected. From 1990 to 1991, the incidence of clinically significant antibodies increased 1.5-fold in our laboratory (unpublished observations). It is also conceivable that antibodies at low titers become undetectable sooner than high-titer antibodies. This could explain why antibodies discovered by a more sensitive technique disappeared earlier and more frequently. We found that Kidd antibodies are detectable for the shortest time. It is well known that the in vitro detection of Kidd antibodies is very difficult and that they frequently (30-60%) play a role in delayed HTRs[10].

When we compared the persistence of existing D antibodies, most probably made after pregnancy, to that of new D antibodies formed after incompatible RBC and/or platelet transfusions, we found a significant difference. This difference might be explained by the fact that in former years women were exposed through pregnancy to D-antigen for a long period (months) and repeatedly (multiple pregnancies, which enhance secondary immune responses), which resulted in very high titers of anti-D. In contrast, an incompatible transfusion is a short, one-time event that may give rise to lower titer anti-D. On the other hand, because the women with anti-D (median age, 59 years) were many years past childbearing, it is possible that, in many women who had anti-D from pregnancy, the antibodies had become undetectable before the women's entry into our study. Only in those women who had a particularly high-titered anti-D did the antibody last until they entered the study population.

For some patients, predominantly those with hematologic disorders, antibody detectability varied. Most of these patients suffered from dysfunctioning of the immune system due to chemotherapy, multiple blood transfusions, or the disease itself. Issitt and Anstee[10] reported that patients with sickle cell anemia make an antibody that is detected for a short time, after which other antibodies are formed, each also detectable for a short time. In the study of Rubenstein et al.[11], it was

shown that the titer of anti-D exhibits cyclical variations, with differences of up to 30 percent. This could also be the case for other antibodies, and the lower levels could result in undetectability.

In view of the observations reported in this and other studies[10] of RBC antibody incidence and detectability, a large prospective study with sensitive screening methods and well-defined patient populations should be undertaken to interpret the differences found. The results of that study could have an impact on the immunogenicity of blood group antigens, as calculated by Giblett[12].

In this study, 18 to 30 percent of all antibodies became undetectable over the course of time, depending on the sensitivity of the antibody detection technique used rather than the antibody specificity. To prevent delayed HTRs due to the transfusion of incompatible blood, accurate blood bank records and precise patient files are of the utmost importance.

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9

RED BLOOD CELL ALLOANTIBODIES AFTER TRANSFUSION: FACTORS INFLUENCING INCIDENCE AND SPECIFICITY

Schonewille H, van de Watering LM, Loomans DS, Brand A. Red blood cell alloantibodies after transfusion: factors influencing incidence and specificity. Transfusion. 2006;46:250-6.

SUMMARY

BACKGROUND: Alloimmunization after exposure to red cell (RBC) alloantigens depends on genetic and acquired patient-related factors, dose and route of administration and the immunogenicity of the antigen, but exact kinetics are still unknown.
STUDY DESIGN AND METHODS: A 5-year retrospective multicenter study analyzing factors influencing the rate and specificity of RBC alloimmunization was performed, with special emphasis on the time interval between transfusion event and antibody detection. Included were clinically significant alloantibodies against the RH, KEL, FY, JK and MNS blood group systems.
RESULTS: Multivariate analysis involving 1710 immunized patients revealed that time interval between transfusion and antibody tests was strongly associated with the antibody specificity. Anti-Jk^a and anti-Jk^b were predominantly found in patients tested within 3 months, whereas anti-K and anti-Fy^a were the most encountered antibodies at more than 5 years after transfusion. Of all immunized patients, new antibodies were detected within 14 days after transfusion in 299 patients (16.8%) and in 1479 patients (83.2%) after more than 14 days.
Fifty percent of transfusion recipients were retested for alloimmunization because of a new transfusion indication. Eleven of 2932 patients (0.4%) retested up to 3 days after transfusion had formed new antibodies.
CONCLUSION: The time interval between transfusion and antibody test was associated with RBC antibody specificity. Because RBC antibody tests after transfusion are not routinely performed, many antibodies may (not) be detected at the time of a new transfusion event, posing the transfusion recipient at risk for transfusion delay or a (delayed) hemolytic transfusion reaction. Routine RBC antibody screening at set time intervals following transfusion would reduce these risks.

INTRODUCTION

Individuals exposed to red blood cell (RBC) alloantigens through transfusion, pregnancy or transplantation may produce antibodies. Whether the recipient's immune system will react depends on genetic or acquired patient-related factors, dose and route of administration and the immunogenicity of the antigen. Immunogenicity is defined as the ability of a given antigen to stimulate antibody production in a patient lacking the antigen. The D antigen is the most immunogenic. After transfusion in immunocompetent patients more than 80 percent of the recipients will ultimately form anti-D, although this incidence is much lower in immunocomprimised patients[1-3]. Because it is common practice to prophylactically match RBC transfusions for the D-negative status, anti-D formation as a consequence of blood transfusion is rare. Such routine is not common to prevent other RBC alloimmunization, for which we rely on serologic screening before transfusion. The increasing number of multi-transfused patients raises the question whether this is a safe approach.

For instance, delayed hemolytic transfusion reactions were reported in 72 patients over a 2-year period in the 2002 and 2003 annual Serious Hazards of Transfusion Reports (SHOT)[4,5]. The authors expressed their concern regarding underrecognition.

The relative immunogenicity of blood group antigens is estimated from the antibody frequency. Giblett[6] calculated the relative immunogenicity for a number of RBC antigens compared to the K antigen. She compared the frequencies in which particular antibodies are encountered with the calculated probability of exposure. Based on her calculations, the relative likelihoods of non-D blood group antibody formation are K (0.05) > c (0.0205) > E (0.0169) > Fy^a (0.0023) > Jk^a (0.0007).

We recently performed a large retrospective multicenter study in order to compare alloimmunization against clinically significant alloantigens before and after universal leucodepletion, which turned out not to be different[7]. Here we report our investigation in this cohort on factors relevant for immunization and specificities of antibodies, with special emphasis on the time interval between transfusion event and antibody testing.

MATERIALS AND METHODS

We examined retrospectively the records of all patients with newly detected RBC antibodies by searching the computer databases from 19 hospitals within the

Dutch Sanquin Bloodbank Southwest region during a 5-year period from 1999 through 2003. The study included all clinically significant alloantibodies against the RH, KEL, FY, JK and MNS blood group systems. Autoantibodies, D antibodies and antibodies to low- and high-frequency antigens were excluded. Only patients of whom the transfusion history was known were included in the study. Data collected included age, sex, transfusion history, and the interval between transfusion and antibody screening. The clinical indications for the immunizing transfusions were not available.
The hospital laboratories used a poluethylene glycol enhancement tube test or a low-ionic-strenght saline enhancement column technique, both of comparable sensitivity for antibody detection and cross-matching.
The relative potency of antigens, compared to K-antigen, was determined with Gibletts equation:

$$\frac{\text{Number of antibodies of interest}}{\text{Number of K-antibodies}} \times \frac{\text{Probability of exposure to K-antigen}}{\text{Probability of exposure to antigen of interest}}$$

Antibody investigation was performed in case a patient was referred for a transfusion indication. The routine pre-transfusion antibody screening practice was studied by investigating the interval between transfusion and antibody screening tests in all transfused patients during the period 1999 through 2003 in one of the two University Hospitals in our region.

Statistical analysis
Statistical software packages (Excel and Access, Microsoft, Redmond, WA; SPSS 11.0, SPSS Inc. Chicago, IL) were used for data management and analysis, respectively. The chi-square or Fisher exact test was used for comparisons of proportions in two groups. Multivariate analysis was used to determine factors that were independently associated with antibody specificity.
Groups were assumed to differ significantly when the probability level was less than 0.05.

RESULTS

Patient characteristics

New antibody specificities (n=2177, Table 1) were found in 1778 patients (female-to-male ratio 2.2; mean age 64 years; range, 1-101 years), including 83 patients (4.7%) with antibodies before entering the study. Multiple new antibody specificities (2-5) were seen in 284 patients (16.0%).

Patients had received 12,379 RBC units (mean 7 units; median 4 units; range, 1-116 units) transfused in 4,050 transfusion events (mean, 2.3 transfusion events; median, 1 transfusion event; range, 1-50 transfusion events). Patients with multiple antibodies had received slightly more transfusions than patients with one antibody specificity (mean, 8.0 units vs. 6.7 units; p=0.018).

Table 1. Antibody specificity and frequency in 1778 alloimmunized patients

Antibody specificity	Frequency	
	Number	% of total
E*	740	34.0
K	541	24.9
Fy^a	195	9.0
c	186	8.5
Jk^a	173	7.9
C*	138	6.3
Jk^b	54	2.5
S	50	2.3
M	45	2.1
e	28	1.3
Fy^b	19	0.9
s	7	0.3
k	1	0.05
Total	2177	

* anti-E including anti-DE (n=14) and anti-C including anti CD (n=52).

Factors associated with antibody specificity

Multivariate analysis (Table 2) revealed that anti-C presence was higher in patients with older age, but when anti-CD was excluded this relation disappeared. Anti-M and anti-S were identified more frequently in younger patients. Anti-Jk^a was seen predominantly in male patients and after a few units transfused, while anti-K was more frequent in multi-transfused patients.

Table 2. Multivariate analysis of factors independently associated with antibody specificity

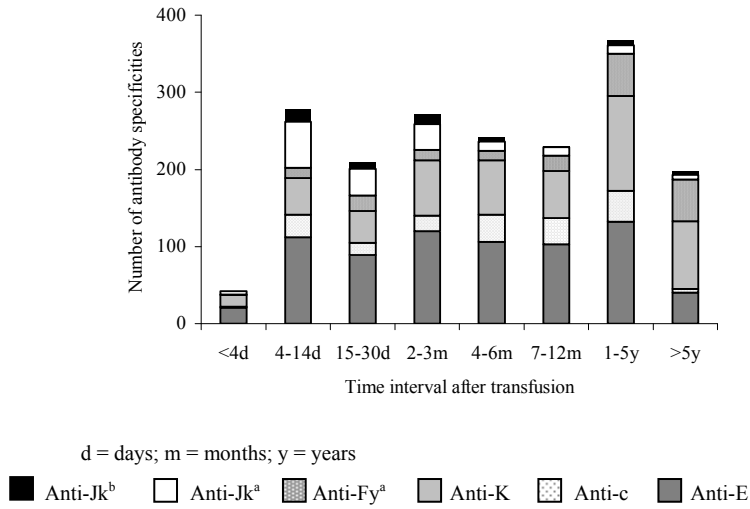
Antibody specificity	P-value of factors included in the multivariate logistic analysis			
	Sex	Age (years)	Interval (months)*	Units transfused
C (+CD)	n.s.	0.035	n.s.	n.s.
C (minus CD)	n.s.	n.s.	0.095	n.s.
E	n.s.	n.s.	<0.001	n.s.
c	n.s.	n.s.	0.017	n.s.
e	n.s.	n.s.	0.086	n.s.
K	n.s.	n.s.	<0.001	0.019
Fy^a	n.s.	n.s.	<0.001	n.s.
Jk^a	0.022	n.s.	<0.001	0.026
Jk^b	0.077	n.s.	0.037	n.s.
M	0.087	0.029	n.s.	n.s.
S	n.s.	0.009	n.s.	n.s.

n.s.: non significant p>0.1; * time interval between transfusion and antibody test; Not included in the table are antibodies with p>0.1 for all factors included in the multivariate analysis (anti-k, anti-Fyb and anti-s).

Interestingly, the interval between transfusion and antibody tests was associated with particular antibody specificities. Anti-E was the most encountered antibody up to 5 year following transfusion, but from 12 months onward declined from 45 to 20 percent of encountered antibodies persisting after more than 5 years. Anti-c presented 10 to 15 percent of antibodies found up to 5 years after transfusion, after which period it decreased to less than 3 percent. Anti-K showed a gradual increase from 20 percent after 1 month to 45 percent after more than 5 years after transfusion. Anti-Fy^a rapidly increased from less than 10 percent after 1 year to 27 percent of antibodies found after more than 5 years. Anti-Jk^a and anti-Jk^b were predominantly found in patients tested during the first 3 months after transfusion; thereafter they decreased from, respectively, 18 and 5 percent to, respectively, 3 and less than 2 percent (Fig. 1).

The type of RBC bloodproduct transfused (leucoreduction by buffycoat depletion vs. prestorage filtration) did not influence the results regarding antibody incidence and specificity, neither was there a relation of the function of interval between transfusion and specific antibody detection.

Fig. 1 Antibody number and specificity in relation to the time interval between transfusion and antibody testing.



Because most E, c, K, Fy^a and Jk^a antibodies (n=936) are formed within 6 months after transfusion, we calculated after this period the immunogenicity relative to the K antigen. This revealed that the K antigen was 1.3 times more immunogenic than the E antigen, 3.5 times more than the Jk^a antigen, 4.9 times more than the c antigen and 11.6 times more immunogenic than the Fy^a antigen. When these antibodies are taken into account when detection tests were performed after a longer period than 6 months (n=787), anti-K was 2.5 times more immunogenic than E antigen, 22.4 times more than Jk^a antigen, 6.5 times more than c antigen, and 5.8 times more immunogenic than Fy^a antigen. Figure 2 depicts the immunogenicity results, showing a considerable variation with time interval.

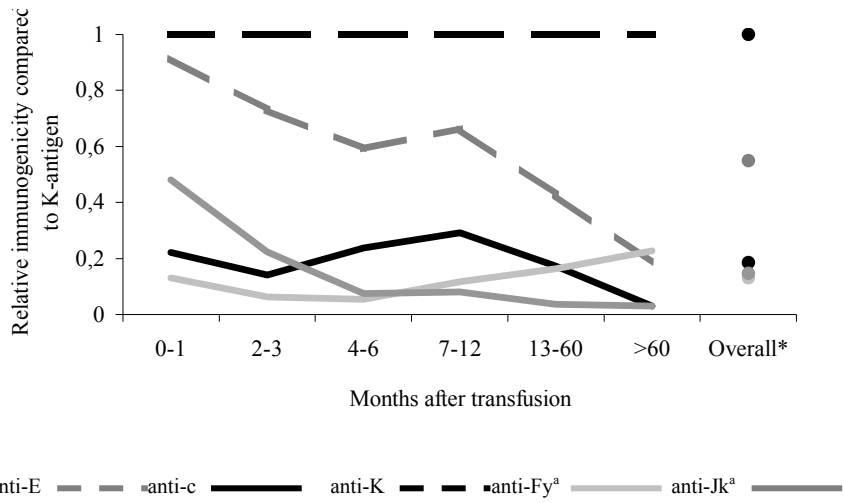
RBC alloimmunization suggesting a secondary immune respons

Of all patients who developed alloantibodies, 299 patients (16.8%) did so within 14 days after transfusion. The blood group specificities involved were RH (n=180; predominantly anti-E, n=128), JK (n=80), K (n=60), MSs (n=21) and FY (n=16). Forty-six antibodies, predominantly anti-E and anti-K (n=21 and n=14, respectively), were found within 3 days post-transfusion in 41 patients (2.3% of all immunized patients).

Comparison of antibody frequency within 14 days versus more than 14 days after transfusion, showed that anti-Jk^a and anti-Jk^b were more often involved within the short interval after transfusion ($p<0.0001$ and $p=0.016$, respectively), while anti-

K and anti-Fy^a were present more often after more than 14 days (p=0.0003 and p=0.0007, respectively).

Fig. 2. Relative potency of E, c, Fy^a and Jk^a antigens to K antigen as a function of time interval between transfusion and antibody test.



* Overall immunogenicity relative to K-antigen (= 0.05): E-antigen = 0.0275; c-antigen = 0.0093; Fy^a-antigen = 0.0066 and Jk^a-antigen = 0.0073

Routine antibody screening practice

Routine antibody screening practice was studied in one of the two participating university hospitals. The interval between transfusion and antibody screening tests in *all* transfused patients during a 5-year period form 1999 through 2003 was investigated.

During this period, 16,126 patients received transfusions. Antibody screens were performed 1 to 2 days before scheduled transfusion in 20 percent and on the day of transfusion in 80 percent. Almost 50 percent of patients (n=8004) were retested for alloimmunization because of a new transfusion indication, resulting in 165 new antibodies in 150 patients. Twelve new antibodies (6x anti-E, 5x anti-K, 1x anti-Jk^a) were found in 11 of 2,932 patients (0.4%) who were retested up to 3 days after transfusion and 7 antibodies (4x anti-E, 2x anti-K and 1x anti-Jk^a) in 7 of 2,386 patients (0.3%) if testing was performed up to 2 days after transfusion.

DISCUSSION

In this large multicenter retrospective study we investigated factors that could influence antibody specificity and found that age, gender, number of transfusions and as most important factor the time interval between transfusion and performance of antibody detection test were independent significant factors for antibody specificity. We did not include genetic or acquired patient related factors in our analysis; therefore our reported associations may also be due to other variables. It has been published that disease and/or treatment are important factors with respect to RBC antibody responses[2,11-14,21,23,28]. The role of the time interval after transfusion before specific antibody detection was never addressed before and only one study reports on the association of disease and antibody specificity[8].
To our knowledge, this is the first study investigating factors in a multivariate analysis. Most studies performed univariate analysis of alloantibody formation, regardless of antibody specificity. Discordant results regarding the influence of age, gender and number of transfusions or transfusion events have been reported[9-14]. In our analysis, gender was correlated with anti-Jk^a, age with anti-M and anti-S, and the number of transfusions with anti-K and anti-Jk^a. Previous studies also showed the higher prevalence of anti-M in younger patients[9,15].
We could not confirm the observations by Winters and coworkers[9] regarding older age with the presence of anti-K and anti-Fy^a, but results concerning increasing age with anti-C (including anti-D) were in agreement. The latter probably reflects CD immunization through pregnancy and the close pair clustering of anti-D and anti-C[9]. The differences found are probably explained by the study design. Except for differences in the study population and in the number of the same antibodies studied (806 vs. 2177 in our series), Winters performed an univariate analysis on RBC antibodies during a 20-year period without information on transfusion history, while we multivariately analyzed RBC alloimmunized patients, including the transfusion history.
The relation between higher number of units transfused and anti-K development can be explained by the chance of K alloantigenic exposure. After 4 units of RBCs were transfused (median number in our study), K-antigen exposure was much less compared to the exposure of all other relevant antigens (K 0.31 vs. 0.74 for E and >0.96 for other RH, FY, JK and MS). The chance of exposure (Exp) per antigen was calculated using the formula:

$$Exp = 1-[\text{chance of matched RBC exposure}]^{\text{median number of units transfused}}.$$

Relatively little is known about the kinetics of red cell alloimmunization within the first months after transfusion. Some information comes from studies that address (delayed) hemolytic transfusion reactions[16-19], but most studies report on antibodies found after a longer period of time[20-23] after transfusion or even regardless the transfusion history[9,24].

RBC alloimmunization investigations are in most cases only performed before a new transfusion event and not after a fixed time following antigenic challenge. Consequently, many antibodies may not be discovered, either because there is no new transfusion indication or because antibodies have disappeared. In one of the two university hospitals in our study, not necessarily representative for all hospitals, owing to the relative predominance of complicated patients experiencing multiple transfusion episodes, 50 percent of patients were retested for alloimmunization because of a new transfusion indication within 5 years.

When patients were retested within one month after transfusion, Kidd antibodies represented more than 20 percent of the antibodies and are more frequent than anti-K, whereas anti-Kidd frequency declined to a relative proportion of 3 to 6 percent when retesting was performed after more than 3 months. It is recognized that Kidd antibodies are frequently involved in delayed hemolytic transfusion reactions[16,17,25], in which a booster respons is held responsible. Although we could not distinguish a primary from a booster reaction, the high frequency in our study, shortly after transfusion, suggests a rapid primary immune response. Heddle and coworkers[18], prospectively studied alloimmunization after transfusion in 2082 patients and found 32 new RH, K, FY, JK or MSs antibody specificities, of which 9 (28%) were anti-Jk^a, detected after a median of 4 days (range, 1-94 days) after transfusion. A positive direct antiglobulin test was found in 31 percent of the new alloimmunized patients. Only 3 patients showed hemolysis, of which 1 was an anti-Jk^a case. In a number of other reports, rapid primary immune responses with or without delayed hemolytic transfusion reactions are described [2,26-31].

Duffy antibodies are seen infrequently (5-8%) in the cases tested the first 6 months after transfusion in our study, but they represent up to 27 percent of antibodies after 6 months and become even more frequent than anti-E after 5 years after transfusion. Slow anti-Fy^a evolving after transfusion as well as disappearance of other antibodies over time could explain the frequency difference. In previous studies 13 to 17 percent of Fy^a antibodies in contrast to 29 to 50 percent of E, K and Jk^a antibodies became undetectable over time[32,33].

Overall, our results regarding relative immunogenicity are similar to those previously reported[9,20,22]. Compared to the original results of Giblett, however, we observed a significant increase ($p<0.0001$) in relative immunogenicity of Fy^a and Jk^a antigens compared to K antigen, probably reflecting the use of more sensitive

antibody screening tests for these antibodies over the years and a decrease in relative immunogenicity of the c antigen ($p<0.0001$). The reason for the decrease in c antigen immunogenicity is not apparent.

Our results illustrate that relative immunogenicity is dependent on the interval of testing after transfusion and calculations can best be performed after 3 to 6 months after transfusion when most antibodies have developed and are still detectable. Such information regarding relative immunogenicity of antigens can support transfusion policy decisions.

According to the Blood Transfusion Guidelines from the Dutch Quality Institute for Healhtcare, CBO, published in 2004, pretransfusion antibody tests are allowed to be performed up to 3 days before scheduled transfusion[34]. This practice implicates a small risk for hemolytic transfusion reactions as we found in our study that 0.3 to 0.4 percent of transfused patients had developed antibodies within 3 days after transfusion.

Other issues concern prevention of hemolytic transfusion reactions. A nationwide database is being developed in the Netherlands, registering patient RBC alloantibody information, to reduce the risk of transfusion reactions and delay of transfusion for patients shifting between hospitals. Such prevention improves when antibody investigations are performed at the appropriate intervals after transfusion.

In the ideal situation, RBC antibody tests after transfusion should be performed twice, the first time shortly after transfusion, to detect boostering of existing antibodies or fast appearing new antibodies (i.e. anti-Jk^a and anti-E), and a second time after a longer period of time, to detect slower developed antibody specificities Our study design does not allow to be more specific on the time points. More prospective investigations are needed to recommend on timing of optimal posttransfusion antibody testing. Finally, because automated medium and high-throughput DNA techniques will soon be available for large-scale donor and patient blood group genotyping, extended matching for the most clinically relevant red blood cell antigens (i.e. RH, K, FY, JK and S) to prevent alloimmunization will become a topic of discussion. The benefits of such a policy, in terms of immunization prevention and costs can only be based on reliable figures of alloimmunization, incidence of repeated transfusions and costs of current policy.

In conclusion, the time interval between transfusion and antibody test is strongly associated with the identified RBC antibody specificity. RBC antibody tests following transfusion are not routinely performed and in case the antibodies are detected for the first time at the time of a new transfusion indication, or have become undetectable, they are posing the transfusion recipient at risk for transfusion delay or a (delayed) hemolytic transfusion reaction. To anticipate

future developments, such as large-scale extended phenotyping of donors, the kinetics and frequency of RBC alloimmunization need to be more precisely determined through prospective studies in which antibody detection tests are performed at set time intervals after transfusion.

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10

GENERAL DISCUSSION

Due to the several hundreds polymorphic blood group epitopes, a red blood cell transfusion is inevitable incompatible. The formation of antibodies against foreign red blood cell antigens is a common side effect of transfusion. Over the years, techniques with increasing sensitivity to detect RBC antibodies during pre-transfusion testing have been implemented in the hospital transfusion laboratories. Low-ionic strenght enhanced column-agglutination techniques or polyethyleneglycol enhanced tube tests, both of comparable sensitivity, together with commercial available test-erythrocytes are nowadays routinely used in most Dutch laboratories. The specificity of these techniques to detect only the clinically relevant RBC antibodies, however, still needs improvement. Panel cells still are dependent on the variability of donor cells. A recent development, using recombinant fusion proteins containing the Fy^a and Fy^b extracellular antigen domain produced by *E. coli* transformed with Duffy expression plasmids, shows promising results[1]. In addition, DNA can be transfected into cells and grown in tissue culture. Kell and Duffy proteins have been expressed in mouse erythroleukemic (MEL) cells or 293T cells and detected by human antibodies[2]. Theoretically, it is possible to produce a panel of cell lines expressing the individual clinically relevant blood group proteins allowing simultaneous single step detection and identification of (multiple) blood group antibodies.

In chronically transfused hemoglobinopathy patients the incidence of RBC antibodies is high, but for other patient populations it is largely unknown. Chronically transfused patients are frequently tested for the presence of red cell antibodies and therefore, despite retrospectively studied, data on alloimmunization risk and the antibody specificities involved are most likely accurate. The vast majority of studies on RBC alloimmunization in non-chronically transfused patients are retrospective or observational cohort studies, in which data are obtained from a single or a few antibody tests per patient. The results from these studies, which show <1-3 percent immunization risks, are hampered by the fact that patients are routinely only tested for RBC antibodies before a new transfusion event and antibodies that may have formed after the last transfusion may have become undetectable before the next transfusion. In chapter 8 we report our results on the persistence of 593 antibodies with various specificities. Overall, 26 percent of antibodies become undetectable. Five years after the initial identification more than 50% of antibodies are no longer detectable and this increases to 70% after more than 10 years. Besides, detectablity varied considerably for the various antibody specificities, although not statistically significant. Especially Kidd antibodies are detectable for the shortest period of time, which is consistent with the fact that they are often involved in delayed transfusion reactions. Due to this disappearance of antibodies the frequency of RBC alloimmunization in non-prospective studies is most likely underestimated, both in general and for certain antibody specificities. This is

confirmed by the few studies that adress alloimmunization in non-chronically transfused patients, prospectively. Hewitt and Heddle both studied the incidence of new antibodies formed within 1 week after a mean of 4.5 units transfused, in cardiac surgery, respectively random patient, populations[3,4]. New antibody formation occurred in 2-2.8% of patients. Most of these patients had a history of transfusion and/or pregnancy and therefore a booster response is most likely. In chapter 9 we report that at least 16.8% of 1778 patients who had formed RBC antibodies did so within 14 days after transfusion and 2.3% already within 3 days. For transfused patients who were retested for antibodies within 7 days after the previous transfusion we found new antibodies in 0.9% (unpublished data), which is not at variance with the results from Hewitt (1,7%) and Heddle (1.5%) when only the clinically important antibodies in these studies are considered.
Redman and colleques performed serial RBC antibody testing during a 9 month period following transfusion in a cohort of 452 elective surgery patients[5]. New antibodies with clear specificity were detected in 8.4% of patients between 2-24 weeks after a mean of 3 RBC units transfused. Van de Watering found new RBC antibodies, after a maximum follow-up of 50 days, in 5.3% of 374 cardiac surgery patients, who had received a mean of 4.8 RBC transfusions[6].
However, all of the aforementioned studies considered all transfused patients in stead of only those who are actually at risk for RBC antibody formation, e.g. patients lacking specific RBC antigens and transfused with units containing these foreign antigens. This discrimination may result in higher individual patient alloimmunization risk and more accurate data on the immunogenicity of the various RBC antigens.

Contrary to the 'disappearance' of antibodies, some antibodies may persist for decades after the immunizing event. In chapter 8 we report that D-antibodies that are formed after pregnancy may persist for longer periods of time than those that are formed after transfusion. Although this observation needs further investigation, one could speculate on the mechanism(s) involved in long-term antibody persistence. One possible explanation may be related to persistent antigen exposure as the result of the long-term survival of allogeneic blood cells in the recipient, e.g. microchimerism. Microchimerism is often the byproduct of organ transplantation, pregnancy or transfusion. Cell traffic between mother and fetus during pregnancy has been found to result in frequent and long-term persistence of fetal cells in the mother and maternal cells in her progeny[7-9] Long-term microchimerism after transfusion has been reported especially in trauma patients and in children that had received intra-uterine transfusions[10-14]. It is conceivable that differentiation of chimeric hematopietic stem cells towards the erytropoietic cell line may result in a continuous production of foreign erythrocytes maintaining antibody production by the host. On the other hand,

maternal or donor hematopoietic progenitor cell chimerism after transfusion early in life may induce tolerance against foreign RBC antigens introduced later in life.

The factors that regulate whether an individual will mount an immune response to transfused RBCs remain largely undetermined. The probably most important is the degree of genetic disparity between the donor and the recipient. Some diseases, restricted to certain ethnic groups, e.g sickle cell disease, exhibit high immunisation risks, especially when the patients are transfused in countries where the ethnic group is underrepresented in the donor population. Prophylactic matching for the most important antigens (RH and K) has reduced RBC antibody formation in these patients substantially and is standard practice in the Netherlands[15]. However, no randomized controlled studies have been performed yet to evaluate this policy.
Immunogenetic factors, such as MHC-type, CD4+ T-cell, B-cell and cytokine polymorphisms can all contribute to the presentation and recognition of foreign antigen and maturation of the immune response resulting in antibody production. Differential immunogenicity of mismatched antigens is, besides non-antigen specific co-stimulatory help, largely dependent on MHC-restricted activation of T-cells. Alarif and colleques showed that HLA-B35 positive sickle cell patients are six times more likely to form RBC alloantibodies with various specificities after transfusion than those lacking that antigen[16]. Recent studies have identified possible relationships between the HLA-DRB-restriction sites and the immune response to a number of RBC antigens. The *HLA-DRB1*1501* allele is significantly overrepresented in D-negative donors who have produced D-antibodies in response to D-positive cells compared to non-responders[17]. Jk^a-immunization has been found associated with the *HLA-DRB1*0101*, *HLA-DRB1*0102* and *HLA-DRB1*1001* alleles, which share a common gene sequence in the P4 pocket of the peptide binding site, which is the major determinant of T cell antigen recognition[18,19]. Also for K and Fy^a immunized persons associations with HLA-DRB1 polymorphisms have recently been described (table 1)[20,21].
However, HLA associations alone can not explain the incidence of specific antibodies. From mouse models, there is evidence that genetically determined immunomodulatory genes outside MHC are also involved in the regulation of the extent of an immune response against sheep red blood cells[22-26]. Recently a transfusion model using donor transgenic mice, expressing Fy^b antigen, and Fy^a positive mice as recipient was developed to study anti-Fy^b alloimmunization after intravenous RBC transfusion[27]. This model may serve to further study the immune mechanisms involved in antibody formation.

Table 1. HLA-DRB1 restriction sites and immunization to RBC antigens

RBC antibody	HLA-restriction site	P4 pocket sequence	Frequency (%)		Odds Ratio (95% CI, p-value)
			Immunized persons	Controls	
Jk[a]	DRB1*0101 DRB1*0102 DRB1*1001	F13/R71/A74	65	19.5	7.7 (2.9-20.5, <0.05)
K	DRB1*11 DRB1*13	S13/D70/A74	83	52	4.5 (2.1-9.7, <0.001)
Fy[a]	DRB1*04	-	100	19	12.9 (8.0-20.7, <0.0001)

Patient related clinical factors will influence the possibility of a patient to respond. A suppressed immune system as a result of either the disease's pathophysiology or severe immunosuppressive therapy may result in a reduced immune response, while on the other hand a normal or enhanced immune status, again related to the disease's pathophysiology or, for instance, the presence of inflammation (danger signal)[28], may result in (enhanced) antibody production. Other, yet unknown, clinical factors may play a role in distinquishing patients that will or will not form antibodies after transfusion.
A higher number of transfusions will increase the possibility of encountering a foreign antigen. Obviously, this is the case for some antigens such as K-antigen and low-frequency antigens. For other common antigens (e.g. non-D RH, FY, JK, S) the theoretical chance of encounter is already more than 90 percent after transfusion of 3-4 RBC units. For anti-D, a single transfusion of D-positive RBCs will mount an immune response with detecable anti-D within a few weeks after the transfusion in most immunocompetent persons, but for other blood groups the number of transfusions to mount an immune response as well as the kinetics involved are largely unknown.

Although RBC antibodies by themselves are not considered harmfull, its presence can evoke serious problems.
Fetal and neonatal hemolytic disease originates from maternal RBC alloimmunization. D-antibodies are the result of a previous pregnancy, but approximately 50 percent (range 10-83%, depending on the antibody specificity) of other blood group antibodies during pregnancy are caused by previous transfusions[29]. Therefore, K-antigen compatible transfusions are given to females of (pre)child bearing age to prevent K-antibody formation[15]. For c and E antigens, these preventive measures are still a matter of debate.

Many blood groups are exposed on tissues other than blood, and corresponding antibodies can facilitate immune damage to transplanted tissues, which may lead to allograft rejection or even poor patient survival[20-32]. As a consequence, the presence of pre-transplant antibodies can complicate allograft selection.

Alloantibody formation can be associated with temporary pan-reactive autoantibody formation triggering a phenomenon known as 'bystander immune cytolysis', where the autologous red cells of the recipients are destroyed and which can result in life-treatening anemia. This phenomenon is estimated to occur in 4-11% of sickle cell patients[33,34], but has recently been described in other patients[35,36] and is probably the result of an antibody booster reponse.

Hemolytic transfusion reactions, after an incompatible transfusion in an immunized patient, can result in serious morbidity and mortality[37-40]. Delayed transfusion reactions (DTR) occur after transfusion of seemingly compatible RBC in previously alloimmunized patients in whom the antibody titers have fallen below serological detectable levels or rarely after an early primary antibody response. In the majority of cases, which are termed 'delayed serological transfusion reactions' (DSTR), the serologic findings are consistent with DHTR but there is no clinical evidence of hemolysis. Delayed serologic reactions appear to occur almost four times more frequent than DHTR, which has a frequency of one per 5400-6700 transfusions[41-43]. According to the 2004 and 2005 annual reports from the UK and Dutch hemovigilance organizations clinically relevant DHTR occur once per 46.000 units transfused[37,38,45,46]. This lower rate compared to previous studies may be explained by factors such as, improved pre-transfusion antibody screening, shortened hospital stay resulting in underrecognition, and a more restrictive transfusion policy. A wide variety of blood group antibodies are involved in DTR, but antibodies against E and Jk^a antigens are involved in more than 50% of cases and compared to other antibody specificities, anti-Jk^a is most frequent present in the hemolytic type. Recently, a nation-wide antibody registration system (TRIX) has been installed and is expected to be implemented for all Dutch hospitals in 2007. The aim of this registration is to provide transfusion laboratories with information regarding (historical) RBC antibodies in patients to prevent transfusion delay and to prevent hemolytic transfusion reactions. Studies given insight into the incidence of antibody based transfusion delay and transfusion reactions are needed to evaluate the efficacy of this registration.

At last, in patients with multiple antibodies or antibodies against high-frequency antigens obtaining compatible blood for future transfusions can be complicated and may result in transfusion delay.

The age of genomics has enabled the application of DNA-based molecular methods to transfusion medicine. Isolation of the molecules that carry blood group antigens, followed by cloning of the genes and the development of DNA sequencing and PCR, have made application of genetic information to blood transfusion possible. Although assays for blood group antigens encoded by single SNPs are highly reproducible and correlate well with RBC genotype, genotyping for ABO and RH systems are still challenging because of the many mutations responsible for A and B subgroups and numerous variant and hybrid *RH* genes.
The development of high-throughput genotyping platforms that utilize micro-array and chip technologies will offer the opportunity to perform large scale testing on numerous antigens simultaneous and this will decrease costs compared to labor-intensive serologic techniques. Centralized data systems containing the complete molecular profile of all donors will give blood banks instant access to many different typed donors allowing an accurate selection of donor units to facilitate matching of donor RBCs to the recipient's blood type. This will create the opportunity to extend routine antigen matching between donor and recipient to prevent alloimmunization to RBC antigens. Matching for C, E, c, e and K antigens will theoretically reduce clinically important antibody formation by 70%, adding Fy^a, Jk^a and S antigens will increase this to almost 90%. Preventive extended matching is currently only applied for females of (pre)child bearing age (K blood group) to prevent hemolytic disease of the fetus and newborne and for patients who suffer from hemoglobinopathies or hemolytic anemias (RH and K blood groups) who are known to ultimately develop antibodies. However, patients with myelodysplastic and myeloproliferative syndromes have shown to also exhibit a high antibody response after transfusion (chapter 5). In addition, patients who have already made RBC antibodies are also high responders towards other blood group antigens after repeat transfusion (chapters 6 and 7). Therefore extended antigen matching for these patients may be considered.
Although RBC antibodies are in most cases only of interest in case of repeat transfusion, longer life expectancy of the population and an increased probability of repeat surgery or diseases requiring blood transfusion increas the clinical relevance in non-chronically transfused patients as well.
The introduction of extending current preventive measures should be preceded by studying its feasibility and cost-effectiveness.

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SUMMARY

Summary

Chapter 1
In this chapter blood group alloimmunization after transfusion is outlined in short, followed by a description of the aim and design of the studies of the thesis.

Chapter 2
Chapter 2 contains a review of the literature on red cell immunization after blood transfusion covering the developments in this field in the past century. The discovery of the ABO-blood group system, the alloimmune origin of hemolytic disease of the fetus and newborne and the antiglobulin test by Coombs are the most important developments. Transfusion safety increased tremendously with the routine introduction of sensitive pretransfusion compatibility testing and current protocols primarily focus on potential clinical significant antibodies that only react in the indirect antiglobulin phase.
After the development of DNA sequenching and amplification techniques the genes encoding almost all blood group antigens were characterized on the molecular level, elucidating that many blood groups arise from single nucleotide polymorphisms. In clinical transfusion practice, genomic typing assays are increasingly being used for donor and patient blood group typing. Studies on the functional aspects of the molecules that express RBC antigens, showed their possible significance in pathophysiology, e.g. red cell abormalities and organ dysfunction.
The evolving field of immunology unravelled processes, immune cells and molecules involved in the alloimmune response against foreign antigens. Antigen presenting cells, T- and B-cells regulated by (co-)stimulatory factors are responsible for the formation of antibodies, whereas inhibitory signals control homeostasis of the immune response. Besides genetic recipient factors, e.g. MHC polymorphisms, several unknown or ill-defined factors may influence the immune response towards red blood cell transfusions and need elucidation in the coming years.
The incidence of RBC alloimmunization after transfusion varies between less than 1% up to over 70%, depending on the population studied. From all studies, it appeared that E and K blood group antigens are the most immunogenic non-D antigens and are responsible for the majority of antibodies found. The high immunization rate in patients with sickle cell disease and thalassemia has led to the policy to prophylactically match donor RBCs for RH and K antigens.

Chapter 3

Chapter 3 describes the results of a retrospective multicenter study, performed on a large non-selected patient cohort in the Sanquin Southwest region, with the aim to establish whether prestorage leucodepletion alters the development of clinically relevant red blood cell antibodies after transfusion. Two periods were investigated, 2 years before and 2 years after the introduction of universal leucodepletion. We compared new antibodies that were detected in patients transfused during the study intervals (antibody incidence) with antibodies in patients admitted to the hospitals in the same period but who had not received transfusions during the study periods (antibody prevalence). A total of 4148 patients possessed 4770 RBC antibodies.

No differences in antibody incidence or prevalence as well as antibody specificities, multiple antibodies, historical antibodies and additional antibodies between the two study periods were found. Similarly, patient cohorts were comparable with regard to age, sex, number of units transfused, transfusion events, interval from transfusion to antibody detection and alloimmunization rate (0.13%, expressed as the number of immunized patients per RBC unit transfused).

From this pre- and post intervention study we concluded that RBC alloimmunization in non-selected random transfused patients was not favourably affected by universal leucodepletion compared to buffy-coat leucoreduced RBC transfusions.

Chapter 4

In chapter 4 we present a study on the frequency of coincidentally discovered antibodies against low incidence antigens in a RBC alloimmunized patient population during a 23 year period. The aim of the study was to investigate whether the type and screen policy, without performing a complete crossmatch before transfusion in non-immunized patients, is a safe procedure, with regard to the risk on transfusion reactions due to antibodies against low incidence antigens. In addition the presence of anti-Wr^a was studied in random hospital patients with and without RBC antibodies, in pregnant women and in blood donors.

Antibodies to low incidence antigens were detected in 50 patients who did not possess other RBC antibodies and in 39 patients together with other RBC antibodies (2.2% of patients with RBC antibodies). Fourteen antibodies to low incidence antigens were found for the first time during 117,000 complete crossmatch tests (0.01%). During the study period, no transfusion reactions were reported due to antibodies against low incidence antigens.

The prospective study on the presence of anti-Wr^a revealed that 4.3% of blood donors, 5.8% of random non-immunizd hospital patients, 10.2% of pregnant women and almost 20% of patients with other antibody specificities possessed this antibody.

From this study we concluded that antibodies to low incidence antigens are often present in patients as well as in healthy persons, but that the risk of a transfusion reaction due to these antibodies is very low and therefore that transfusion without complete crossmatch is a safe procedure.

Chapter 5

In this chapter, red blood cell antibodies found after transfusion in various malignant myelo- and lymphoproliferative diseases are described from a 10-year retrospective study. The 564 evaluable patients received a total of 15,287 RBC units in 6182 transfusion episodes. Patients with myeloproliferative diseases required transfusion support for a twice as longer period resulting in almost 3 times more RBC units transfused compared to patients with lymphoproliferative diseases. A total of 71 allo-antibodies, predominantly anti-E and -K, were found in 51 patients. Multiple antibody specificities were present in 33% of immunized patients. Fifty percent of antibodies were already formed after 13 RBC transfusions, indicating that the majority of antibodies are formed early during the course of transfusions. The alloimmunization rate was 0.5% per RBC unit transfused. Patients who received intensive chemotherapy produced RBC antibodies at a much lower rate than those who are not treated intensively. This was further confirmed by the fact that only three patients formed anti-D after 244 D-incompatible platelet transfusions.

From this study, we concluded that antibody formation in patients with malignant hematologic diseases is comparable to that for other diseases requiring multiple transfusions. Therefore prophylactic antigen matching to prevent antibody formation seems unnecessary in this clinical setting.

Chapter 6

The aim of the 20-year retrospective multicenter study described in this chapter was to explore to what extend non-chronically transfused allo-immunized patients are prone to form additional clinically significant RBC antibodies upon repeat transfusions. A total of 653 patients who presented with 772 RBC antibodies received a median of five additional RBC transfusions during median two transfusion episodes. A total of 140 patients formed 157 additional antibodies and 57% did so after a single additional transfusion episode. At the end of the study almost 34% of patients had multiple antibodies. The odds ratio for additional antibody formation compared to primary antibody formation (see chapter 3) was 29 (95% CI, 22.7-37.2). The calculated risk on additional immunization per unit transfused, for 316 patients from whom the RBC antigen profile was known, showed considerable variation between specific RBC antigens; K-antigen exhibit the highest immunization risk of 9%, RH-antigens between 1.9 and 3.6% and other antigens between 0.2 and 1.7%.

The theoretical exercise regarding extended matching, in patients with RBC antibodies, showed that donor feasibility should be no problem in the Netherlands if matching is restricted to the most immunogenic antigens (RH, K, Fy^a and Jk^a).
This study showed that non-chronically transfused patients who formed RBC antibodies after a previous transfusion are high-antibody responders to subsequent alloantigenic challenges. Extended antigen matching seems feasible and should be considered for subsequent transfusions in this patient group.

Chapter 7
This chapter describes the results of a study, performed at Leiden Medical University Center, the Dutch national reference center for intrauterine transfusion (IUT) treatment, with the aim to evaluate maternal additional clinically significant antibody formation after introduction of preventive D, C, c, E, e and K matching of IUT donors. During an 11-year period 686 IUTs were performed during 233 pregnancies in 212 women. Before IUT treatment the women possessed 242 antibodies and multiple antibodies, mainly anti-CD, were present in 59% of pregnancies. After IUT treatment fifty-three women (25%) had formed 64 new additional antibodies and in 11 women historical antibodies that were no longer detectable before IUT treatment had reappeared. The percentage of women with multiple antibodies increased from 59% at admission to 72% after delivery and non-RH/non-K antibodies increased from 12% to 25%. The mean immunization rate (new antibodies per IUT) was 18% after the first IUT and declined to about 5.5% after subsequent IUTs.
Multivariate analysis revealed that additional antibody formation was predominantly associated with transplacental puncture to administer the transfusion.
Rh- and K-matching of the IUT donor did not prevent the formation of RH and K antibodies in 31% of pregnancies at risk, because the fetal RBCs carried the antigens. In 11 of 17 (65%) evaluable cases the additional *non*-RH/*non*-K antibodies were induced by the donor and not by fetal RBC antigens.
We concluded from this study that additional antibody formation during IUT therapy is common, associated with transplacental puncture and not reduced, despite RH- and K-matching of the IUT donors. To reduce the high rate of alloimmunization in these patients we propose to extend matching of IUT donors also for JK, FY and S antigens.

Chapter 8
The purpose of the study, of which the results are described in chapter 8, was to investigate the persistence of clinically significant RBC alloantibodies during a 20-year period. We identified 593 antibodies in 480 patients whose sera were screened multiple times for antibodies after first antibody detection. Overall, 153 antibodies (26%) became undetectable over time. Four years after the first

detection of newly formed antibodies 50% had become undetectable and this increased to more than 70% after 16 years. Although there were no statistically significant differences in the rates with which the various antibody specificities became undetectable, Kidd antibodies were detectable for the shortest period of time, which may explain their frequent cause of delayed hemolytic transfusion reactions.
We concluded from this study that it is common for RBC alloantibodies to become undetectable over time and that accurate blood bank records and precise patient files are of importance to prevent (delayed) hemolytic transfusion reactions due to the transfusion of incompatible blood.

Chapter 9
Chapter 9 describes the results of a 5-year retrospective multicenter study on factors relevant for immunization and specificities of clinically significant alloantibodies, with special emphasis on the time interval between transfusion event and antibody testing. Two thousand one-hundred and seventy-seven new antibody specificities were found in 1778 patients in the years 1999 through 2003. Patients had received 12,379 RBC units in 4,050 transfusion events.
Male gender was correleated with anti-Jk^a, younger age with anti-M and anti-S and the number of RBC transfusions with anti-K and anti-Jk^a. The most important independent factor associated with particular antibody specificities was the time interval between transfusion and antibody tests. Anti-E was the most encountered antibody up to 5 year following transfusion, but from 12 months onward declined from 45 to 20 percent of encountered antibodies persisting after more than 5 years. Anti-c presented 10 to 15 percent of antibodies found up to 5 years after transfusion, after which period it decreased to less than 3 percent. Anti-K showed a gradual increase from 20 percent after 1 month to 45 percent after more than 5 years after transfusion. Anti-Fy^a rapidly increased from less than 10 percent after 1 year to 27 percent of antibodies found after more than 5 years. Anti-Jk^a and anti-Jk^b were predominantly found during the first 3 months after transfusion; thereafter they decreased from, respectively, 18 and 5 percent to, respectively, 3 and less than 2 percent.
In 299 patients alloantibodies were detected within 14 days after transfusion, either due to a secondary or to a rapid primary immune response.
We concluded that the time interval between transfusion and antibody tests is strongly associated with the number and RBC antibody specificity. Because RBC antibody tests are not routinely perfomed after transfusion, many antibodies may (not) be detected at the time of a new transfusion event, posing the recipient at risk for transfusion delay or a hemolytic transfusion reaction. The frequency and kinetics of RBC alloimmunization need further study, to determine the optimal moment for antibody testing and to anticipate on possible future developments regarding extended donor RBC matching.

Chapter 10

This chapter contains a discussion on the significance of the results of the studies included in the thesis and directions for future research in the field of RBC alloimmunization after transfusion.

From our studies, we conclude that certain patients groups are at increased risk for RBC alloimmunization, and should be considered for extended donor RBC antigen matching. For other transfusion populations the risk is still largely unknown, but most probably underestimated. The main reason for discripancies found are related to the study designs, which are hampered by the fact that not all patients are routinely tested after a transfusion event and antibodies may have become undetectable before the next transfusion. Prospective studies should ideally focus on patients actually at risk for antibody formation, e.g. patients who have received RBC transfusions containing foreign antigens.

Contrary to the disappearance of antibodies, some antibodies persist for decades. The mechanisms involved are yet largely unknown, but persistent microchimerism of donor cells may be one explanation.

The immune mechanisms and patient related clinical factors involved in RBC alloimmunization will be the subject of studies in the coming years and may define patients at increased risk for antibody formation after transfusion.

Finally, the development of high-throughput genotyping platforms will offer the opportunity to perform large scale testing on numerous antigens simultaneously, and will create the possibility to extend routine donor antigen matching for patients at risk for alloimmunization.

SAMENVATTING

Samenvatting

Hoofdstuk 1
Dit hoofdstuk bevat een korte beschrijving van alloimmunisatie na bloed transfusie, gevolgd door een beschrijving van opzet en doel van de studies van dit proefschrift.

Hoofdstuk 2
Hoofdstuk 2 bevat een samenvatting van de literatuur betreffende rode bloedcel immunisatie na bloed transfusie, met inbegrip van de ontwikkelingen op dit gebied in de afgelopen eeuw. De ontdekking van het ABO bloedgroep systeem, de alloimmuun oorzaak van hemolytische ziekte van de foetus en pasgeborene en de antiglobuline test van Coombs zijn de belangrijkste mijlpalen geweest. De veiligheid van transfusies verbeterde enorm na de routinematige toepassing van de indirecte antiglobuline in pretransfusie compatibiliteit onderzoek, en de huidige protocollen zijn primair gericht op de detectie van klinisch belangrijke alloantistoffen in de indirecte antiglobuline fase.
Na de ontwikkeling van DNA sequentie en amplificatie technieken werden de genen coderend voor bloedgroep antigenen op moleculair niveau gekarakteriseerd en bleek dat veel bloedgroepen het gevolg zijn van enkelvoudige nucleotide polymorfismen. In de klinische transfusie geneeskunde wordt genotypering in toenemende mate gebruikt voor bloedgroep bepaling van donor en patiënt. Uit studies naar de functie van bloedgroep moleculen is gebleken dat zij een rol spelen in de pathofysiologie, zoals erytrocyten afwijkingen en orgaan dysfuncties.
Ontwikkelingen in de immunologie hebben inzicht gegeven in de processen, de immuuncellen en de moleculen die betrokken zijn bij de alloimmuun respons tegen lichaamsvreemde antigenen. Antigeen presenterende cellen, T- en B-cellen gereguleerd door (co)stimulatoire signalen zijn verantwoordelijk voor de vorming van antistoffen, terwijl inhiberende factoren de homeostase van het immuunsysteem controleren.
Naast genetische factoren bij de ontvanger, zoals MCH-polymorfismen, spelen nog andere (onbekende) factoren een rol bij de immuunrespons tegen rode bloed cel transfusies. Toekomstig onderzoek zal de rol van deze factoren ophelderen.
De incidentie van RBC alloimmunisatie na transfusie ligt tussen minder dan 1% en meer dan 70% en is afhankelijk van de onderzochte populatie. Uit alle studies blijkt dat, van de non-D antigenen, de E en K bloedgroep antigenen het meest immunogeen zijn en verantwoordelijk zijn voor het merendeel van de gevonden antistoffen. Het hoge immunisatie risico bij patiënten met sikkelcel ziekte en thalassemien heeft ertoe geleid dat bloedtransfusies bij deze patiënten preventief gematcht worden voor de RH en K antigenen.

Hoofdstuk 3

Hoofdstuk 3 beschrijft de resultaten van een retrospectieve multicentrum studie, uitgevoerd in een groot ongeselecteerd patiënten cohort in de Sanquin Zuidwest regio, met als doel vast te stellen of algehele leucodepletie van RBC producten invloed heeft op de vorming van klinisch relevante RBC antistoffen na transfusie. Er werden 2 perioden onderzocht, 2 jaar voor en 2 jaar na de implementatie van algehele leucodepletie. We hebben het aantal nieuw gevormde antistoffen die gevormd waren door patiënten die in de studieperiode getransfundeerd zijn (antistof incidentie) vergeleken met antistoffen die gevormd waren gedurende dezelfde periode bij patiënten die niet getransfundeerd waren in het betreffende ziekenhuis (antistof prevalentie). In totaal werden 4770 RBC antistoffen aangetoond bij 4148 patienten.

Er werden geen verschillen aangetoond tussen de twee studie perioden met betrekking tot antistof incidentie, antistof prevalentie, antistof specificiteiten, historische antistoffen en additionele antistoffen.

De patiënten cohorts waren vergelijkbaar voor leeftijd, geslacht, aantal transfusies, aantal transfusie episoden, interval tussen transfusie en antistof detectie en het alloimmunisatie risico (0.13%, weergegeven als het aantal patiënten met antistoffen per getransfundeerde RBC eenheid).

Op grond van deze pre- en post interventie studie kan worden geconcludeerd dat RBC alloimmunisatie in een ongeselecteerde transfusie populatie niet gunstig beïnvloed word na universele leucodepletie van RBC producten in vergelijking met buffy-coat gedepleteerde RBC producten.

Hoofdstuk 4

In hoofdstuk 4 presenteren we een studie naar de frequentie van bij toeval gevonden antistoffen gericht tegen laag frequente RBC antigenen in een RBC geïmmuniseerde patientenpopulatie, gedurende een periode van 23 jaar. Het doel van de studie was te onderzoeken of het type en screen beleid, waarbij geen complete kruisproef wordt uitgevoerd voor transfusie bij patiënten zonder RBC alloantistoffen, veilig is met betrekking tot het risico op een transfusiereactie ten gevolge van antistoffen tegen laag frequente antigenen. Tevens werd de frequentie van Wr^a antistoffen onderzocht in niet-geselecteerde ziekenhuis patiënten met en zonder RBC antistoffen, in zwangere vrouwen en in bloed donoren.

Atistoffen tegen laag frequente antigenen werden gevonden in 50 patienten zonder andere RBC antistoffen en in 39 patienten waarbij ook andere RBC antistoffen werden aangetoond (2.2% van de patiënten met RBC antistoffen). Tijdens 117.000 kruisproeven uitgevoerd in de indirecte antiglobuline test werden 14 antistoffen tegen laag frequente antigenen gevonden (0.01%). Gedurende de studie periode werden geen transfusiereacties gemeld ten gevolge van antistoffen tegen laag frequente antigenen.

In het prospectieve onderzoek werden Wr^a antistoffen aangetoond bij 4,3% van de bloed donoren, bij 5,8% van de patiënten zonder andere RBC antistoffen, bij 10,2% van de zwangere vrouwen en bij bijna 20% van de patiënten waarbij ook andere RBC antistoffen waren aangetoond.
Op basis van de resultaten van dit onderzoek is geconcludeerd dat antistoffen tegen laag frequente RBC antigenen vaak voorkomen in patiënten en in gezonde personen, maar dat het risico op een transfusiereactie ten gevolge van deze antistoffen zeer laag is en dat daarom transfusie zonder een kruisproef in de indirecte antiglobuline test veilig is.

Hoofdstuk 5
In dit hoofdstuk worden de RBC antistofvorming na transfusie beschreven in patiënten met maligne myelo- en lymfoproliferatieve ziekten. Gedurende een periode van 10 jaar hadden 564 evalueerbare patiënten in totaal 15.287 RBC transfusies ontvangen in 6182 transfusie-episoden. Patiënten met myeloproliferatieve ziekten hadden 2 keer langer transfusie ondersteuning nodig, resulterend in bijna 3 keer meer RBC transfusies dan patiënten met lymfoproliferatieve ziekten. In totaal werden 71 alloantistoffen, voornamelijk anti-E en anti-K, aangetoond in 51 patienten. Bij 33% van de RBC geïmmuniseerde patiënten werden meerdere antistoffen gevonden. Vijftig procent van de antistoffen waren al gevormd na 13 RBC transfusies, hetgeen aangeeft dat het merendeel van de antistoffen al gevormd worden in het begin van de transfusie therapie. Het immunisatie risico was 0,5% per getransfundeerde eenheid voor de totale bestudeerde populatie. Patiënten die intensief met chemotherapie werden behandeld vormden minder RBC antistoffen dan patiënten die niet intensief chemotherapeutisch werden behandeld. Dit verlaagde immunisatie risico werd bevestigd doordat maar 3 patienten anti-D vormden na 244 D-incompatibele trombocyten transfusies.
De resultaten van deze studie laten zien dat antistofvorming door patiënten met maligne hematologische ziekten vergelijkbaar is met andere ziekten waarbij de patiënten frequent getransfundeerd worden. Er is in deze populatie vooralsnog geen reden om antistof vorming te voorkomen door preventief donor antigeen matchen.

Hoofdstuk 6
Het doel van deze multicenter retrospective studie was te onderzoeken in hoeverre niet-chronisch getransfundeerde patiënten met RBC antistoffen additionele klinisch belangrijke antistoffen maken nadat zij opnieuw transfusies hebben ontvangen. In een periode van 20 jaar werden 653 patienten met 772 eerste antistoffen getransfundeerd met mediaan 5 RBC eenheden in mediaan 2 transfusie-episoden. Additionele antistoffen met 157 specificiteiten werden gevormd door 140 patienten en bij 57% van de patiënten werden deze antistoffen

gevormd na één transfusie episode. Aan het einde van de studie hadden 34% van de patiënten multiple antistoffen. Het risico op additionele antistofvorming is, vergeleken met primiare antistofvorming (hoofdstuk 3) een factor 29 hoger (95% BI, 22,7-37,2). Het berekende risico op additionele antistofvorming, in 316 patienten waarvan het antigeenprofiel bekend was, liet behoorlijke verschillen zien tussen specifieke RBC antigenen; het K-antigeen heeft het hoogste immunisatie risico van 9%, RH-antigenen tussen 1,9% en 3,6% en de andere antigenen tussen 0,2% en 1,7%.
Een theoretische exercitie naar de beschikbaarheid van getypeerde donoren voor uitgebreid matchen voor deze patiënten, liet zien dat dit in Nederland geen probleem is als matchen beperkt wordt tot de meest immunogene antigenen (RH, K, Fy^a en Jk^a).
In deze studie werd duidelijk dat patiënten die na voorgaande transfisies antistoffen hebben gevormd 'high-responders' zijn na een nieuwe antigeen expositie. Uitgebreid matchen voor deze patiënten lijkt mogelijk en zou overwogen moeten worden.

Hoofdstuk 7
Dit hoofdstuk beschrijft de resultaten van een studie verricht in het Leids Universitair Medisch Centrum, het nationale behandelcentrum voor intrauteriene transfusies. Het doel van het onderzoek was het effect van preventief D, C, c, E, e en K matchen van de IUT donors en moeders, met betrekking tot maternale additionele antistofvorming, te evalueren. Gedurende een periode van 11 jaar werden in 233 zwangerschappen bij 212 vrouwen 686 IUTs gegeven. Voor IUT behandeling hadden de vrouwen 242 antistoffen en multiple antistoffen, voornamelijk anti-CD, bij 59% van de vrouwen. Na de behandeling hadden 53 vrouwen (25%) 64 nieuwe antistoffen gevormd en bij 11 vrouwen werden historische antistoffen die voor de eerste IUT niet meer aantoonbaar waren, weer aangetoond. Het percentage vrouwen met multiple antistoffen nam toe van 59% tot 72% na de bevalling en non-RH/non-K antistoffen van 12% naar 25%. Het gemiddelde immunisatie risico (aantal nieuwe antistoffen per IUT) was 18% na de eerste IUT en daalde naar ongeveer 5,5% na volgende IUTs.
Multivariaat analyse toonde aan dat additionele antistofvorming voornamelijk geassocieerd was met de transplacentale transfusieroute.
Matchen voor de RH en K antigenen voorkwam niet dat RH en K antistoffen gevormd werden in 31% van de zwangerschappen waarbij deze antigenen voorkwamen op de foetale erytrocyten. In 11 van de 17 evalueerbare (65%) patiënten waren additionele non-RH/non-K antistoffen het gevolg van donor en niet van foetale antigenen.
De conclusies van deze studie zijn dat additionele antsitofvorming tijdens IUT behandeling frequent voorkomt, geassocieerd is met transplacentair puncteren en niet verminderd is ondanks RH en K matchen van de IUT donors. Om het hoge

risico op alloimmunisatie in deze patiënten te verminderen, wordt voorgesteld om de IUT donors ook te matchen voor JK, FY en S antigenen.

Hoofdstuk 8
Het doel van deze studie, waarvan de resulaten beschreven zijn in hoofdstuk 8, was de persistentie van klinisch belangrijke RBC alloantistoffen geduirende een periode van 20 jaar te onderzoeken. We identificeerden 593 antistoffen in 480 patienten waarvan het bloed, na de eerste antistof detectie, meerdere keren was onderzocht op antistoffen. In totaal waren 153 antistoffen (26%) na verloop van tijd niet meer aantoonbaar. Vier jaar na de eerste detectie van nieuw gevormde antistoffen was 50% niet meer aantoonbaar en dit steeg tot meer dan 70% na een periode van 16 jaar. Ondanks dat er geen significante verschillen konden worden aangetoond in de snelheid waarmee de verschillende antistof specificiteiten onaantoonbaar werden, waren Kidd antistoffen maar zeer kort aantoonbaar. Dit kan verklaren waarom deze antistoffen zeer frequent betrokken zijn bij uitgestelde hemolytische transfusiereacties.
We concludeerden dat het RBC alloantistoffen frequent onaantoonbaar worden na verloop van tijd en dat accurate registratie van antistoffen in bloedbank en patienten dossiers van het grootste belang om (uitgestelde) hemolytische transfusiereacties, ten gevolge van transfusie met incompatibel bloed, te voorkomen.

Hoofdstuk 9
In hoofdstuk 9 beschrijven we de resultaten van een 5 jaars retrospectieve studie naar relevante factoren voor immunisatie en specifiteiten van klinisch relevante alloantistoffen, met speciale nadruk op het tijdsinterval tussen transfusie en antistofscreening. In de periode 1999 t/m 2003 werden 2177 nieuwe antistoffen gevonden bij 1778 patienten na een totaal van 12379 RBC transfusies in 4050 transfusie episoden.
Mannelijk geslacht was gecorreleerd met anti-Jk^{a}, lagere leeftijd met anti-M en anti-S en het aantal RBC transfusies met anti-K en anti-Jk^{a}. De belangrijkste onafhankelijke factor geassocieerd met bepaalde antistofspecificiteit was het interval tussen transfusie en daaropvolgende antistofscreening. Anti-E was de meest frequent voorkomende antistof tot 5 jaar na transfusie, maar nam vanaf 12 maanden na transfusie af van 45% tot 20% van het totaal aantal gevonden antistoffen dat aanwezig was tot 5 jaar na transfusie. Anti-c betrof 10-15 procent van de antistoffen tot 5 jaar na transfusie, en daalde daarna tot minder dan 3 procent. Anti-K toonde een geleidelijke toename van 20 procent na 1 maand tot meer dan 45% van alle antistoffen na meer van 5 jaar na transfusie. Anti-Fy^{a} steeg snel van minder dan 10 procent na 1 jaar tot 27 procent van alle antistoffen na 5 jaar. Anti-Jk^{a} en anti-Jk^{b} werden voornamelijk aangetoond in de eerste 3

maanden na transfusie; daarna nam hun aantal af van, respectievelijk 18 en 5 procent naar resp. 3 en minder dan 2 procent.
Bij 299 patienten werden antistoffen aangetoond binnen 14 dagen na transfusie, ten gevolge van een secundaire of een snelle primaire immuun respons.
De conclusie van dit onderzoek is dat het tijdsinterval tussen transfusie en antistofonderzoek sterk geassocieerd is met het aantal en de specificiteit van RBC antistoffen. Omdat RBC antistofonderzoek niet routinematig gedaan wordt na een transfusie, is het mogelijk dat antistoffen (niet) gevonden worden voor een volgende transfusie. Hierdoor loopt de patient het risico op enerzijds transfusie uitstel of anderzijds op een hemolytische transfusiereactie. De frequentie en de kinetiek van RBC alloimmunisatie dient verder onderzocht te worden, om zo het optimale moment voor antistofscreening te bepalen en te anticiperen op toekomstige ontwikkelingen met betrekking tot preventief donor matchen.

Hoofdstuk 10
Dit hoofdstuk bevat naast een algemene discussie over de betekenis van de resultaten van de studies in dit proefschrift ook aanbevelingen voor toekomstig onderzoek op het gebied van RBC alloimmunisatie na bloed transfusie.
De conclusie van onze studies is dat sommige patiënten een verhoogd risico hebben op RBC antistofvorming en dat overwogen kan worden om transfusies uitgebreid te matchen tussen patient en donor. Voor andere populaties is het immunisatie risico nagenoeg onbekend, maar waarschijnlijk onderschat. De voornaamste reden voor de gevonden discrepanties is gerelateerd aan het feit dat niet alle patiënten routinematig getest worden op antistofvorming na transfusie en antistoffen alweer onaantoonbaar kunnen zijn voor de volgende transfusie. Prospectief onderzoek zou idealiter gericht moeten zijn op de patiënten die ‘at risk’ zijn voor antistofvorming, t.w. patiënten die transfusies hebben ontvangen met een alloantigeen.
In tegenstelling tot het verdwijnen van antistoffen persisteren sommige antistoffen tientallen jaren na het ontstaan. De mechanismen die hierbij een rol spelen zijn nog grotendeels onbekend, maar persisterend microchimerisme van donor cellen zou één van de verklaringen kunnen zijn.
De immunologische mechanismen en patient gerelateerde factoren betrokken bij RBC alloimmunisatie zullen onderwerp van studies zijn in de komende jaren en mogelijk dat hierdoor patienten met een verhoogd risico op antistovorming geïdentificeerd kunnen worden.
De ontwikkeling van ‘high-throughput’ genotyperings systemen zullen het mogelijk maken om vele antigenen tegelijkertijd te typeren bij zowel donoren als patiënten, waardoor routinematig uitgebreid matchen tussen donor en patiënten met risico op antistofvorming mogelijk wordt.

CURRICULUM VITAE

Henk Schonewille werd op 6 mei 1959 geboren te Vlijmen. In 1977 behaalde hij het HAVO diploma aan het Groen van Prinsterercollege te 's-Gravenhage. Na een jaar wis- en natuurkunde aan de lerarenopleiding te Delft is hij gestart met de analistenopleiding te Scheveningen. In 1980 werd het HBO-A diploma Klinisch Chemisch Analist behaald en werd hij kwaliteitsmedewerker bij Bloedbank den Haag (Dr. JA. van der Does). Na ruim een jaar werd deze functie verruild voor een (research) analisten baan bij het hematologisch laboratorium van het Leyenburg ziekenhuis te 's-Gravenhage (Dr. HL. Haak). In 1984 werd het HBO-B diploma Medisch Chemisch Analist via avondstudie aan de analistenschool te Scheveningen behaald. Van 1998 tot 2001 is hij kwaliteitsfunctionaris geweest bij de afdeling hematologie van het Leyenburg ziekenhuis en van 2001 tot 2004 hoofd van het transfusielaboratorium aldaar (Dr. PW. Wijermans). Tijdens deze periode werd het niet-klinische deel van de opleiding tot Bloedtransfusiegeneeskundige (Sanquin/LUMC) te Leiden gevolgd. Momenteel is de auteur van dit proefschrift werkzaam als onderzoeker bij de afdeling O&O van Sanquin Bloedbank regio Zuidwest te Leiden (Prof. Dr. A. Brand).

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