Development of Novel Carbohydrate Blood Group Related Kodecyte Assays

Elizabeth Holly Perry

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Contents

Attestation of authorship

"I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgements), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning".

Elizabeth Holly Perry
17th June 2019

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Ethical approval

Procurement of donor and patient samples was approved by the Auckland University of Technology Ethics Committee: AUTEC#15/366 and AUTEC#16/175.

Abstract

The carbohydrate blood group systems are collectively considered important in human blood transfusion and organ transplantation. Characterised by antibodies with an ability to activate complement, these carbohydrate blood group systems are implicated in the rejection of transplanted organs, and in potentially fatal transfusion reactions. Robust laboratory assays to successfully match patients and donors are imperative to avoid such consequences. Laboratory assays have evolved considerably since the first discovery of a blood group system in 1900; the carbohydrate ABO blood group system described by Landsteiner. However some assays are outdated, and limited by their reliance on having to use human red blood cells as assay reagents. Such reagents suffer from constraints due to natural biological variation and availability, and consequently inter-laboratory variation for these assays is common. This research aimed to apply an alternative set of laboratory reagents, to provide alternative assays to the inaccurate and sometimes imprecise historical tests.

Kode™ Technology is an approach to modify surfaces including human red blood cells, allowing the cells to express blood group antigens including those which are biologically foreign to them. This allows production of cell-based reagents which are absolutely standardized both in terms of the antigen they express and the quantity in which they express it.

This research used Kode™ Technology to develop two new assays. Firstly, an assay to investigate the stability of complement in stored human serum, and secondly a set of assays to evaluate levels of antibodies to carbohydrate blood group antigens.

Results showed that complement in stored human serum is at least twice as stable as previously believed. This is significant for all laboratories who have to store human samples.

Antibody levels to four different carbohydrate antigens were established in samples from a healthy New Zealand population, and showed high correlation with antibody titres in the case of ABO antibodies. This work may prove useful to two groups of patients; those preparing for ABO incompatible kidney transplantation, and those having cancer therapy of a type which utilizes their own natural antibodies.

List of abbreviations

A	Absorbance
ABOiKT	ABO incompatible kidney transplantation
ad	adipate
AHG	Anti-human globulin
AUTEC	Auckland University of Technology Ethics Comittee
CAT	Column agglutination technology
CB	Copper Biomedical (brand of antisera)
CH_{50}	50% haemolytic complement activity
CMG	Carboxymethylglycine
DOPE	1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine
DTT	Dithiothreitol
EDTA	Ethylene-diamine-tetra-acetic acid
EIA	Enzyme immunosorbent assay
Fs	Forssman
FSL	Function spacer lipid-Kode™ Technology construct
HTLA	High-titre, low-avidity
Ig	Immunoglobulin
IAT	Indirect antiglobulin test
Lab	Laboratory
LISS	Low ionic strength saline
MAC	Membrane attack complex
MCV	Mean cell volume
Mw	Molecular weight
NZBS	New Zealand Blood Service
PBS	Phosphate buffered saline (0.9% sodium chloride, pH 7.4)
PP	Plasmapheresis
QC	Quality control
RBC	Red blood cell
RCD	Red cell diluent
TBS	Tris buffered saline (0.9% sodium chloride, pH 9.0)
v/v	Volume to volume
WHO	World Health Organization

Chapter One: Introduction

This chapter introduces the natural antibodies of the carbohydrate blood group systems, together with their clinical significance and interaction with complement.
A number of assays traditionally used *in vitro* to assess carbohydrate antigen-antibody and complement interactions are described, together with their limitations. Principles and advantages of Kode™ Technology are introduced, together with a brief outline of the way this technology was applied in this research.

1.1 Carbohydrate Blood Group Systems

In 1901, Karl Landsteiner described the ABO blood group system [1]. With the cooperation of his colleagues, Landsteiner collected a blood sample from each of his team and mixed the red blood cells (RBC) of each person with the serum of each of the other people. He then reversed the test series, mixing the serum of each person with the RBC of each of the others. Some combinations of RBC and serum produced a clumping of cells (agglutination), and others did not. Calling the clumped reactions positive, and the non-clumped negative, Landsteiner observed three different patterns which he designated blood groups A, B and 0 (the 0 describing no agglutination). The 0 (zero) later became known as the O blood group. The fourth blood group AB was discovered within Landsteiner's group in 1902 [2]. Landsteiner's discovery of ABO was the first blood group system to be recognised. His work revolutionized the practice and safety of human to human blood transfusion. ABO testing of patients and blood donors remains the single most important test of blood transfusion compatibility.

Following the discoveries of Landsteiner and his group [1], scientists across the world began to search for other blood group systems in humans. This work continues to this day, although very few new systems are realised. Recent advances in molecular techniques have led to elucidation of blood group genes, allowing new systems to be described and others to be re-classified or assigned to existing blood group systems as necessary [3,4].

Of the 36 blood group systems currently recognised [5], seven are classified as carbohydrate or "glycan", and consist of backbone protein or lipid chains, to which sugars are added in sequence by enzymes known as glycosyltransferases. The

carbohydrate blood group systems are ABO, H, Lewis, I, P1PK, GLOB and FORS. Independent of other blood group systems, Sd^a is a high frequency carbohydrate blood group antigen [6]. Within one system different phenotypes are classified by the presence or absence of antigen (a moiety to which a specific antibody can bind). For example when Landsteiner observed agglutination with the RBC he called group A, it was the A antigen on the RBC of one person reacting with anti-A antibody in the serum of another person that was responsible for the agglutination. Although the functions of the carbohydrate blood group systems are not fully understood, the rich and diverse array of cell markers that they provide is known to be important for interactions with human bacterial flora [7-10] . For example, as a result of polymorphisms in the carbohydrate blood group systems, different antigen profiles may render individual humans more or less susceptible to particular bacterial, parasitic or viral pathogens [10]. The carbohydrate blood group systems do not exist in isolation, rather expression of our complete glycan blood group profile is the result of interaction and competition between enzymes of several blood group systems [9]. Nor are the carbohydrate blood group systems restricted to red cell lineages, as they are widely distributed throughout the human body as histo blood group antigens on surfaces of the digestive tract and in bodily secretions [11,12]. Different chain types, or "precursors" are present in different body sites (Table 1), and form the backbones to which sugars are added sequentially to form antigens of the ABO, H, Lewis, I, P1PK, GLOB and FORS systems. Chain types are classified as types 1 through 6, with chains differing in monosaccharide composition, and in linkages between monosaccharides (Table 1). Different chain types are found in both soluble and non-soluble membrane bound forms, and as both glycoproteins and glycolipids [12]. The complex pathways which form the antigens of the carbohydrate blood group systems have been exemplified by Svensson et al [13] (Figure 1).

Table 1. Location and terminal saccharides of human carbohydrate blood group precursor chains by chain type. Compiled from references [7,11,12]

Chain type	Structure	Location
1	Galβ1-3GlcNAcβ1-R	secretions, plasma, endodermally derived tissues, RBC
2	Galβ1-4GlcNAcβ1-R	RBC, plasma, ectodermally or mesodermally derived tissues
3	Galβ1-3GalNAcα1-R	endodermally derived tissues, RBC
4	Galβ1-3GalNAcβ1-R	RBC, kidney
6	Galβ1-4Glcβ1-R	endodermally derived tissues, breast milk, urine

Gal = D-galactose, GlcNAc = N-acetyl-D-glucosamine, GalNAc = N-acetyl-D-galactosamine, Glc = D-glucose, R = remainder of structure.

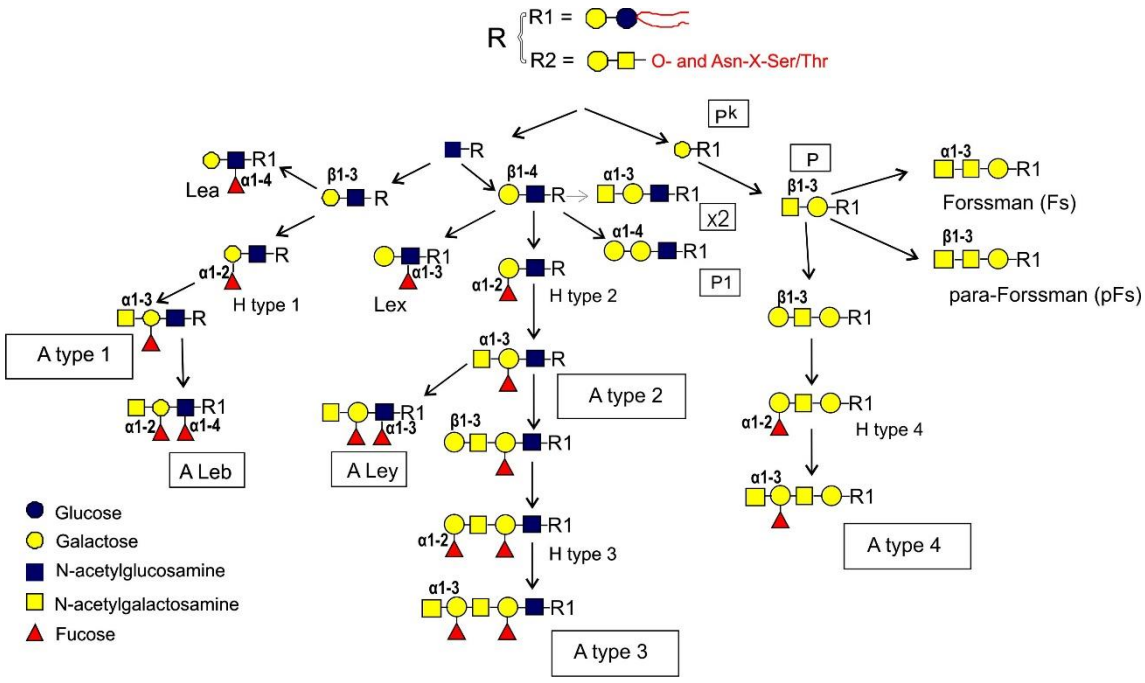


Figure 1. Interaction of carbohydrate blood group pathways on type 1-4 precursors in a blood group A individual. Chain types are detailed in Table 1. Reproduced with permission from Svensson, Pérez and Henry, 2011 [13].

Membrane-bound carbohydrate blood group antigens occur on various cell lineages including RBC [11,12], platelets [14], mucous membranes and gut epithelium [9]. Type 2, 3 and 4 chains predominate on RBCs, whereas types 1, 3 and 6 are dominant in mucous membranes and the gastrointestinal tract [9,12]. Type 2 antigen chains can be straight or branched, with a high degree of branching seen on the I antigen of the I blood group system [15]. In secretions (for example saliva, tears, urine, breast milk and semen) soluble

type 1 and type 2 chains are present [12]. Type 6 chain oligosaccharides have also been identified in human milk and urine [16,17].

1.2 Carbohydrate antigens and antibodies *in vivo*

Glycan-terminating antigens, including blood group antigens on the bodily mucosal surfaces, protect humans from bacterial pathogens [8,10] and are involved in both the innate and humoral immune responses. Firstly, they form part of the dense, negatively charged layer of endothelial cells known as the glycocalyx [8]. The glycocalyx consists of proteoglycans, glycoproteins and glycolipids [18] and provides an innate physical barrier to potentially pathogenic bacteria [8]. The thickness of the glycocalyx varies according to cell type [19] (Figure 2).

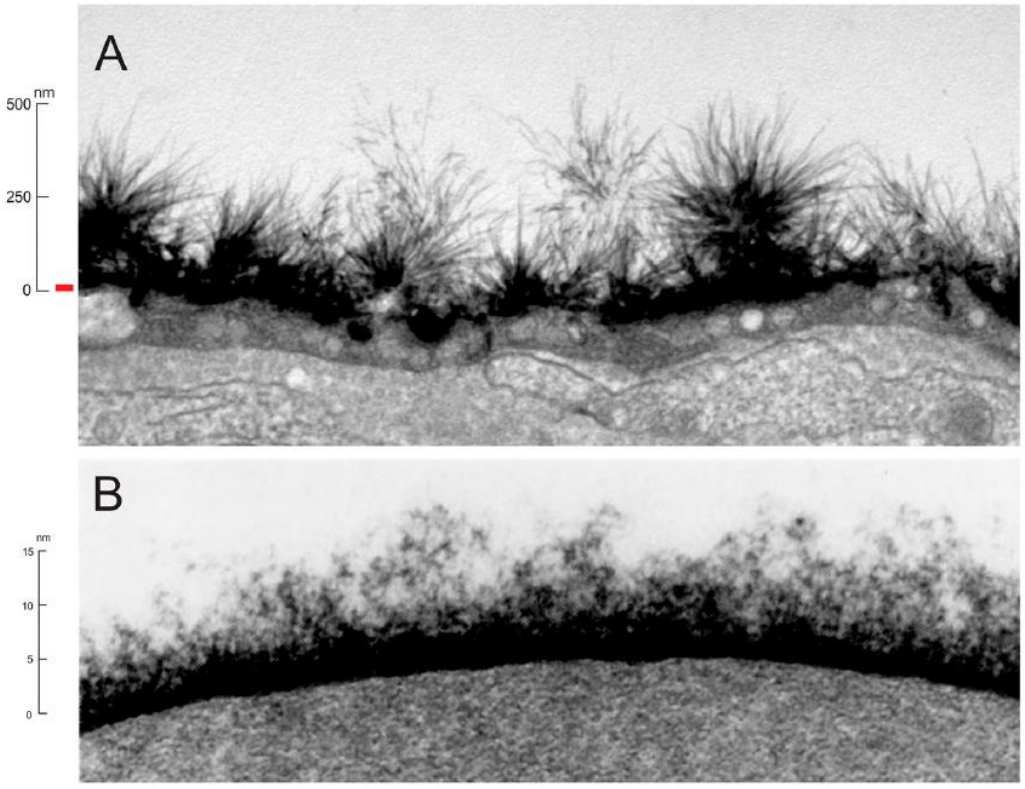


Figure 2. Glycocalyx of renal endothelial cell, and RBC. **A** Glycocalyx of a renal endothelial cell, with a large 500 nm glycocalyx. **B** In contrast, the RBC glycocalyx is much smaller at only 15-20 nm (for comparative purposes a 15 nm high red bar has been placed adjacent to the scale). Reproduced from Henry and Bovin, 2018 [19] who reproduced from Dane et al, 2015 [20] (**A**), and Voet and Voet, 2011 [21] (**B**) with permission of the publisher John Wiley & Sons. Images remain subject to the copyright conditions of John Wiley & Sons.

Bacteria also express carbohydrate antigens on the surface of their cell wall [22-26]. During molecular interactions occurring when bacteria bind to the glycocalyx of human cells [27], the human humoral immune system distinguishes between self and non-self carbohydrate antigens. The resulting production of antibodies to foreign bacterial carbohydrates [28] (largely of the bacterial family *Enterobacteriaceae*) [23], and further to glycans present in pollen [29], parasites [30], and insects [31] results in humans having a vast array of antibodies to carbohydrate antigens [32-35]. This antibody production begins at an early age and is ubiquitous in all humans with a healthy immune system [23,28,32], thus these antibodies are often referred to as naturally occurring or natural antibodies. Naturally occurring antibodies may be defined as those antibodies produced as a consequence of exposure to an antigen; where the antigen comes from an unknown environmental stimulus [36]. These naturally occurring antibodies are considered part of both the innate and adaptive immune system of humans [34,37] and the genes encoding them are highly conserved [36]. Naturally occurring antibodies may be directed to carbohydrate (glycan) or peptide moieties [32]. Biological functions of natural antibodies are still being elucidated, but include pathogen clearance, cancer cell surveillance and involvement in the inflammatory response [28,32,34,37-40]. ABO antibodies are examples of naturally occurring antibodies [23,28,41].

Most naturally occurring carbohydrate antibodies are capable of activating complement by the classical pathway [37]. When carbohydrate blood group antibodies bind to their target RBC antigen, the resulting complex may activate complement proteins [6,42,43], and the classical pathway then proceeds through a series of steps, with each activated complement protein acting as a trigger for activation of the next protein in the cascade. Final assembly of the membrane attack complex (MAC) causes cell rupture [44].

Due to the fact that ABO antibodies are ubiquitous, high-level, able to bind to specific antigens at body temperature, and have the power to activate the classical complement pathway, these antibodies can cause severe transfusion reactions and rejection of transplanted organs unless blood and tissues are ABO compatible [6]. The ABO system must therefore be considered in transfusion practice, and when selecting donors for patients requiring solid organ transplantation (for example kidneys), as ABO non-matched organs are prone to acute rejection by the recipient [45]. Historically, it was not considered advisable to transplant kidneys across the ABO blood group barrier.

However, due to a shortage of organs for transplant, crossing the ABO barrier has been actively pursued, and ABO incompatible transplantation is now practised in many countries, particularly for kidneys [45-63], but also for paediatric hearts [64-67].
The ABO barrier can be crossed when the level of ABO incompatible antibody is naturally low [64-67], or has been reduced to a low level prior to transplantation [46,50,52-55].

High-level IgG anti-A, anti-B or anti-A,B is capable of causing *in vivo* destruction of patient red cells following transfusion of non-identical ABO blood components. Most commonly this occurs when O group blood components are transfused to non-O group patients [6,68-79]. Similarly, high ABO antibody levels can cause incompatibility problems in patients receiving blood products prepared from pools of donors of different ABO groups [80-82].

Haemolysis is due to complement activation through the classical pathway, with progression to the end of the complement cascade, assembly of the membrane attack complex and resulting lysis of patient RBCs [42].

High-level (high-titre) ABO antibodies (sometimes called “haemolysins”) may be found in many blood donors [6,68-79,83-85]. These high-level ABO antibodies jeopardise the safety of using Group O as the universal donor of RBCs, as anti-A, anti-B and anti-A,B present in residual plasma on the RBC in high concentrations can cause lysis of patient RBC in patients of groups A, B and AB [6]. The ability of an antibody to act as a haemolysin *in vitro* and *in vivo* is concentration dependent, but there is no internationally agreed minimum level of antibody at which an ABO antibody is declared dangerous [69-74]. Historically, blood centres around the world screened all donors for high-titre ABO antibodies, but this practice became less common as whole blood transfusion was replaced with the use of blood components targeted to clinical indication. Replacement of plasma in red cell components with additive solutions reduces the likelihood of the quantity of passively transferred antibody being sufficient to cause haemolysis [86]. New Zealand exclude high titre ABO donors as universal RBC and platelet donors [87]. Joint United Kingdom Blood Transfusion and Tissue Transplantation Services Professional Advisory Committee (JPAC) advocate exclusion of high-titre anti-A or anti-B Group O components for non-group O higher risk recipients (primarily neonatal patients) [88]. American Association of Blood Banks requires blood centres to have a policy “concerning transfusion of

components containing significant amounts of incompatible ABO antibodies", and as a result some centres exclude group O high titre ABO antibody donations as universal platelet donors [78]. Legislation in some countries (for example Brazil) does not require screening of group O blood donors for ABO antibody levels [70]. In countries where haemolysin levels are high, and/or continuous blood supply of ABO-identical products is not guaranteed, and/or replacement of plasma with red cell additives is not common, it is desirable to exclude high titre group O donations as universal red cell donors [84,85].

Another group of naturally occurring human carbohydrate antibodies is that produced in response to foreign species antigens; so called "xeno-antibodies". For example antibody anti-Galα is specific for the antigen Galα1-3Galβ1-4GlcNAc; an antigen found in abundance in all non-primate mammals but absent in humans [89]. This antibody has long been considered a major barrier for non-human to human organ transplantation [89]. Other carbohydrate antibodies feature in contemporary studies to profile human populations in order to predict and improve response to vaccines [40,90], identify natural antibodies useful in immuno-oncotherapy [40,89,91-95] and study disease progression and management [96,97]. This is a large group of more than 100 antibodies [98], which includes three antibodies profiled in population studies [33,35,99] , namely anti- Galα, anti-Rhaα and anti-GalNAcα.

In this study, the following naturally occurring antibodies to carbohydrate antigens were studied in immunological assays:

- anti-A and anti-A,B of the ABO system
- anti-Galα
- anti-Rhaα
- anti-GalNAcα

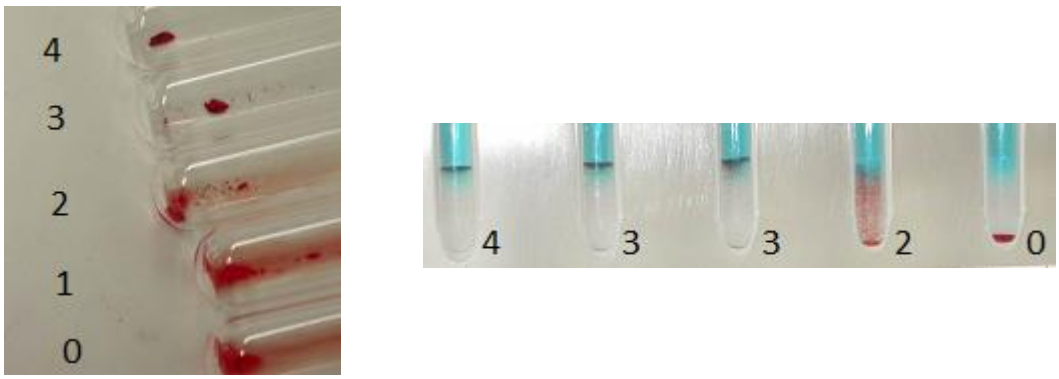
1.3 Carbohydrate antigen-antibody interactions *in vitro*

Landsteiner's original experiments formed the basis of the methods we still use for ABO typing. Today, monoclonal anti-A and anti-B are used to test for the presence or absence of ABO antigens on the RBC rather than human sera [6], but a patient's own serum (or plasma) is still used to define their ABO antibodies, to confirm their ABO group (so called "reverse grouping").

More recently, molecular typing methods, which describe and compare the sequence of blood group genes, have become available [3,4]. However, these methods are currently expensive, relatively time consuming and provide more information than is needed for most routine clinical work. Haemagglutination remains the preferred method to determine ABO blood group in routine patient situations, with molecular methods currently reserved for resolving anomalous blood groups [3,4].

1.3.1 Haemagglutination methods

Haemagglutination can be defined as the clumping together of RBC to form an agglutinate, which is the result of specific interaction between antigen and antibody and is visible without magnification aids. The predominant class of antibody affects whether a "direct" or "indirect" haemagglutination technique is used [6]. Anti-carbohydrate antibodies often possess a significant quantity of the immunoglobulin class M (IgM), and lesser quantities of class G (IgG) [32]. Due to their large pentameric structure, IgM antibodies are able to agglutinate red cells expressing the corresponding antigen when the RBC are suspended in saline. This method is known as the direct haemagglutination method, "direct" since RBC are able to agglutinate directly, when in contact with specific IgM antibody. Briefly, red cells are suspended to 5% in phosphate buffered saline (PBS) at 0.9%, and mixed together with serum in a test tube. The tube is centrifuged and the RBC button is observed for agglutination. The method can also be conducted in column agglutination technology (CAT) [100], where the red cells are suspended to 0.8% in a low ionic strength buffered saline, and the typing sera is incorporated in a matrix in a miniaturised tube. Centrifugation forces RBC into the matrix, where agglutinated cells are trapped at the top of the column (strong positive reaction), or near the top of the column (weaker positive reaction), whilst non-agglutinated cells travel to the bottom of the tube (negative reaction). Direct haemagglutination of RBC in tube and CAT is illustrated in Figure 3.



A **B**

Figure 3. Direct haemagglutination method in **A** tube and **B** CAT. Positive reactions are depicted in each at grades 4 (all RBC agglutinated), 3 (majority of RBC agglutinated), and 1 and 2 (some cells agglutinated). The negative reaction is graded 0 and shows no agglutination [101].

IgG antibodies can be detected using the antiglobulin technique, first described by Moreschi in 1908 [102], and re-discovered by Coombs in 1945 [103]. This test uses anti-human globulin (AHG) to bridge the gap between IgG sensitized but non-agglutinated cells, allowing them to agglutinate. This is often referred to as an indirect haemagglutination method, or indirect antiglobulin test (IAT). The term indirect was coined because the antigen-antibody reaction of agglutination can only be visualized after the addition of a secondary antibody. The IAT can also be conducted in tube or CAT platforms. In tube IAT, it is necessary to manually wash away unbound immunoglobulins found in patient plasma, as these bind to AHG and can invalidate the test [103]. This technical drawback is eliminated in CAT [100], as RBC pass into the matrix containing the AHG reagent during centrifugation, whilst any unbound antibody is unable to pass, thus effectively "washing" the cells free of unbound antibody by centrifugal forces.

These haemagglutination methods (the direct saline test and the indirect antiglobulin tests) are simple, reliable and reasonably inexpensive, and remain the methods of choice for routine blood grouping, including determination of an individual's carbohydrate blood group system antigen profile.

The ability of carbohydrate blood group antibodies to fix complement [42] is also exploited in specialised laboratory tests. The technique is no longer routine as plasma is used in most assays, and plasma contains chelators that inhibit complement [104]. However, when serum is used in direct saline assays, lysis of RBC may be observed. In addition to

agglutination, haemolysis demonstrates specific antigen-antibody binding *in vitro*. The appearance of RBC lysis in tube tests is illustrated in Figure 4.

Figure 4. Direct haemagglutination methods may show haemolysis. The figure shows visual differences between no haemolysis (**A**), partial haemolysis where some of the RBC have been lysed (**B**), and complete haemolysis with all RBC lysed (**C**).

In this study, the following assays were utilised to study carbohydrate antigen : antibody interactions *in vitro*:

<u>Haemagglutination</u>

- direct saline (both tube and CAT platforms)
- indirect antiglobulin test (both tube and CAT platforms)

<u>Haemolysis</u>

- classical pathway complement activation (tube only)

1.3.2 Antibody quantification methods

In some clinical scenarios it is desirable to quantify the level of blood group antibodies using a laboratory assay. Quantification of antibodies in patients preparing for ABO incompatible organ transplantation is important to inform clinical decisions, as ABO antibodies must be reduced to a low level to successfully transplant organs across the ABO barrier [46,50,52-55]. In addition, ABO antibodies at high levels in group O blood products present risks to transfused non-O patients [6,68-79] and blood services use quantification assays to identify these donors [69,70,72,83-85].

The most common method used to measure ABO antibody levels is titration, either in tube or CAT platforms. Antibody titration involves preparing serial dilutions of human sera or plasma, and testing these dilutions against target RBC, using direct and/or indirect haemagglutination tests. The highest dilution of serum to show a positive reaction of specified grade is the end point of the titre [101]. For example, in Figure 5 the

end point of the titre is seen with the 1 in 1024 dilution of the serum. The titre is reported as the reciprocal of the dilution; in this case 1024.

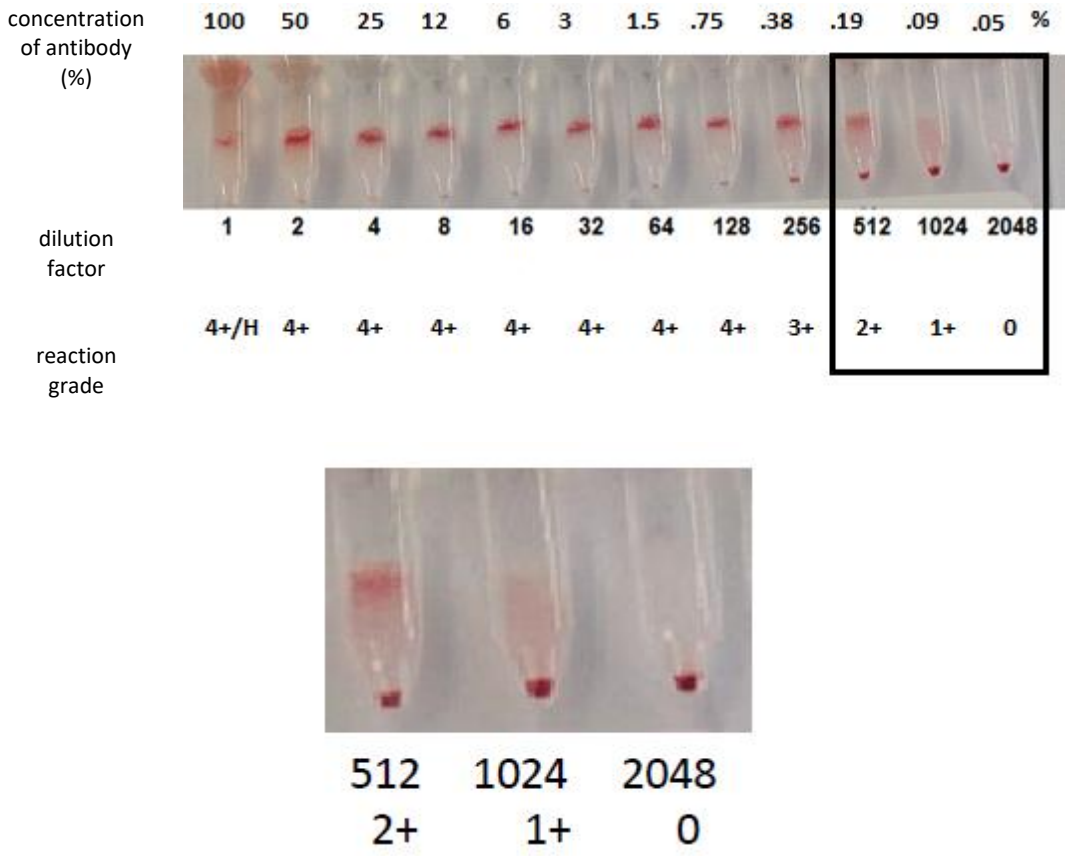


Figure 5. Titration of a serum performed in CAT. Dilution factors are labelled 1 (undiluted) to 2048. In the upper panel, the percentage of antibody compared to the undiluted sample is shown for each dilution. Grades of reaction are labelled below each dilution factor. 3+ and 4+ grades represent strong positive reactions, 2+ and 1+ weaker reactions. 0 is negative (no agglutination). In the undiluted tube (1 or neat serum), haemolysis can also be observed. This is indicated as H in the grading. The enlarged panel of the titration series shows the endpoint of the titre as 1024, where the final positive reaction is observed.

1.3.3 Assay Limitations

Limitations in haemagglutination assays can affect the validity and consistency of results. In chemistry laboratories, stringent standards are expected of chemical compound reagents and their lot to lot consistency [105]. In laboratory assays which characterize blood group antigens and antibodies, it is not always possible to adopt this stringent chemistry approach. Although human RBC are used as a source of antigen for laboratory reagents, the actual amount of antigen or its type cannot be controlled, and instead the reagent must rely on “what nature provided”. The standards applied by

International Standards Organisation (ISO) to medical laboratory testing [106] are more difficult to apply when human bodily fluids (for example RBC from blood) are used as reagents, due to their natural genetic variation.

Because human individuals have different alleles in their genomes, human RBC phenotypes differ considerably. For example, due to polymorphism of alleles at the ABO locus [107] blood group A individuals naturally fall into two main subgroups; A_1 and A_2 [108]. Since A_1 RBC have higher numbers of A antigen than A_2, anti-A antibodies may bind at different rates and in different quantities to A_1 and A_2 RBC. For characterization of ABO antibodies, A_1 cells are preferred as the reagent RBC, because they have less heterogeneity of A antigen number on the RBC than A_2 cells [109,110] and they are the strongest form of A phenotype [111]. Reagent A_1 RBC are frequently pooled from two or more individuals in an attempt to overcome natural variation between individuals [69]. Nevertheless, variation in different lots of reagent RBC is common and has been cited as a possible reason for differences in laboratory results [69].

A second problem associated with human RBC is that they naturally express not only the antigen leading to their selection as a reagent RBC, but also antigens of other blood group systems. For example, an A_1 RBC is selected as a reagent RBC for the presence of its A antigen, but it also possesses many other antigens from other blood group systems. The laboratory scientist considers a positive reaction with an A_1 reagent RBC as an indication that the patient has antibody A (anti-A) in their plasma. However, if the patient plasma under test contains antibodies to antigens outside the ABO system, and the A_1 reagent RBC expresses the corresponding antigen, then false positives occur and cause grouping anomalies. This is most often due to antibodies M and P_1 in patient plasma, with presence of corresponding M or P_1 antigen on the reagent RBC [112].

Finally, RBC are perishable and therefore it is not possible to prepare a standard batch of reagent cells with a long shelf life.

The inaccuracies associated with traditional titration methods for quantification of ABO antibodies have been well described [56,57,69,113-119]. Firstly, because the nature of the assay involves serially diluting serum or plasma, any error in volume delivery is doubled with each sample transfer, and assays are susceptible to “carry-over” (transfer of more

antibody than intended between tube transfers). Secondly, the assay is affected by batch to batch variation of target RBC, in the same way that blood grouping assays are affected. However, this problem is more evident in titration assays, where the variable of serial plasma dilution compounds the errors. Cooling *et al* reported significant difference ($p<0.0001$) in titre results conducted in the same laboratory over extended time intervals, and attributed this difference to variation in the number of RBC target antigens [69]. Technical aspects of titration assay performance can be monitored with a commercially available reference reagent of known antibody titre, such as those assessed by Thorpe [113,118]. Nevertheless, the natural variation in antigen copy number on human RBC cannot be controlled and will always vary between RBC batches. Thirdly, differences in grading ability amongst individuals performing the test are acknowledged to produce discrepancies in the tube titration assay in particular, but also in haemagglutination assays in general [120-122].

Differences between testing platforms produce further titration variables, and survey results show high inter-laboratory variation when different centres test the same samples [113,114,116-119]. CAT is replacing the conventional tube platform in many countries, and there is evidence that CAT reduces titration variation [114,116]. However, studies to investigate whether tube or CAT technique is more sensitive do not show universal agreement [68,69,114,116,123-125]. There is clearly a need for standardization of ABO antibody titration [56,113,114].

1.4 Immunological reactions work in concert

In immunohaematology and transfusion medicine there are two key components. The first is antigen and the second antibody, and together these two determine the ability to react; either in a laboratory assay or in the human body. It must be appreciated that antigen-antibody reactions are dynamic; that is an equilibrium exists between free antigen and antibody, versus antigen-antibody complex. Several factors affect the equilibrium constant to determine whether the equilibrium favours the formation of antigen-antibody complex; these include type and class of antigen, level of antibody, and environmental factors of temperature, pH, ionic strength and contact time between antigen and antibody [126].

The immune system is complex, and reaction dynamics depend not only on antigens and antibodies, but also on other factors such as the complement cascade, which can cause lysis of RBC. These factors work in concert to contribute to *in vitro* and *in vivo* consequences. The research in this thesis examined several key aspects of immunological reactions, namely:

- antigenicity
- actions and stability of complement
- measurement of antibody levels

1.5 Complement

1.5.1 The biological function of complement

The human immune system is a complex array of elements designed for survival of the species by protecting against infectious agents. In order to protect the species, the immune system of human individuals performs continuous surveillance; searching the body for foreign or "non-self" intruders, and taking action to eliminate them. Elements of the immune system work in concert rather than in isolation. One vital part of the human immune surveillance system is complement; a series of plasma proteins existing in the circulation as separate inactive components [42]. Through exposure to antibodies that have bound to antigen, or directly to foreign surfaces, these components come together in a cascade of activated building blocks, to ultimately form a complex which damages the cellular integrity of invading pathogens [44]. Complement assists the immune system to provide protection not only from invading micro-organisms, but also foreign tissues and materials [127].

Complement is found in a wide variety of animals, including humans, and is ancient in evolutionary terms [128]. In humans, complement proteins constitute 10% of total serum proteins, and of these complement proteins C3 is the most abundant and is central to all pathways of complement activation [129].

An important role of complement is cell lysis in the innate immune system. Through formation of a membrane-attack-complex (MAC), the cell membranes of microbes can be punctured, resulting in the death of a pathogen [44]. Complement also participates in the immune response in many other ways, grouped together by Muller-Eberhard as

"activation of specialized cell functions" [130]. However, these specialized functions are outside the scope of this study, and will not be further discussed.

1.5.2 The complement cascade

There are three pathways of complement activation: classical, alternative and lectin. Activation of the classical pathway is antibody-dependent, whereas this is not the case for the alternative and lectin pathways [129]. As the alternative and lectin pathways were not utilised in this study, they are not further discussed here.

1.5.3 Nomenclature and classification

Knowledge of the plasma proteins constituting the complement system is still evolving. There are currently over 35 recognised proteins which comprise the human complement system [44]. Complement proteins are designated with the prefix C and numbered C1 to C9, in the order of their discovery. They are grouped in three families; the recognition unit C1 (C1q, C1r and C1s), the activation unit (C4, C2, C3) and the membrane attack unit (C5, C6, C7, C8 and C9) [130].

In the classical pathway, complement activation proceeds by a series of enzymatic protein cleavages. The presence of magnesium and calcium metals is required for activation and cleavage [131]. One complement protein acts as a trigger for cleavage of a subsequent complement protein. This stepwise nature of the pathway, whereby one activated protein binds more than one subsequent protein, means that a relatively small initiating event can result in a significantly larger effect, and therefore the complement cascade constitutes a powerful immune response [42,43,132].
In common nomenclature, cleaved subunits are differentiated from intact proteins (for example C3) by the use of lower case letters (for example C3a and C3b). The smaller molecular weight fragment is given the "a" designation, and the larger the "b" [133].

Because the complement system is potentially very destructive, it is also paired with an inactivation system [42,132,134], which constantly checks and inactivates complexes as required (see further detail in section 1.5.5 Pathway regulation).

1.5.4 Pathway Activators

Different substances, both biological and non-biological act as activators of the three complement pathways. Activators of the classical pathway include antibodies, some viruses, some bacterial species and polyanions [6,132,135,136]. When an antibody binds to a specific antigen, complement may or may not be activated via the classical pathway. Complement activation requires a shape change in the complement protein C1 [132]. C1 consists of three sub-units; C1q, C1r, and C1s. Figure 6 shows intact C1, with C1r and C1s protectively positioned within a ring of six C1q molecules. The complement receptors for the fragment crystallizable region of antibodies (Fc) are located within the C1q globular head. Each head group consists of six subunits. The activation shape change in C1 can only be effected by two Fc fragments binding to one or more C1q sub-unit. Therefore, complement activation is more likely to occur with antibodies present in higher concentrations, and is also more likely with antibodies of the subclass IgM than IgG [137], because IgM have pentameric structure and contain 5 Fc fragments. The IgG, with its single Fc, can only effect the C1 shape change when it is present in high concentration, aggregated with other IgG molecules, and/or contained in an immune complex [42].

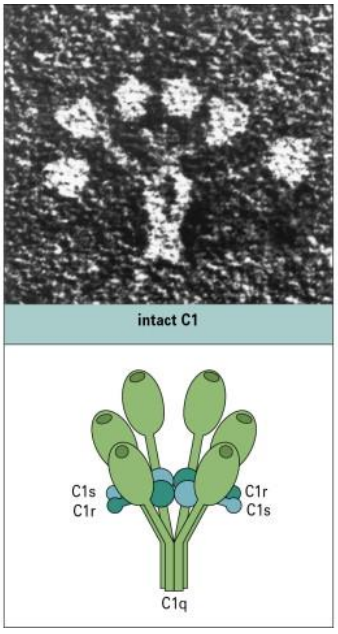


Figure 6. Intact C1 molecule of human complement. The top picture shows an electron micrograph, whilst the lower schematic is modelled, and shows the positioning of the C1s and C1r pro-enzymes lying within the ring of six heads. The heads contain the receptor sites for Fc regions of immunoglobulins. This figure was published in Immunology, seventh edition, Male, Brostoff, Roth & Roitt, p91, Copyright Elsevier (2006) [129]. Reproduced with permission.

C1 activation also requires Ca^{++} ions [42,131]. Once bound to Fc in the presence of calcium, intact C1 cleaves C1s from the molecule, thus activating it and exposing receptors for subsequent complement proteins. Activated C1s can bind to two further molecules, C4 and C2. Activated C1s cleaves C4 to produce C4a (a smaller molecule) and C4b (a larger molecule). Once this has happened, a thioester bond within C4b becomes unstable and prompts the binding of C4b to C2. The C4b2a complex cleaves C3 to subunits C3a and C3b. Like C4, C3 contains a thioester bond, which becomes unstable once C3b is exposed. In an attempt to restabilise the bonding, C3b binds to activated C4b2a forming active C4b2a3b. The formation of this C4b2a3b complex is critical to the progression or cessation of the rest of the complement cascade. If not bound at this point, C3b degrades to C3d. This binding or non-binding gateway makes it difficult to detect stable C3b on human red cells *in vitro*. It is for this reason that commercially produced antibodies in AHG to detect the activation of complement on human red cells contain anti-C3d rather than anti-C3b [138].

After C4b2a3b is formed, the cascade proceeds to the binding of C5. C4b2a3b cleaves C5 to C5a and C5b. C5a is released, C5b is bound. This cleavage of C5 is the final enzymatic step in the classical pathway, with the subsequent steps relying on assembly (rather than cleavage) of proteins. From C5, the pathway may be referred to as the "terminal pathway". Complement proteins C5b, C6, 7, 8 and 9 units assemble to form a membrane attack complex (MAC). As in the first part of the pathway, MAC assembly is believed to take place in a stepwise fashion, with protein binding producing conformational changes in proteins [44]. C5b binds to C6 and then C7. This complex is labile and hydrophobic. Hydrophobicity contributes to the eventual insertion into a cell membrane, as lipid-rich cell membranes comprise a more hospitable environment to the hydrophobic MAC. Membrane bound C5b67 recruits C8, and finally multiple copies of C9. The C5b6789 complex is a rigid protein-lined channel which is able to puncture the cell membrane of the cell. Once cell membrane puncture has occurred, the osmotic balance is disrupted, and the cell is lysed [42,44]. This classical complement pathway is illustrated in Figure 7.

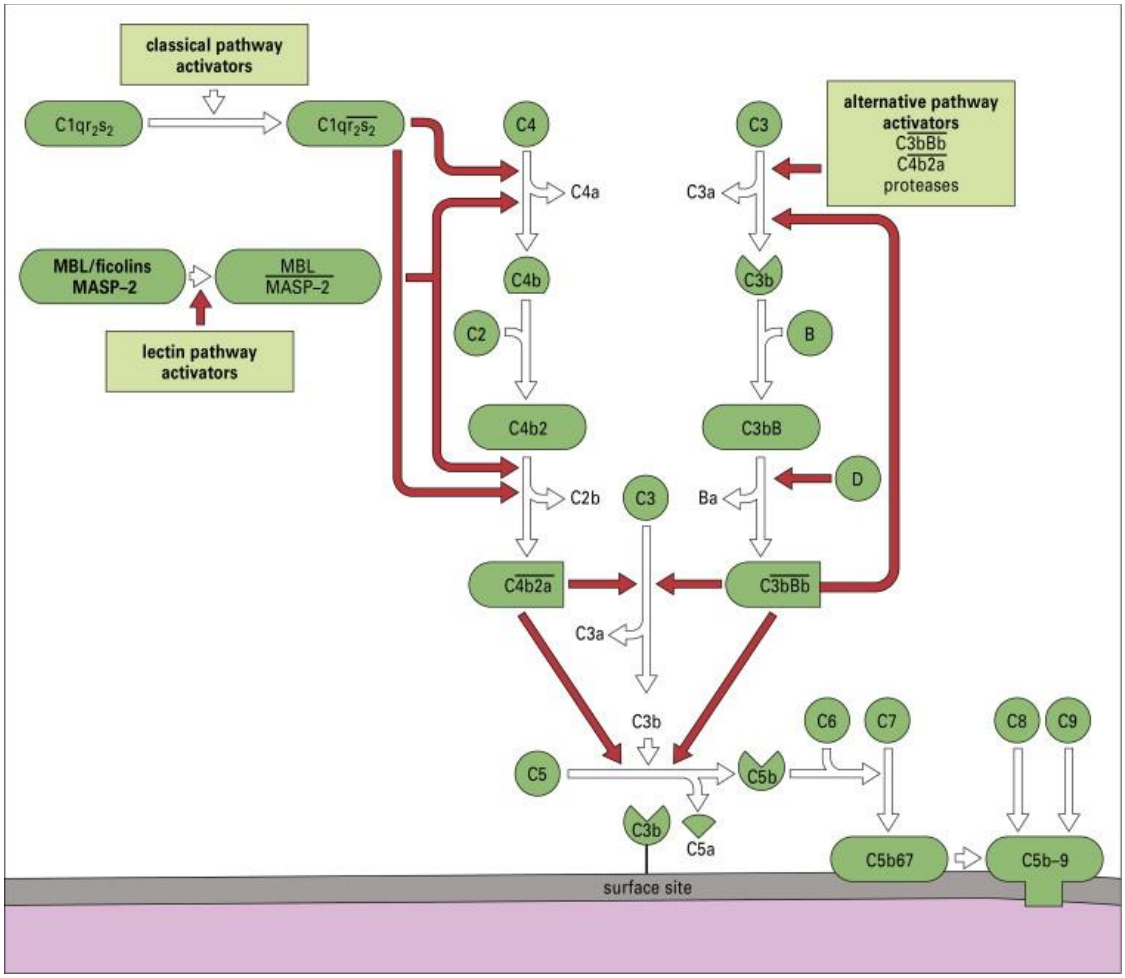


Figure 7. Classical, alternative and lectin pathways of complement activation. Reproduced with permission. This figure was published in Immunology, seventh edition, Male, Brostoff, Roth & Roitt, p90, Copyright Elsevier (2006).

Within human blood group systems, antibodies of some systems are able to activate complement. Carbohydrate blood group systems ABO, Lewis, P and I are notable in this regard because IgM antibodies predominate [6]. Complement-activating antibodies can produce catastrophic consequences for patients transfused with incompatible blood, by virtue of the fact that incompatible red cells are lysed. Free haemoglobin, alongside red cell membrane debris and immune complexes and in human circulation leads to potentially fatal complications including renal failure [6,139] and disseminated intravascular coagulation [6]. Antibodies in non-carbohydrate blood group systems vary in their ability to activate complement. Not all blood group systems activate the complement cascade [42]. Rh system antibodies only rarely activate complement [42,140]

whereas Kidd system antibodies regularly do so [141,142]. This depends not on the class of immunoglobulin, since both are predominantly IgG [6,141], but on the location of the corresponding antigen on the red cell surface. Rh antigens are too far apart on the RBC surface for IgG molecules to bind in close proximity and activate C1 [140], whilst those of the Kidd system, although much fewer in number, occur in clusters of high density.

1.5.5 Pathway regulation

Due to the powerful nature of complement activation, there is a high need for control and regulation as unchecked, the pathway would continue until C3 supply was exhausted, a circumstance which could prove clinically disastrous [42,132,134]. Not only would target cells be eliminated by MAC, so also would cells of the host. This is illustrated in the ABO-incompatible blood transfusion, whereby lysis of the host's own RBC occurs, in addition to initial lysis of the incompatible, antibody-coated transfused cells. This is sometimes called "innocent bystander" haemolysis [43,134], where C1q lands on all cells in near proximity, as well as the target cells, and thus all cells in a localised area are subject to lysis by complement activation.

The control mechanisms to keep the complement cascade in check are many. Firstly, many of the complement complexes formed are unstable and therefore reversible up to a point [42,130]. Factors such as concentration of complement proteins, pH and ionic strength of the environment influence the dynamics of the reaction and consequent complex stability [143].

Secondly, regulators exist, both as soluble plasma proteins or molecules bound to human cell membranes. These have the specific function of regulating activation of the complement cascade [144]. One of these is complement regulatory protein (CD59) which blocks the assembly of the MAC very late in the cascade. CD59 is found in plasma and tissues and can bind to C8 within the C5b6789 complex and prevent formation of the final protein-lined channel which pierces the cell membrane. It does this by preventing "unfolding and polymerisation of the final component C9" [144].

Complement activation can be stopped *in vitro* by the addition of chelating agents such as ethylenediamine tetraacetic acid (EDTA). EDTA is commonly used as a pre-analytical anticoagulant during phlebotomy at a concentration of 120 mmol/L, to produce plasma

rather than serum for medical laboratory assays [104,145]. Because EDTA chelates calcium it also inhibits complement activity, preventing the formation of catalytic C(1qr2s2) in the classical pathway. Sometimes it is desirable to stop complement activation in an assay utilising antibody-induced complement-mediated haemolysis (for example to provide a timed stop point in an assay quantitatively measuring free haemoglobin). In this case EDTA at a final concentration of 100 µmol/L in assay samples will effectively halt in vitro complement activation [136].

1.5.6 Complement stability

As with any laboratory assay, the methods for measuring complement are subject to consideration of pre-analytical factors. Loss of complement, or *in vitro* complement activation, can affect final results. *In vitro* activation of complement, and deterioration of complement is minimised at low storage temperatures (≤ minus 70°C), allowing storage periods for samples containing complement of three to six years [127,146]. For assays that rely on complement in the patient sample, for example ABO haemolysin detection [72,79,83-85], assessment of complement deficiency [147,148] and auto-immunity studies [149-152], scientists rely on storage guidelines to ensure samples are stored at the correct temperature for no longer than the stated time. The detection of some blood group antibodies (notably those of the Kell, Kidd and Lewis systems) can be enhanced in serum-based methods [6,145], and it was for this reason that George Garratty was interested in establishing storage guidelines for sera samples [145]. Accordingly, Garratty designed a study in which he tested aliquots of human serum subjected to different storage times and temperatures in a modified 50% haemolytic complement activity (CH_{50}) assay. He reported his findings quantitatively from spectrophotometry readings, and calculated percentages of complement activity. Garratty's study was published in 1970 [145]. Some years earlier, Polley & Mollison conducted a similar study, but their study differed from Garratty's in that they reported their data qualitatively, recording visual observations in place of spectrophotometric readings [153].

The findings of Garratty [145] and Polley & Mollison [153] had not been revisited but are worth revisiting for several reasons. Firstly, the stability of complement appears to be poorly understood in the wider context of laboratory testing [146,154] with Lachmann stating that it "is, perhaps, surprising that there is relatively little literature on the topic

of how serum should be prepared in order to perform functional complement assays. It is apparent that good practice in this area is not always followed, even by complementologists, let alone by companies developing diagnostic tests [154] ". Although the topic of complement stability has been revisited by Yang [146] at very low storage temperatures (minus 80°C), storage temperatures of minus 20°C and 4°C were not assessed. Others have looked at the storage of complement in serum and found evidence of complement activation during storage, but did not undertake any functional assays based on lysis of RBC with stored sera [146,155].

1.5.7 CH_{50} assay

Amongst assay screens for human complement deficiencies, the CH_{50} is the oldest method [156]. Although there are many more sophisticated assays available for the measurement of complement and its components today [127,147], the CH_{50} assay is still used in many diagnostic laboratories as a first-pass screening method to assess functional activity [127,147,148]. This assay involves incubating dilutions of fresh human serum with sheep RBC. To overcome the confounding variable of natural differences in human anti-Galα and anti-Forssman (Fs) levels, the sheep RBC are traditionally coated with an anti-sheep complement-activating antibody produced in rabbits [148], with specificity for the Fs and Galα1-3Galβ1-4GlcNAc antigens [157]. Upon incubation with fresh human serum containing complement (and xeno-antibodies anti-Galα and anti-Fs), complement is activated by the classical pathway. The classical pathway proceeds and ultimately produces lysis of the sheep RBC, and the degree of haemolysis is considered to be proportional to the total level of complement available for classical pathway activity [147,148]. Released haemoglobin is measured by spectrophotometry, and the reciprocal of the dilution required to lyse 50% of a specified amount of RBC is reported, producing a semi-quantitative result of complement activity [148]. The endpoint of 50% haemolysis was chosen because early work demonstrated that the amount of complement, and the proportion of cells lysed is not linear outside the 30-80% range (Figure 8) [156,158]. Dilutions of serum are necessary because undiluted serum in individuals with normal or high complement levels usually produces 100% cell lysis, which is outside accurate analysis limits of the assay. Thus the assay is not informative to quantify complement levels without dilution of serum.

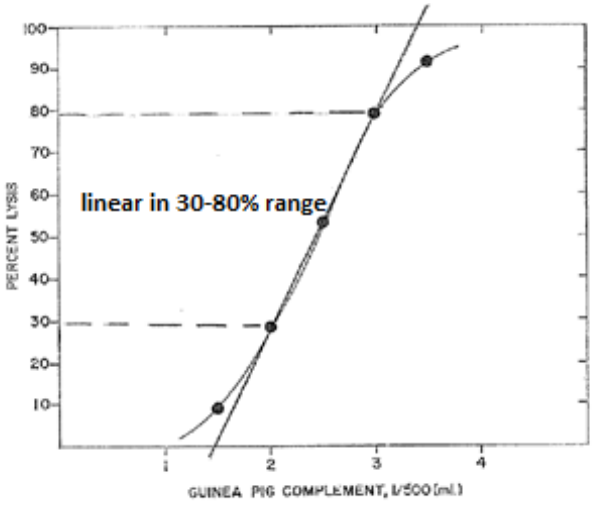


Figure 8. Percentage of lysis of sensitized sheep RBC as a function of the volume of diluted serum complement in a functional assay to measure RBC lysis. A sigmoidal curve is produced, with linearity demonstrated in the 30-80% lysis range. Reproduced and adapted from Experimental Immunochemistry (p135), by E.A Kabat & M.M Mayer, Springfield, USA. Charles C Thomas. (1961).[156]

In early studies of complement stability in stored human sera samples, Garratty [145], and Polley & Mollison [153] used modified CH_{50} assays to measure lytic complement activity. Despite its currency as a functional complement assay, several problems exist with the CH_{50} assay:

- The method relies on serum dilution, which is problematic for standardization and reproducibility, and introduces method error [147]. Assay reproducibility is subject to dilution of the lowest active reaction component in a multifactorial system; that is the reaction endpoint is reached when the weakest link is broken
- The CH_{50} utilises sheep RBC as indicator cells. It is widely acknowledged that batch to batch variation of sheep cells can affect results [145,148,153,156,158,159]. This batch to batch variation is due to natural genetic diversity amongst sheep, resulting in differences in number of expressed antigens (zygosity) on the RBC surface. This affects the accuracy of the assay, because antigen number affects number of antibody molecules that bind [126], in turn affecting the amount of complement bound [156]
- The method uses non-human complement-activating heterophile antibodies to presensitize the sheep RBC, usually from rabbits. Batch variation in heterophile

antibodies is known to be problematic [148]. Further, presensitization adds a further test variable, since efficiency of sensitization depends not only on batch consistency of antibody, but also on experimental variables of temperature, time and ionic strength [126]

The components of the CH_{50} assay are illustrated in Figure 9, to highlight the multiple antigens and antibodies taking part in the reaction.

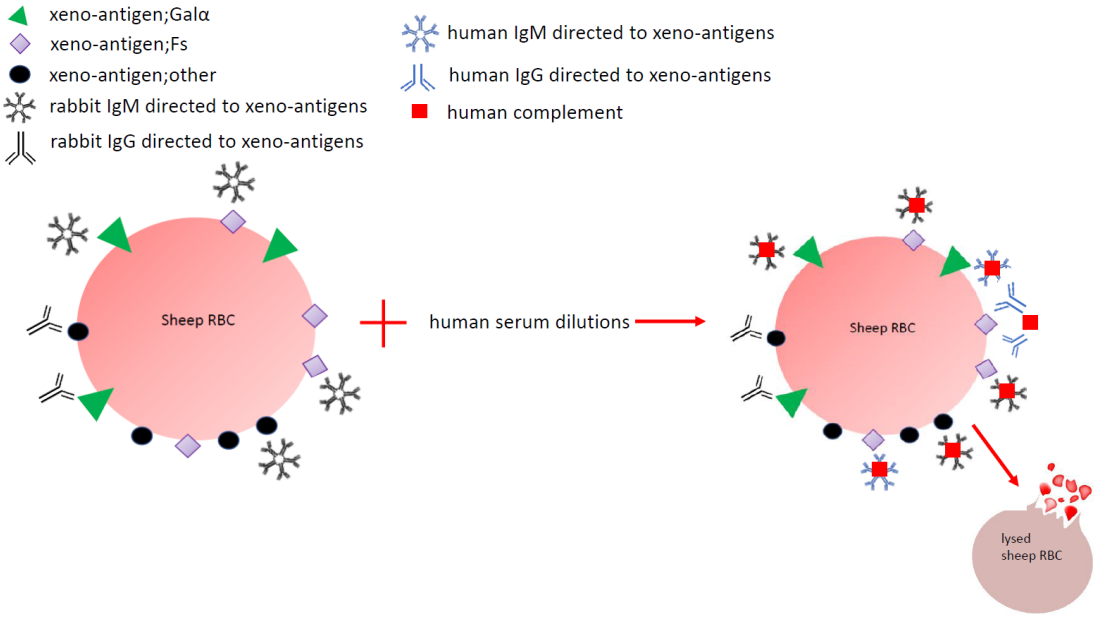


Figure 9. Diagrammatic representation of the CH_{50} assay. Sheep RBC are presensitized with antibodies with specificity for sheep antigens, in serum produced in rabbits. Sheep RBC express target antigens Forssman and Galα1-3Galβ1-4GlcNAc, along with other sheep antigens. Rabbit serum contains a mixture of antibodies, including IgM and IgG forms of anti-Forssman and anti-Galα, but also antibodies to other sheep antigens (both IgG and IgM forms). Human serum also contains IgM and IgG forms of anti-Forssman and anti-Galα, in addition to other naturally occurring antibodies, and complement (red box); the only actual element of interest in the CH_{50} assay.

1.6 Quantification of ABO antibodies

There is a wide range of titres reported for natural ABO antibodies, both in children [160] and adults [161]. There are also different levels observed in different ethnic groups and geographical regions [161,162], and it is normal for an individual's levels to fluctuate throughout a lifetime [163] depending on which bacteria individuals are exposed to [23]. ABO antibody levels can be boosted as a result of active and specific immunisation events such as pregnancy with an ABO incompatible fetus, and some vaccinations [163]. Bird, as cited by Mathai *et al* [83] also noted that mosquito bites and intestinal parasitic infections can boost titres of ABO antibodies. Antibodies boosted as a result of active and specific

immunisation events, described by Race and Sanger as “hyper-immune” [163], present as IgG warm-reactive antibodies [164]. Like natural ABO antibodies, immune ABO antibodies may demonstrate an ability to activate complement and lyse red cells in laboratory tests or *in vivo* [42,163]. Consequently it is difficult to assign any sort of normal range to quantities of ABO antibodies, despite the fact that they are one of the dominant antibodies in human blood [32].

Natural ABO antibodies may be of immunoglobulin class M (IgM), G (IgG) or A (IgA) [32,164]. Blood group A and B individuals produce predominantly IgM anti-B and anti-A respectively. However group O people have been noted for having IgG anti-A, anti-B and anti-A,B [115,165,166].

Carbohydrate blood group antibody quantification is not a straightforward exercise. For some antibodies, for example anti-D in the Rh blood group system, it has been possible to develop flow-cytometry methods to measure the concentration of antibody in nanograms per millilitre, and these methods have been adopted for quantification in clinical situations such as assessing risk to a fetus from haemolytic disease of the fetus and newborn [167]. However, methods for accurately quantifying ABO antibodies are difficult, due to the plethora of blood group A and B structures (Table 1) against which ABO antibodies are produced [41]. Furthermore, methods which determine only the concentration of ABO antibodies may not be clinically useful, as they do not necessarily reflect the *in vivo* affinity of antibodies for RBC [168,169].

The two most common diagnostic methods used by blood transfusion services to quantify ABO antibodies are titration [45-50,56-58,60-62,72,79,113-119,125], a semi-quantitative assay with haemagglutination as the end point, and the haemolysin test [72,79,83-85], a qualitative assay with haemolysis as the end point. The haemolysin test relies on activation of the classical pathway of complement, whereas the titration method does not involve complement. The lack of a standard method to quantify ABO antibodies is widely commented on in the literature [56,57,69,72,74,77,79,113-115].

1.6.1 ABO antibody titration

The principle of this test is that serum or plasma is diluted in a doubling dilution series in 0.9% saline, from dilution factors of 1 (undiluted) to 2048 or greater. Aliquots of this dilution series are then incubated with target A or B cells, and examined for agglutination in either the direct or indirect haemagglutination assay. The titre is reported as the highest dilution causing agglutination [101].

Whereas the application of the haemolysin test is limited to detection of high level antibody in blood components [72,79,83-85], applications of antibody titration are broader. In addition to detecting high level ABO antibody in blood donors, applications of ABO antibody titration include monitoring of levels in patients preparing for ABO incompatible kidney or pediatric heart transplantation [45-67], monitoring ABO mismatched bone marrow transplantation [170] and predicting and monitoring hemolytic disease of the fetus and newborn [171]. Although widely used, antibody titration suffers from lack of precision and accuracy to a remarkable degree [69,113-119].

1.6.2 The haemolysin test

The principle of this test is that fresh donor serum (either neat or as a series of dilutions) is incubated at 37°C, with both group A and B RBC [83-85]. After incubation, the supernatant is examined visually or spectrophotometrically for haemolysis. ABO antibodies capable of activating complement and causing haemolysis are deemed high-level. The test is elegant in its simplicity, however, its performance as a predictor for clinical haemolysis has been remarkably under investigated [72,79]. Landim *et al* (2015) found poor correlation between the haemolysin test and antibody titres, as did Mathai, at least for Anti-A [79,83].

1.6.3 ABO antibody quantification for monitoring ABO incompatible kidney transplantation (ABOiKT) patients

As stated earlier, it is necessary to reduce the level of ABO antibodies in ABOiKT recipients to avoid kidney rejection and graft loss, using strategies including adsorption of ABO antibodies onto synthetic ABO antigen loaded columns [46,52-55], plasmapheresis (PP) [46,50,51,63] and suppression of antibody producing cells with anti-CD20 medications such as rituximab [46,47,50-52,61,63]. In New Zealand, four rounds of PP and rituximab therapy are applied to patients prior to transplantation [50], and anti-A and anti-B must be reduced

to an antiglobulin titre of ≤16 for transplantation to proceed (I. Dittmer, personal communication October 18 2016). ABO antibody titres are monitored immediately before and after each round of PP, using conventional serological titration methods (L.Wall, personal communication November 11 2016). Although there had been no antibody mediated rejection at the time of communicating with the local renal transplantation unit [50], the prospect of an alternative laboratory assay to monitor antibody level in place of, or in addition to titration, was of interest to the clinical team (I. Dittmer, personal communication October 18 2016).

A definitive ABOiKT pre-transplantation antibody titre has not been defined internationally [60], probably due to the significant variance observed between laboratories when assaying the same sample [113,114,116-119]. Therefore different transplantation centres have different acceptable titre cut-offs to allow transplantation to proceed, ranging from ≤4 to ≤ 32 in the IAT titre [45,46,48-50,60,61]. This variation makes international comparison of ABOiKT outcomes somewhat difficult, and together with acknowledged inaccuracies of titration, potentially jeopardises patient safety and transplantation outcomes [56,57,60,113,119,169].

1.7 Kode™ Technology

1.7.1 Technology overview

The basis of Kode™ Technology is cell-surface modification, based on the insertion or attachment of chemically synthesised molecules to a wide variety of non-biological and biological surfaces, including RBC [19,172,173]. The Kode™ molecules have a common design, and are collectively known as Function Spacer Lipids constructs (FSL). There are currently over 60 FSL, with more in development [174]. Each FSL has a common design consisting of three units; a lipid tail (either 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), ceramide or sterol), a spacer; usually carboxymethylglycine (CMG) and a functional small molecule head [174] (Figure 10).

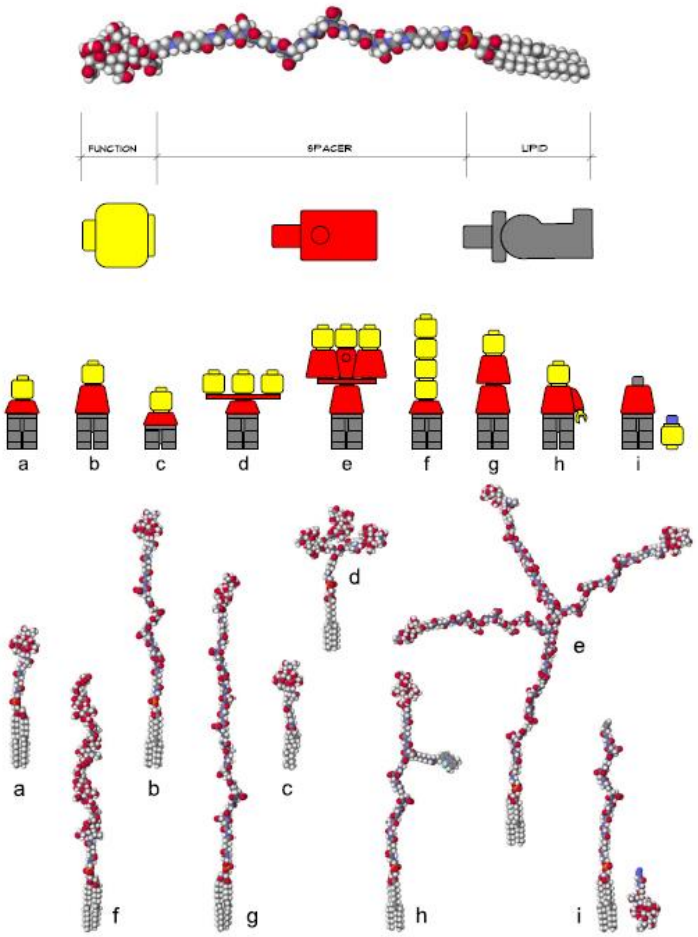


Figure 10. Schematic representation of different FSL Kode™ Technology construct presentations. The building block toy figure representations show a yellow functional head, the red body represents the spacer and the grey legs represent the lipid tail. Assembled building block figures a - i show the flexibility of mixing and matching functional head, spacer and lipid tail types. The same structures a - i are also modelled to show chemical structures. Structures a - e, and g - i each have the same tetrasaccharide blood group A functional head, whereas f has a hyaluronic acid functional head. Variation representations are (a) short 1 nm adipate spacer (b) CMG 7 nm spacer (c) sterol lipid instead of DOPE (d) clustered head (e) trimeric CMG spacer (f) linear repeating functional heads (g) double CMG spacer (h) functionalized CMG spacer (i) secondarily attached functional head. Reproduced from the author's work [174].

The functional head can be one of a wide variety of carbohydrates, peptides or other small molecules. A large proportion of the 60 FSL are blood group antigen-related structures. The spacer is a biologically inert structure unique to Kode™ Technology. The spacer allows presentation of antigen away from a surface without steric hindrance, by virtue of its length (about 7 nm), which separates the antigenic head from the attachment surface. The spacer also aids with rendering FSL dispersible in water [172]. Water dispersibility is an attractive feature for simplicity of use of the molecules, and

means that the technology is suitable for use with living cells, unlike organic solvents required to dissolve some other glycosphingolipids [175].

The lipid tail is hydrophobic, which allows rapid and stable insertion of FSL molecules into lipid bilayers such as the RBC glycocalyx. Functional heads, spacers and lipid tails can be interchanged to provide FSL with a wide variety of designs and maximum functionality [174]. The antigenic head can be monomeric, dimeric or trimeric. Trimeric (branched) heads have three copies of the antigen per molecule, to increase antibody binding capacity. Spacers can be flexible or rigid, with the design depending on the application. Figure 10 shows design combinations that have been synthesised. Carbohydrate FSL range from simple monosaccharides to large polysaccharides [176].

Modification of RBC with FSL is a very simple process involving incubation of cells with a solution of FSL of a specified concentration for one to two hours [19]. During the incubation time, the lipid tail of the FSL inserts itself into the glycocalyx of the RBC, leaving the antigenic head uppermost and therefore well positioned for antigen-antibody interactions (Figure 11). RBC thus modified display normal functionality in *in vitro* assays [177].

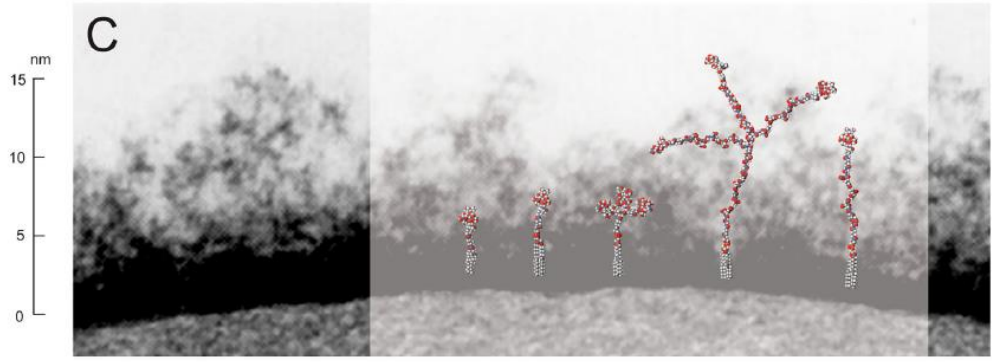


Figure 11. Positioning of FSL constructs of various designs defined in Figure 10 in RBC glycocalyx. Reproduced from Henry and Bovin, 2018 [19] modified and reproduced from Voet and Voet, 2011 [21] with permission of the publisher John Wiley & Sons. Images remain subject to the copyright conditions of John Wiley & Sons.

1.7.2 Kode™ Terminology

Terminology adopted in this research to describe processes utilising FSL has been published [19,177,178] and is as follows:

1. Modification of surfaces or cells with FSL is called koding
2. The resulting cell following koding is called a kodecyte
3. RBCs koded with FSL at a given concentration are known as "concentration-FSL name-kodecytes", for example RBCs koded with FSL-Galα at 100µg/mL are named 100-Galα-kodecytes.

1.7.3 Kode™ Technology advantages

Kodecytes are superior to natural human RBC in diagnostic and research assays for several reasons. Firstly, because kodecyte antigens are spaced away from the RBC surface by virtue of the FSL spacer, antigen is presented to specific antibody with less steric hindrance than may be present with natural RBC [179]. Secondly, there is no variation in antigen zygosity as there is on natural RBC of the same phenotype [109-111], because antigen in the form of FSL is added to the cell at a controlled concentration [176]. This feature of Kode™ Technology therefore eliminates the batch to batch variation associated with assays using natural RBC as antigen targets [69,145,148,153,156,158,159]. Each batch of kodecytes is created in exactly the same way, whereas natural RBC are sourced from different individuals at different times. Finally, the ability to control the quantity of FSL inserted in kodecytes means it is possible to create a panel of RBC expressing the same antigen over a range of concentrations; a concept of "antigen dilution". In the past, it has not been possible to control the level of antigen expression on human RBC used as blood grouping reagents in the laboratory. As mentioned earlier, A_1 cells are selected by commercial RBC distributors for reagent RBC of blood group A, but it is well known that the number of A antigens in A_1 individuals still shows considerable variation between individuals [109-111]. Also, the average A antigen number in A_1 individuals is very high (800,000 on average) [111], and the majority of group B and O plasmas will agglutinate the RBCs when the plasma is undiluted. Therefore no information is gained about the quantity of antibody present. In order to differentiate between an individual with high level antibody and one with low level antibody when testing with natural RBC, it is necessary to dilute the plasma to see a difference in quantity.

As discussed earlier, there is a high degree of inter-laboratory variation in assays utilising natural RBC and plasma dilution [57,69,113-119,145,148,153,156,158,159]. With Kode™ Technology, it was possible to move away from plasma dilution, and use undiluted plasma in both the alternative assay to the CH_{50}, and in the quantification of natural antibodies (ABO antibodies, anti-Galα, anti-Rhaα and anti-GalNAcα). In the antibody quantification studies, Kode™ Technology allowed the antigen concentration rather than the antibody concentration to be varied, by the insertion of FSL in known and different concentrations in a panel of kodecytes.

1.7.4 Overview of FSL used in this study

FSL-A type 2

The FSL of choice for quantification of anti-A and anti-A,B was A type 2, which has the same tetrasaccharide antigenic head (GalNAcα1-3(Fucα1-2)Galβ1-4GlcNAc) as natural A type 2 chains do [12,180,181]. The nomenclature of A type 2 refers to the chain type (type 2) and should not be confused with the natural A_2 RBC phenotype. FSL A type 2 is depicted in Figure 12. The formula is $C_{118}H_{197}N_{18}O_{52}P$, and the molecular weight is 2731.

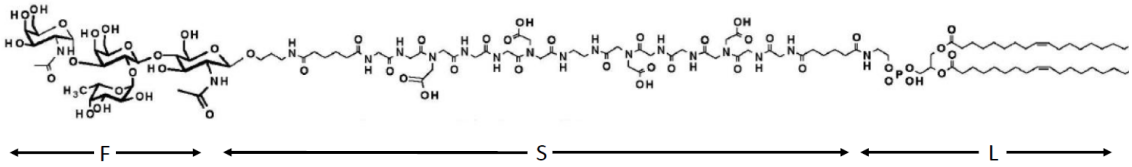
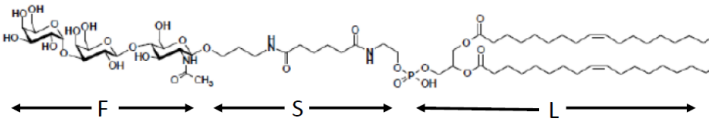


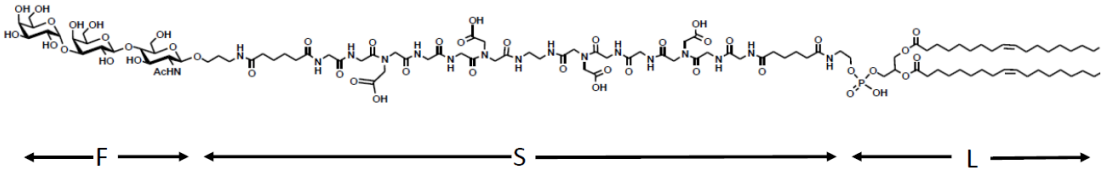
Figure 12. Chemical structure of FSL-A type 2. F = functional head (A antigen), S = CMG_2 spacer, L = lipid (DOPE)

FSL-Galα

Galα1-3Galβ1-4GlcNAc is an antigen that occurs naturally on many cells of non-primate mammals, prosimians, and New World monkeys, but not in apes or humans [182]. It is possible to modify human RBC to express the Galα1-3Galβ1-4GlcNAc antigen (Galα) by koding them with FSL-Galα [174,178,183]. The FSL functional head is identical to the animal Galα antigen (type 2 chains) [89]. FSL-Galα designs, formulae and molecular weights for FSL with spacers adipate and carboxymethylglycine (CMG) are depicted in Figure 13.



a) FSL-Galα adipate spacer design. $C_{70}H_{126}N_3O_{26}P$, Mw 1457



b) FSL-Galα CMG_2 spacer design. $C_{110}H_{179}N_{17}Na_5O_{48}P$, Mw 2654

Figure 13. Chemical structures of FSL-Galα with a) adipate and b) CMG_2 spacers. The functional head is Galα1-3Galβ1-4GlcNAc.

FSL-GalNAcα

The GalNAcα1-3Galβ1-4GlcNAc glycan is not found in animals. It is therefore considered a xeno-antigen by humans. The functional head group of FSL-GalNAcα is the trisaccharide GalNAcα1-3Galβ1-4GlcNAc. This FSL is depicted in Figure 14. The formula is $C_{112}H_{187}N_{18}O_{48}P$, and the molecular weight is 2585.

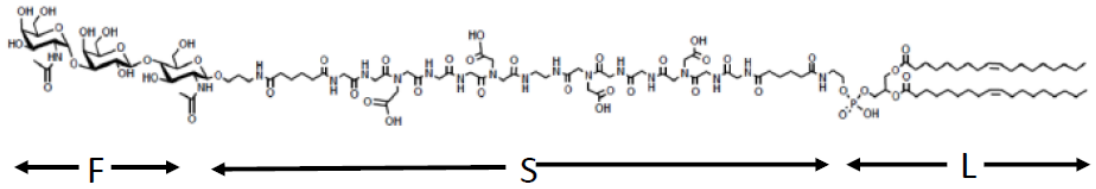


Figure 14. Chemical structure of FSL-GalNAcα, with functional head GalNAcα1-3Galβ1-4GlcNAc labelled F, CMG_2 spacer labelled S, and DOPE tail labelled L.

FSL-Rhaα

Rhamnose (Rha) is a common glycan found in bacteria [24-26], fungi [184], viruses [185] and plants [186]. The functional head group of FSL-Rhaα is monosaccharide Rhamnose.

FSL-Rhaα is depicted in Figure 15. The formula is $C_{96}H_{161}N_{16}O_{37}P$, and the molecular weight is 2162.

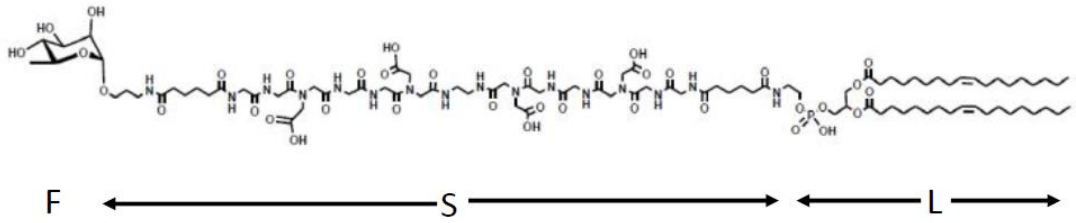


Figure 15. Chemical structure of FSL-Rhaα with functional head Rha labelled F, CMG_2 spacer labelled S, and DOPE tail labelled L.

1.7.5 Antibodies to FSL antigens used in this study

Blood group B individuals have anti-A in their plasma, whilst blood group O individuals have both anti-A and anti-A,B [6]. These two antibodies of the ABO system were quantified using A type 2 kodecytes in this study.

Nearly all human sera contain antibodies to the three glycan antigens expressed on FSL Galα, GalNAcα and Rhaα [33,35,99]. Antibodies to many glycans are capable of activating complement [89,91,178]. This has been of interest for some years in the field of immuno-oncotherapy, whereby researchers aim to decorate cancer cells with specific antigen motifs, to induce antibody-mediated-complement destruction of tumour cells [40,89,91-95]. FSL have the potential to decorate cancer cells [92-94,174]. Although immuno-oncotherapy is outside the scope of this thesis research, the understanding of *in-vitro* antigen-antibody binding on kodecytes, with subsequent haemagglutination and lysis by complement activation, can inform work in the field.

In addition to cell-based assays, solid-phase assays are popular for identification and quantification of natural antibodies to carbohydrates [33,35,38,91,96,97,99,187,188]. FSL have been successfully attached to an extensive range of solid surfaces by Barr [189], with subsequent binding of antibody with specificity for the FSL antigen. In this research, Barr's work was extended to investigate whether it is possible to deposit complement on the surfaces of paper, glass and plastic by antibody-mediated complement activation.

The FSL candidates Galα, GalNAcα and Rhaα in this research were chosen for their high levels of corresponding antibodies (relative to other glycan antibodies) in most individuals in population studies [33,35,99], as individuals with low level antibodies are less

likely to respond when challenged with the corresponding antigen [190,191]. In addition, studies with Galα-kodecytes could be compared with historic studies of complement stability [145,153], by exploiting the fact that anti-Galα is ubiquitous in human sera [35,89,99,192].

1.8 Research Aims

Kode™ Technology provided contemporary tools to design new assays without some of the described limitations.

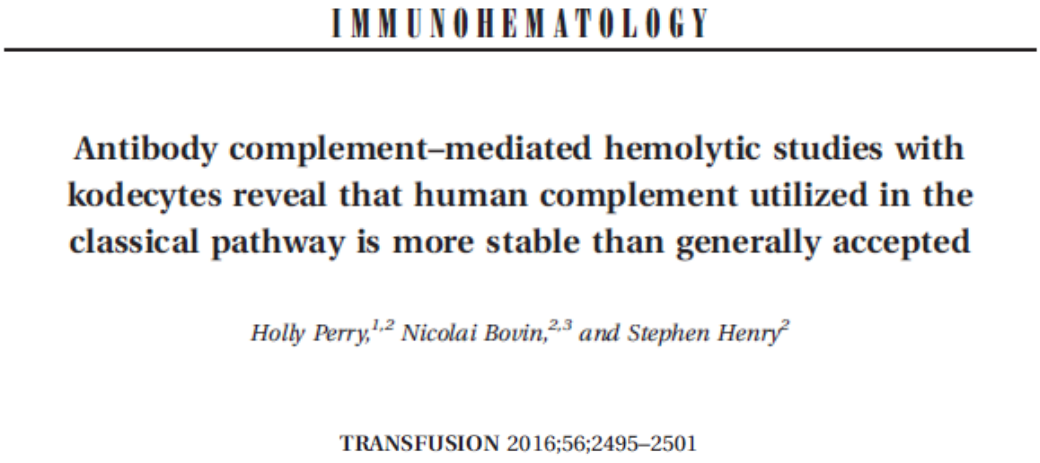
Research aims were as follows:

1. Prove that a range of kodecytes are susceptible to lysis by antibody-mediated complement activation, with a range of natural antibodies to glycans
2. Revisit the topic of complement stability, using kodecytes instead of animal cells
3. Design an alternative assay to antibody titration for quantification of ABO antibodies, using undiluted plasma
4. Evaluate xeno-antigens and antibodies in human plasma
5. Investigate FSL-antibody mediated complement attachment to non-biological solid phase surfaces of paper, glass and plastic.

Chapter Two: Methods and Results: activation of complement by classical pathway with human antibodies to carbohydrate antigens

2.1 Overview of method principles

This research explored the interaction of antibodies to natural human glycans with kodecytes, with the aim of activating complement via the classical pathway to lyse the kodecytes. Panels of kodecytes were prepared with four different glycan FSL and were optimized for complement activation and kodecyte lysis. Methods were developed to quantify lysis, utilising haemolysis standards and a reference set of complement standards. From the haemolysis standards, a standard curve allowed absorbance readings to be converted to a percentage of kodecytes lysed. The stability of complement in stored serum, and sera subjected to seven freeze-thaw cycles was evaluated in comparison with historical methods [145,153]. The surfaces of paper, glass and plastic were also investigated as mediums to which complement could be attached, by interactions of FSL with human glycan antibodies. Portions of this chapter have been published [178] (Figure 16) and the full publication is in Appendix A Publications.



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Antibody complement–mediated hemolytic studies with kodecytes reveal that human complement utilized in the classical pathway is more stable than generally accepted

Holly Perry,[1,2] Nicolai Bovin,[2,3] and Stephen Henry[2]

TRANSFUSION 2016;56;2495–2501

Figure 16. Front-page title information of publication arising from work reported in Chapter 2.

2.2 Materials and Samples

2.2.1 Kodecytes

FSL constructs (Kode Biotech Materials, Auckland New Zealand) were obtained as 0.5mg vials and reconstituted in red cell preservative solution Celpresol (Immulab, Melbourne, Australia) to prepare stock FSL solutions. Dilutions of stock FSL were prepared in Celpresol to produce working concentrations. Kodecytes were prepared by incubating one volume of FSL at a given working concentration with one volume of packed group O RBCs for 2 hours at 37°C [19,178]. After incubation, kodecytes were resuspended to 5% v/v in Celpresol, and left to rest at 4°C for a minimum of 24 hours. Kodecytes were stored at 4°C for up to four weeks.

Details of kodecytes and their nomenclature are shown in Table 2.

Table 2. Details of FSL and resulting kodecytes used extensively in this research.

FSL	F	S	L	kodecyte
FSL-Galα	Galα1-3Galβ1-4GlcNAc	ad	DOPE	Galα-kodecytes (ad spacer)
FSL-Galα	Galα1-3Galβ1-4GlcNAc	CMG_2	DOPE	Galα-kodecytes (CMG_2 spacer)
FSL-GalNAcα	GalNAcα1-3Galβ1-4GlcNAc	CMG_2	DOPE	GalNAcα-kodecytes
FSL-Rhaα	Rhaα	CMG_2	DOPE	Rhaα-kodecytes

CMG = carboxymethylglycine, DOPE = 1,2-di-O-oleoyl-sn-glycero-3-phosphoethanolamine, ad = adipate.

Stability of Galα-kodecytes (ad spacer) was assessed by storing aliquots of 5% v/v kodecytes in red cell preservative solution at 4°C for a period of four weeks, and analysing the aliquots for red cell lysis at weekly intervals.

2.2.2 Natural O cells

A single O phenotype RBC individual was used throughout to prepare all kodecytes and IgG sensitized cells. RBC from this individual were stored in red cell preservative solution Celpresol for up to four weeks.

2.2.3 Sheep RBC

One donation of fresh sheep RBCs from a single animal (South Pacific Sera Ltd, Timaru, New Zealand) was used to replicate a historical study [145]. These RBC were subsequently

sensitized with anti-sheep RBC stroma antibody produced in rabbits (Sigma-Aldrich, St Louis, USA).

2.2.4 IgG sensitized cells

Group O Rh(D) positive RBC suspended at 5% v/v in PBS were incubated for 15 minutes at 37°C with an equal volume of a 1 in 200 dilution of concentrated human IgG anti-D (MCAD6, bioCSL, Melbourne, Australia). Sensitized cells were washed four times in PBS, and resuspended to 5% v/v in red cell preservative solution. Sensitization was validated by testing with AHG, with a valid preparation producing agglutination of IgG sensitized cells, but no agglutination with the original RBC.

2.2.5 Sera and plasma

Pilot study sera

Sera from five healthy individuals of different ABO blood groups were used in a pilot study.

Standard serum index sample

One standard sera pool was used in the assessment of stability of complement in stored and frozen-thawed serum, as a source of both complement and antibody to anti-Galα. Blood group AB was selected to avoid potential interference from ABO antibodies. This sera pool (hereafter referred to as the standard serum index sample) was sourced from New Zealand Blood Service (NZBS) as a 200 mL pool prepared from two blood donations. At NZBS, the whole blood was collected into donor bags without anticoagulant and allowed to clot for 2 hours at 37°C. Serum from each donation was separated, pooled, and frozen at minus 80°C within six hours of collection. In this research, the pooled serum was subsequently thawed and separated into one mL aliquots. Aliquots were stored at temperatures of minus 85°C, minus 20°C, 4°C, 22°C and 37°C for time periods of 1, 2, 3, 7, 14, 21, 28, 60, 90, 120 and 150 days. As each storage time period was reached, the sample was placed back at minus 85°C, to await testing with all other samples. The aliquot stored throughout at minus 85°C is known hereafter as the “day zero” sample. No azide treatment of the standard serum index sample was applied, as sodium azide can interfere with complement activation [193]. To test whether any aliquot had become bacterially contaminated during storage, sera were gram stained [194] and

examined by light microscopy following storage. No bacteria were seen in any gram stain preparation.

A further seven one mL aliquots of the standard serum index sample were frozen at minus 85°C. At weekly intervals the aliquots were removed and thawed at room temperature for 30 minutes, then re-frozen at minus 85°C. This produced aliquots thawed and frozen up to seven times (Table 3).

Table 3. Freeze-thaw (FT) cycles for seven aliquots of standard serum index sample.

Sample ID	# of FT cycles	Days of FT, where day 1 is the day of the first thaw						
1	1	1						
2	2	1	8					
3	3	1	8	15				
4	4	1	8	15	22			
5	5	1	8	15	22	29		
6	6	1	8	15	22	29	36	
7	7	1	8	15	22	29	36	43

2.3 Pilot study

A pilot study was undertaken to select the FSL used in work for this chapter. Three FSL were tested in the pilot:

- FSL-Galα with adipate spacer (details as per Table 2)
- FSL-CMV. This is a peptide FSL, with the antigen comprising an epitope of a cytomegalovirus (CMV) surface protein [183] which has previously demonstrated antibody-mediated complement activation with antibodies to CMV in human sera [183]. The formula is $C_{179}H_{280}N_{38}O_{62}PSNa$ and the molecular weight 4043
- FSL-Fs5. This FSL pentasaccharide antigen mimics the Forssman (Fs) antigen. FORS blood group system is a very rare human histo-blood group glycan antigen generally absent from human populations, but frequently found in animals [195]. The formula is $C_{84}H_{148}N_4NaO_{36}P$ and the molecular weight 1844

The structures of FSL-CMV and FSL-Fs5 are shown in Figure 17 and Figure 18 respectively.

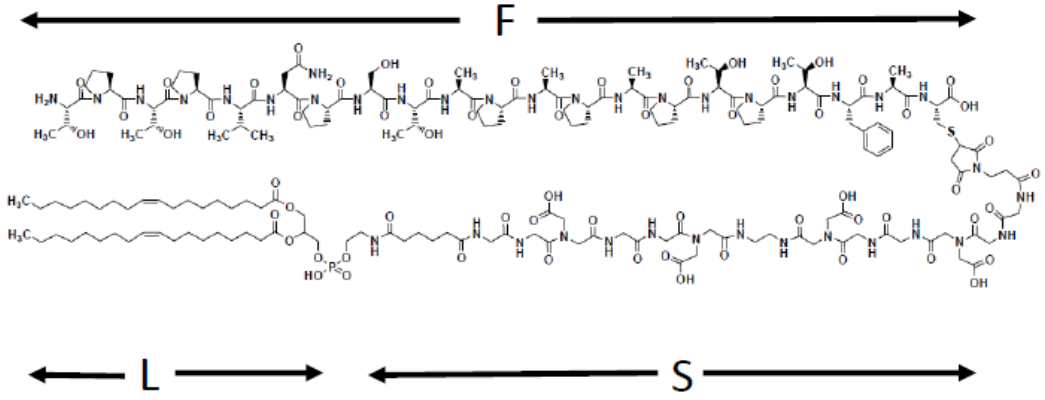


Figure 17. Chemical structure of FSL-CMV. F = CMV peptide functional head, S= CMG_2 spacer, L = lipid (DOPE).

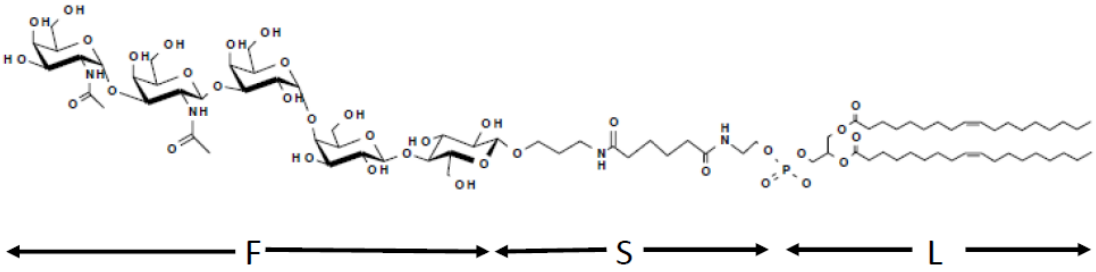


Figure 18. Chemical structure of FSL-Fs5. F = functional head (Fs antigen), S = ad spacer, L = lipid (DOPE)

These pilot study FSL were investigated as potential targets for studies with complement-activating antibodies, as some or all individuals possess corresponding antibodies with potential to activate complement by the classical pathway [89,157,196]. In addition, two of these three FSL had been explored previously in studies with complement-activating antibodies [183].

Kodecytes were prepared with FSL-Galα, FSL-CMV and FSL-Fs5 at a range of concentrations; 500, 200, 100, 80, 60, 40, 20 and 10 µg/mL (representing molarity ranges from 300 - 3 µmol/L). Resulting kodecytes were then prepared at 5% v/v in PBS. A non-koded O RBC (the same cell used to prepare the kodecytes) was also treated in the same way, to control for reactions due to antibodies against other antigens on this RBC. In individual tubes, 50 µl of 5% v/v kodecytes or non-koded control RBC were added to 100 µL of five human sera (Section 2.2.5 Sera and plasma, Pilot study sera). Tube sets were incubated for 60 minutes at 37°C. Tubes were briefly centrifuged to sediment cells,

then observed with a 10 x magnification eyepiece, and graded for agglutination and/or haemolysis (0-4 grading scale) [101].

Results of the pilot study are shown in Table 4. CMV-kodecytes failed to react with any sera, failing to show either agglutination or lysis with a high concentration (500 μg/mL) of FSL. Because CMV antibodies are not ubiquitous [196], sera 1 - 4 were subsequently tested for the presence of both IgG and IgM antibodies to CMV, using chemiluminescent enzyme immunosorbent (EIA) assay kits CMV IgG and CMV IgM (Roche, Basel, Switzerland). Sera 1, 3 and 4 were positive for IgG CMV antibodies, and negative for IgM CMV antibodies. Serum 2 was negative for both IgG and IgM antibodies. Because the results shown in Table 4 are from a direct agglutination method, which generally only detects IgM antibodies [6], it was therefore not surprising that there was no agglutination with CMV-kodecytes. Sera 4 and 5 were subsequently tested in an antiglobulin test with 500-CMV-kodecytes and 200-CMV-kodecytes, using anti-human-IgG and anti-human-C3d (Immulab, Scoresby, Australia) to test sensitization of RBC with IgG and C3 respectively. Both sera did produce positive reactions with 500-CMV-kodecytes and 200-CMV-kodecytes in an antiglobulin test (2+ agglutination with anti-IgG and 4+ agglutination with anti-C3d). All five sera lysed Galα-kodecytes over the full FSL-Galα range 500 - 10 μg/mL, and complete lysis of cells was seen with FSL concentrations as low as 40 μg/mL of FSL-Galα with all five sera (Table 4).

500-Fs-kodecytes and 200-Fs-kodecytes were tested with sera 1 to 4, and completely lysed with all four sera. One serum was tested with Fs kodecytes over the full FSL concentration range. There was partial lysis and agglutination of kodecytes with FSL concentrations 100 – 40 μg/mL, and agglutination without lysis with 20-Fs-kodecytes and 10-Fs-kodecytes (Table 4).

Table 4. Reactions of five human sera with three kodecyte types produced with different FSL concentrations in a direct haemagglutination tube (saline 37°C) pilot study.

	Sera and blood group				
kodecyte	1 O	2 A	3 AB	4 AB	5 B
500-CMV	0	0	0	0	0
200-CMV			0	0	
500-Galα	H	H	H	H	H
200-Gal α	H	H	H	H	H
100-Gal α	H	H	H	H	H
80-Gal α	H	H	H	H	H
60-Gal α	H	H	H	H	H
40-Gal α	H	H	H	H	H
20-Gal α	H,2	H	H	H,2	H,2
10-Gal α	H,2	H,2	H,2	H,2	H,2
500-Fs5	H	H	H	H	
200-Fs5	H	H	H	H	
100-Fs5				H,1	
80-Fs5				H,1	
60-Fs5				H,1	
40-Fs5				H,1	
20-Fs5				1	
10-Fs5				1	
control RBC (non-koded)	0	0	0	0	0

Grading reactions were reported on 0-4 scale [101]. Where both agglutination and lysis were present, both were noted, for example "H,2" indicates partial lysis of cells, with agglutination of remaining cells. Complete lysis is indicated by "H" only. Empty fields indicate there was insufficient serum available to test.

Of the kodecytes tested in the pilot, Galα-kodecytes proved to be the most promising candidate for activation of complement in the classical pathway, as they produced the most lysis over a wide FSL concentration range (Table 4).

Consideration was also given to studying kodecyte lysis due to complement activation via the lectin pathway (Figure 7), by attempting studies with trisaccharide FSL-Man_3 (functional head Manα6Manα3Manβ ; Figure 19) . It was hoped to show activity with Manα-kodecytes and human serum due to interaction between kodecytes and mannose binding lectin (MBL). MBL occurs in all human sera [129]. However, due to limited availability of FSL and initial disappointing results (not shown), it was decided to pursue only FSL with specificity for classical pathway complement-activating antibodies.

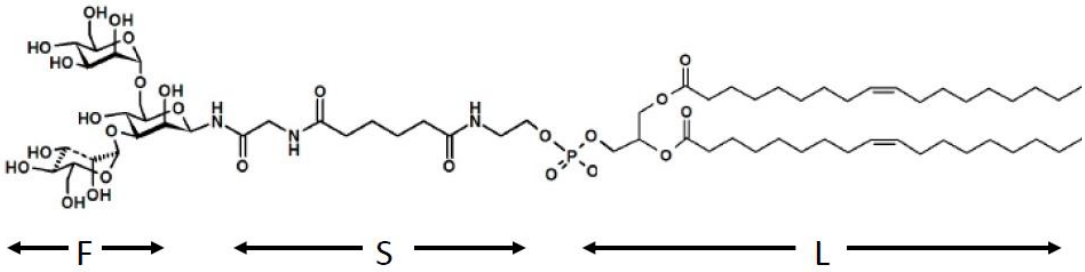


Figure 19. Chemical structure of FSL-Man3. F=functional head (Mannose trisaccharide antigen), S = ad spacer, L=lipid (DOPE)

Following the pilot study, FSL-Galα (ad spacer) (Table 2) was selected as the FSL of choice to re-visit historical complement stability studies [145,153]. FSL-Galα (CMG_2 spacer), FSL-GalNAcα and FSL-Rhaα (Table 2) were added later in the study (i.e they were not included in the pilot).

2.4 Preparation of haemolysis standards

As subsequent methods would rely on comparing quantities of lysis, reference standards were required in order to relate lysis in an experiment to a known standard.

2.4.1 Qualitative haemolysis standards

The first attempt to quantify red cell lysis produced a subjective qualitative scale. The mechanism of haemolysis was freezing and thawing of RBCs. Natural RBC (non-koded) were frozen at minus 85°C for 10 minutes, and thawed at 22°C until liquid. The freeze-thaw cycle was repeated once. Resulting lysate was added to non-lysed suspensions of RBC (volumes as per Table 5), to artificially create a series of different amounts of lysis. Tubes were mixed well, then centrifuged to sediment the RBC. The appearance of resulting lysate mixtures is shown in Figure 20. An attempt to quantify the lysis using a subjective 1 plus (+) to 4 plus (++++) scale as reported by Polley & Mollison [153] was developed (Figure 20). However, this scale proved too subjective to be useful.

Table 5. Artificially created series of red cell lysis.

lysed cells (%)	lysate (µL)	non-lysed (µL)
100	200	0
80	160	40
60	120	80
40	80	120
20	40	160
10	20	180
5	10	190
2	5	195
0	0	200

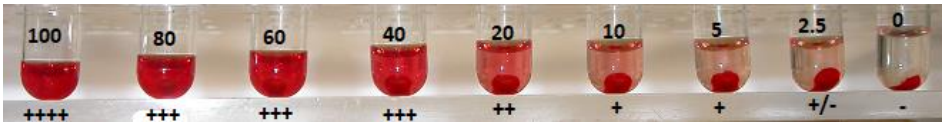


Figure 20. Different percentages of lysed cells in an artificially created panel of lysis. Each tube consists of a volume of lysed cells produced by two freeze-thaw cycles of RBC mixed with an appropriate volume of intact RBC as per Table 5. ++++ = complete lysis (100%), +++ = high degree of lysis (40 – 80%), but some intact RBC visible, ++ = medium degree of lysis (20%) with intact RBC visible, + = small amount of lysis (5 – 10%) with intact RBC visible, +/- = questionable lysis (2.5%), - = no lysis (0%).

2.4.2 Quantitative haemolysis standards

In order to produce a less subjective haemolysis scale for the haemolysis standards than that shown in Figure 20, a more quantitative approach was developed. The mechanism of haemolysis was changed from freezing and thawing to classical pathway complement activation produced by interactions of Galα-kodecytes with human sera containing the antibody anti-Galα, as this would be representative of future experiments with kodecytes. Absorbance readings at 540 nm (A540nm) were taken by sampling 160 µL of supernatant lysate into 1 mL of Drabkin solution [150].

To produce a standard curve to quantify lysis, two series of tubes were prepared from one initial series; a lysis series and a non-lysis series. Following preparation, these two series were re-combined to produce a quantifiable spectrum of lysis. This experiment was conducted on three separate occasions, and results averaged to reduce inter-experimental variation.

Initial series

Volumes of 500 µL of 750-Galα-kodecytes (5% v/v in PBS) were added to eleven separate tubes labelled 0 % to 100 % lysis.

Lysis series

Eleven clean tubes were prepared. Increasing volumes of kodecytes were added from the initial series, as per Table 6. Standard serum index sample, in a serum:cell ratio of 2:1 was added in each tube (Table 6). The lysis series of tubes was incubated for 1 hour at 37°C and produced lysis of 100% of the kodecytes in each tube.

Non-Lysis series

A volume of standard serum index sample was treated with ethylene-diamine-tetra-acetic acid (K_2H_2EDTA) to give a final EDTA concentration of 120 mmol/L [145].

The volumes of 750-Galα-kodecytes remaining in the initial series after the lysis series volumes had been removed were incubated with EDTA treated standard serum index sample, in a serum-cell ratio of 2:1 (Table 6). This series was also incubated for 1 hour at 37°C, but produced no lysis of kodecytes, due to the effect of EDTA [104].

Final series

Following incubation, the lysis and non-lysis series mixtures were re-combined in eleven separate tubes. In this way the total volume was kept constant at 1500 µL, but different percentages of cells were lysed (Table 6 and Figure 21). The absorbance of supernatant in each tube was measured at A540 nm (160 µL supernatant in 1 mL of Drabkin solution), blanked on a sample of 160 µL standard serum index sample in 1 mL of Drabkin to account for serum colour. A standard curve was plotted as a result of averaging A540nm readings of experiments undertaken on three separate occasions.

The appearance of the final series of haemolysis standards on one occasion is shown in Figure 21. Absorbance readings from testing on three separate occasions are shown in Table 7 and the resulting standard curve in Figure 22. The r value (0.99) of the line relating absorbance to percentage of cells lysed showed a high degree of correlation. This meant that a given A540nm reading in future experiments could be equated to a percentage of kodecytes lysed, provided that the following conditions were met:

1. The pre-experimental RBC (or kodecyte) number was kept constant
2. A standard volume of supernatant lysate (160 µL) was sampled in a standard volume of Drabkin solution (1 mL)
3. The serum:cell ratio was constant at 2:1

Table 6. Preparation of lysis percentage mixtures by combining volumes of lysed 750-Galα-kodecytes with non-lysed 750-Galα-kodecytes.

Lysis (%)	lysis series		non-lysis series		final series
	kodecyte (µl)	standard serum (µl)	kodecyte (µl)	EDTA standard serum (µl)	total (µl)
0	0	0	500	1000	1500
10	50	100	450	900	1500
20	100	200	400	800	1500
30	150	300	350	700	1500
40	200	400	300	600	1500
50	250	500	250	500	1500
60	300	600	200	400	1500
70	350	700	150	300	1500
80	400	800	100	200	1500
90	450	900	50	100	1500
100	500	1000	0	0	1500

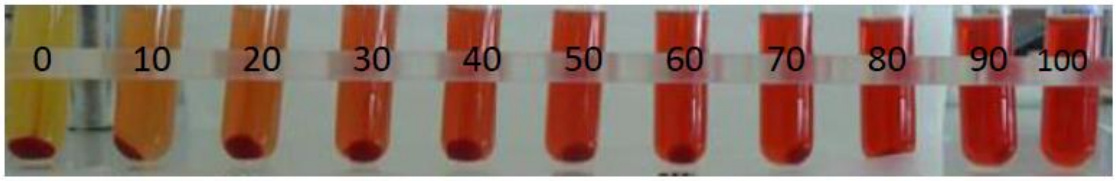


Figure 21. Appearance of set of haemolysis standards, showing the appearance of each percentage of lysis in 10% increments.

A540nm readings from the haemolysis standards (Table 7) were subsequently used to calculate percentages of kodecytes lysed. For example, an absorbance of 0.3 in a future experiment would be calculated as 80% (± error) of a kodecyte sample lysed (Figure 22).

Table 7. Absorbance readings (A540nm) of haemolysis standards in triplicate.

% lysis	Set 1	Set2	Set3	Mean	Error*
0	0	0	0	0	0
10	.029	.020	.038	.029	.01
20	.049	.049	.074	.057	.01
30	.097	.093	.116	.101	.01
40	.134	.132	.144	.137	.01
50	.157	.171	.195	.174	.02
60	.202	.208	.232	.213	.02
70	.235	.247	.285	.256	.03
80	.273	.291	.337	.300	.03
90 -100	.308	.306	.368	.327	.03

*Error = (maximum reading – minimum reading) divided by 2.

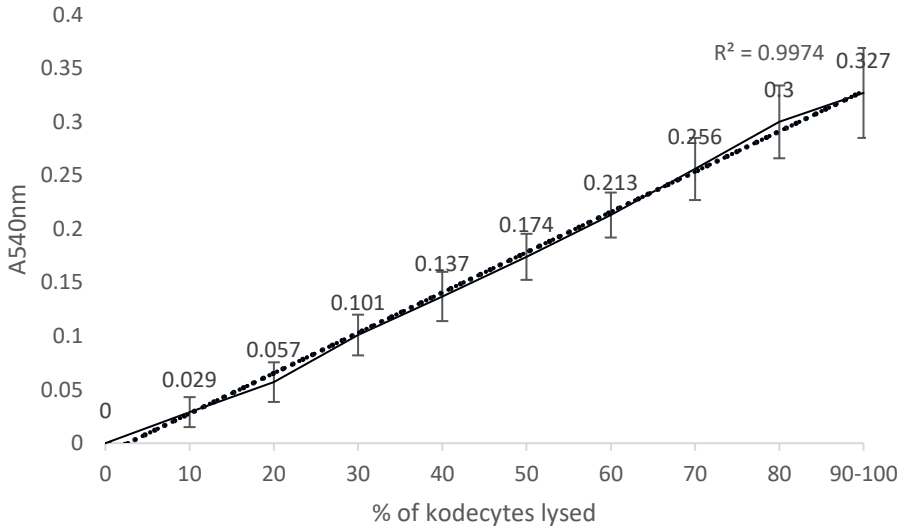


Figure 22. Absorbance (A540nm) versus percentage of 750-Galα-kodecytes lysed. Absorbance readings were calculated means of readings from three separate experiments. Error bars were applied in both directions (±) and show inter assay error calculated using the formula (maximum reading – minimum reading) divided by 2.

2.5 Standardization of RBC number

Before starting an experiment using multiple RBC samples, all kodecyte and control samples were adjusted to a standard A540nm to reflect a constant RBC number. Allowance was made for ±10% variation in A540nm between samples, which represented a low error percentage and was also achievable. RBC were prepared at 5% v/v in PBS by pipetting an appropriate volume of concentrated RBC, wiping the tip before dispensing into in an appropriate volume of PBS, then rinsing the pipette tip seven times in the resulting solution. For example, to make 200 µL of 5% suspension,

10 µL of concentrated RBC were pipetted to 190 µL of PBS; tip wiping and rinsing as described. Suspensions were then well mixed, and 40 µL pipetted into a tube containing 1 mL of Drabkin solution, again ensuring that the pipette tip was wiped before entering the Drabkin solution. Tubes were well mixed and left to rest for 30 minutes, to allow complete conversion of all forms of haemoglobin to cyanmethaemoglobin [150]. Absorbance was measured at 540 nm in spectrophotometric cuvettes, blanking on Drabkin solution. The mean absorbance reading from a minimum of 10 individually prepared RBC suspensions to be used in any experiment was calculated, and a ±10% range from the mean was then calculated. Samples whose absorbance reading fell within the ±10% range were considered to have an equal RBC number, and were therefore suitable to use in any experiment. Although it was time consuming to ensure that RBC numbers were in the ±10% pre-analytical range before any experiment commenced, it added accuracy to the experiments. It was noted that the larger the concentrated RBC reservoir used to prepare the suspensions, the more consistent the absorbance readings were between samples.

2.6 Stability studies of Galα-kodecytes (ad spacer)

Stability of Galα-kodecytes (ad spacer) was undertaken by preparing kodecytes over the range 100-10 µg/mL (100, 80, 60, 40, 20 and 10-Galα-kodecytes), as described in section 2.2.1 Kodecytes. A non-koded O RBC was subjected to the same treatment as the kodecyte preparations. After washing once in PBS and once in Celpresol, all kodecytes and RBC were stored as 5% v/v suspensions in Celpresol at 4°C for four weeks. At one, two and four weeks storage, supernatants (formed by settling of RBC during storage) were examined and graded for lysis using the qualitative haemolysis scale depicted in Figure 20. Suspensions were then re-mixed by inversion and left to re-settle for further weekly intervals, with observations repeated each week.

As shown in Table 8 and Figure 23 RBC koded with FSL-Galα (ad spacer) at concentrations above 40 µg/mL demonstrated more haemolysis than the non-koded cell, with the greatest lysis (a visually estimated 60% at four weeks) seen with the highest FSL concentration of 100 µg/mL. This suggested that FSL insertion, particularly when the FSL is at higher concentration, may damage the RBC membrane in some way during cold storage.

Table 8. Stability of Galα-kodecytes (ad spacer) over four weeks as 5% v/v suspensions in red cell preservative solution, stored at 4°C. Haemolysis is visually scored using the qualitative haemolysis scale depicted in Figure 20.

	FSL-Galα (ad spacer) concentration													
µg/mL	100		80		60		40		20		10		0	
µmol/L	68		55		41		28		14		7		0	
weeks	degree of lysis (+ to ++++ scale, and estimated percentage lysis)													
1	+	10	±	2.5	±	2.5	-	0	-	0	-	0	-	0
2	++	20	±	2.5	++	20	±	2.5	-	0	+	10	-	0
4	+++	60	++	20	++	40	±	2.5	±	2.5	+	10	±	2.5

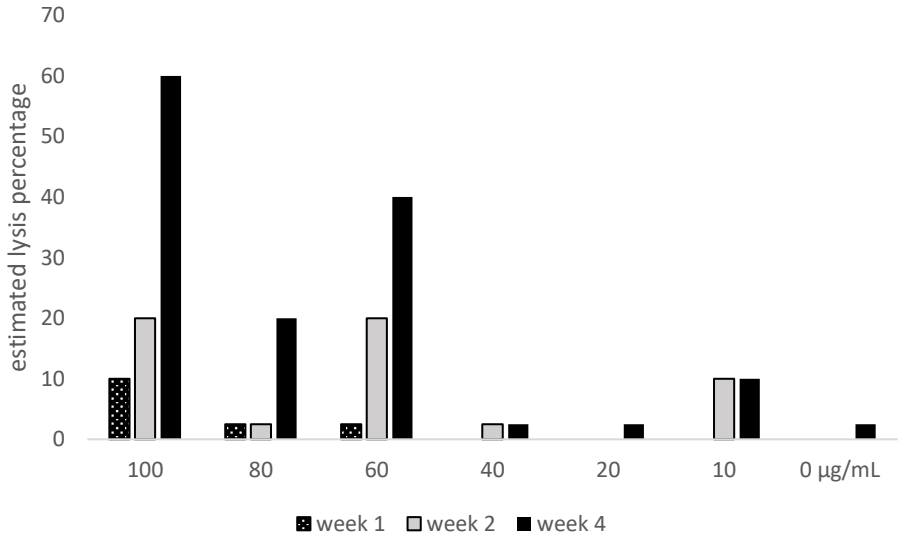


Figure 23. Estimated percentage of lysis in stored Galα kodecytes. 100, 80, 60, 40, 20 and 10-Galα-kodecytes stored in red cell preservative solution, as compared to a non-koded cell (zero), at one, two and four weeks storage at 4°C.

2.7 Preparation of complement activity standards

Complement standards were prepared from fresh AB serum from a healthy individual. This sample was assumed to have 100% complement activity. Complement inactivated serum was prepared by heating a portion of this serum at 56°C for 30 minutes [197,198]. Complement standards were prepared in 10% increments of active complement by mixing appropriate volumes of native fresh serum (100% complement activity) with heat-treated (0% complement activity) serum (Table 9).

Table 9. Preparation of complement activity standards.

% active complement	100% C serum* (mL)	of 0% C serum† (mL)	Total (mL)
100	3.0	0.0	3
90	2.7	0.3	3
80	2.4	0.6	3
70	2.1	0.9	3
60	1.8	1.2	3
50	1.5	1.5	3
40	1.2	1.8	3
20	0.6	2.4	3
0	0.0	3.0	3

*from fresh AB serum, †from AB serum heated to 56°C for 30 minutes

2.8 Concentration of FSL-Galα required to prepare Galα-kodecytes, by which 50% of kodecytes lyse with the standard serum index sample

In order to design experiments to test complement stability in the classical pathway, and to compare results to earlier studies [145,153], it was desirable to design an assay where 50% of a sample of Galα-kodecytes lysed, following incubation with the standard serum index sample. In this way, results from this study using undiluted serum and kodecytes could be compared to those attained in a CH_{50} assay, which also relies on lysis of 50% of a cell population [145,147,148]. The CH_{50} assay relies on antibody dilution in the serum to attain 50% lysis, but in order to avoid the problems associated with serum dilution, the aim in this research was to use undiluted serum. Therefore the antigen concentration that would produce lysis of 50% of a kodecyte population was investigated. Galα-kodecytes (ad spacer) were prepared with FSL-Galα (Table 2) at concentrations of 500, 200, 100, 80, 60, 40 20 and 10 µg/mL. After resting the kodecytes for 24 hours, washed 5% v/v suspensions of each Galα-kodecyte were prepared in PBS. Kodecyte numbers were assessed and standardised by absorbance measurements as described in section 2.5 Standardization of RBC number. Preparations of 200 µL of standard serum index sample and 100µL of Galα-kodecytes (5% v/v) were mixed and incubated for 60 minutes at 37°C. A non-koded O cell (the same RBC used to prepare the kodecytes) was tested in the same way as each kodecyte as a negative control. Lysis was stopped after 60 minutes by the addition of 1 µL of K_2EDTA at 120mmol/L. Tubes were mixed briefly, then centrifuged to separate intact kodecytes from supernatant. Cell free supernatant (160 µL) was sampled into 1 mL Drabkin solution, and after 30 minutes absorbance was measured at A540 nm against a blank of 160 µL standard serum index sample in 1 mL of

Drabkin to account for serum colour. The percentage of kodecytes that lysed was calculated by expressing the absorbance of each concentration-Galα-kodecyte as a percentage of the 500-Galα-kodecyte absorbance (100% lysis). A540nm readings and calculated percentages of kodecytes lysed are shown in Table 10. The correlation coefficient r value was acceptable at 0.95, however the non-koded control cell did not produce an absorbance reading of zero or close to zero (Table 10). Therefore this assay could not be reliably used to quantify lysis of less than 10% of a cell population. If only the absorbances and the calculated percentage of kodecytes lysed for the 500-, 200-, 100- and 80-Galα-kodecytes were plotted, the r value was 0.99, and it was determined that an FSL-Galα concentration of 100 µg/mL produced lysis of 55% of the kodecyte population (Figure 24).

Table 10. Absorbances and percentages (%) of kodecytes lysed for Galα-kodecytes incubated with standard serum index sample.

Galα-kodecyte	A540nm	kodecytes lysed (calculated %)
500	.257	100
200	.197	77
100	.142	55
80	.105	41
60	.097	38
40	.090	35
20	.075	29
10	.095	37
0 (non-koded)	.072	28

Percent lysis was calculated by expressing the absorbance of each kodecyte as a percentage of the absorbance of the 500-Galα-kodecyte.

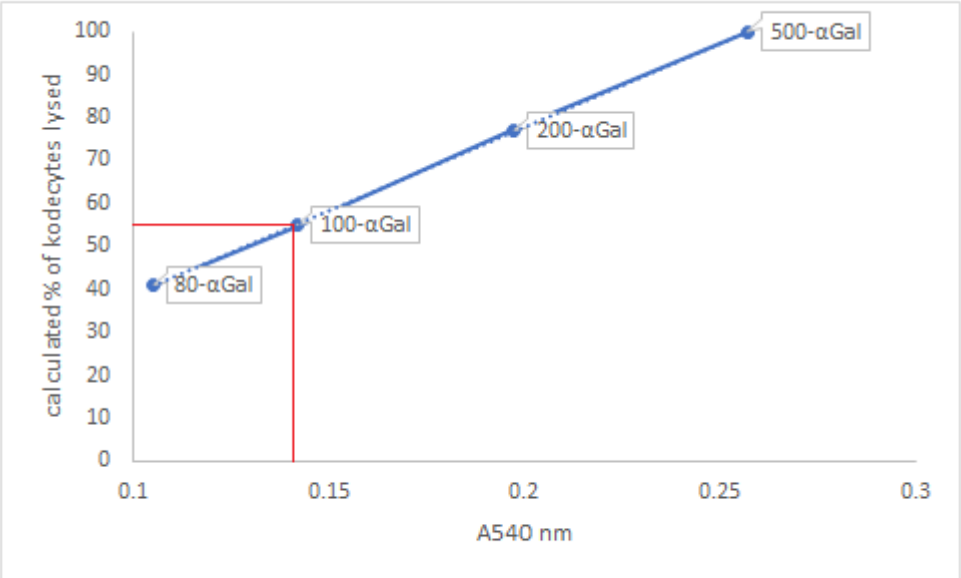


Figure 24. Curve of absorbance (A540nm) from standard serum index sample and Galα-kodecytes prepared with a range of concentrations of FSL-Galα. 100-Galα-kodecytes produced 55% lysis of the kodecyte sample (marked with a red line).

For validation, the absorbance value of 0.142 obtained with the 100-Galα-kodecyte (Table 10) was also compared to the absorbance value of 0.174 obtained with the 50% lysis standard (Figure 22). Allowing for the experimental error range (error bars Figure 22) of of the absorbance reading of the 50% lysis standard, which allowed for 50% lysis to be in the range 0.174 ± 0.02, it was considered valid to state that the standard index serum sample lysed approximately 50% of 100-Galα-kodecytes. Therefore the concentration of 100 µg/mL of FSL-Galα (ad spacer) was selected for use in experiments in which results were later compared with CH_{50} assays, or modified CH_{50} assays such as that used by Garratty in 1970 [145].

2.9 Complement stability in stored standard serum index sample

2.9.1 Background

Galα-kodecytes were used to re-examine findings from studies of Polley & Mollison [153] and Garratty [145], with regard to complement stability in stored samples. Specifically, 100-Galα-kodecytes were tested with aliquots of the standard serum index sample stored at a range of temperatures for various time periods (see section 2.2.5 Sera and plasma; Standard serum index sample). All testing was conducted on undiluted serum.

The differences in the approaches to sample and reagent selection and preparation of RBC by Polley & Mollison [153], Garratty [145] and approaches in this research [178] are summarised in Table 11.

Table 11. Comparison of sera and RBC sources used in three studies to test complement stability.

		Polley & Mollison 1961 [153]	Garratty 1970 [145]	This research [178]
Sera				
storage	-90°C to -85°C*		√	√
	-55°C to -50°C*	√	√	
	-20°C	√	√	√
	4°C	√	√	√
	20 - 24°C	√	√	√
	37°C	√	√	√
storage time (days)	1	√	√	√
	2		√	√
	3		√	√
	7	√	√	√
	14		√	√
	21		√	√
	28	√	√	√
	60	√	√	√
	90		√	√
	120			√
	150			√
freeze thaw		-	-	√
number of human sera tested		1	12 tested singly	2 pooled
complement-activating antibody		bovine anti-sheep RBC membrane (anti-Fs)	bovine anti-sheep RBC membrane (anti-Fs)	human anti-Galα
RBC				
antigen target		sheep cells presensitized with Fs antibody	sheep cells presensitized with Fs antibody	koded human RBC (100-Galα-kodecytes)
antigen levels		natural and variable	natural and variable	synthetic and standardised
RBC source		Sheep RBC (fresh) in anticoagulant citrate dextrose (ACD)	Sheep RBC stored in red cell preservative for up to 2 weeks	human RBC (kodecytes) stored in red cell preservative for up to 48 hours

Fs = Forssman. * choice of storage temperatures was limited by access to equipment

Methodological approaches were also somewhat different between the three studies, as summarised in Table 12. A more comprehensive range of control samples was incorporated in this research in order to control test variables related to both serum and kodecytes (Table 12).

Table 12. Comparison of designs of methods and controls of three studies to test complement stability.

	Polley & Mollison 1961 [153]	Garratty 1970 [145]	This research [178]
method approach	stored and fresh sera at four dilutions	serum at dilution lysing 50% of RBC when complement activity = 100%.	undiluted serum lysing 50% of 100-Galα-kodecytes when complement activity = 100%.
serum: cell ratio	not stated	1 volume of serum to 1 volume of 2.5% cells	2 volumes of serum to 1 volume of 5% cells
incubation	20 minutes at 37°C	30 minutes at 37°C	30 minutes at 37°C EDTA added to stop haemolysis at end of incubation
detection	qualitative visual	absorbance measured in ammonia with a yellow-green filter	absorbance measured in Drabkin solution at 540 nm
100% lysis control	not stated	1 part sheep RBC incubated with 100 parts detergent	750-Galα-kodecytes incubated with 100% complement activity standard (Table 9)
50% lysis controls	not stated	not stated	100-Galα-kodecytes incubated with 100% complement activity standard (Table 9)
10% lysis control	not stated	not stated	750-Galα-kodecytes incubated with 1 volume of 100% complement activity serum, and 9 volumes EDTA-treated standard index serum sample
0% lysis controls	not stated	not stated	A. 100-Galα-kodecytes incubated with 0% complement activity standard (Table 9) B. 750-Galα-kodecytes incubated with 0% complement activity standard (Table 9) C. Non-koded O cells, incubated with 100% complement activity standard (Table 9) D. Non-koded O cells, incubated with 0% complement activity standard (Table 9)

2.9.2 Complement stability lysis assay

This assay was based on the observation that a sample with 100% complement activity produced lysis of approximately 50% of a sample of 100-Galα-kodecytes. Garratty's study [145] and this research [178] were both tuned to samples of 100% complement activity lysing 50% of a target RBC population.

All testing was undertaken in duplicate on stored samples thawed at the same time, and testing commenced within one hour of thawing in the same experiment, in order to eliminate inter-experimental variation. A single large volume of 100-Galα-kodecytes sufficient to test all stored and frozen-thawed aliquots of the standard index serum sample was prepared on the day before the assay, thus eliminating the variables of kodecyte number and mechanical lysis due to storage. This volume of kodecytes was washed in PBS and standardised for RBC number using the methodology described in section 2.5 Standardization of RBC number, so that A540nm readings could be used to compare percentages of kodecytes lysed, and related to the haemolysis standard values (Figure 22).

The sample that was stored throughout at minus 85°C was referred to as the "day zero" sample and was regarded as having 100 % complement activity. The "day zero" sample was in fact stored for the entire storage period of 150 days, but as minus 85°C is a temperature known to preserve complement for at least six years [146], it was regarded as a fresh or "day zero" sample.

Duplicates of 200 µL of each serum aliquot and 100 µL of 100-Galα-kodecytes (5% v/v in PBS) were mixed together and incubated for 30 minutes at 37°C. Lysis reactions were stopped with the addition of 1 µL of K_2EDTA at 120 mmol/L. Tubes were mixed, centrifuged to sediment kodecytes and 160 µL of cell-free supernatant was sampled to 1 mL of Drabkin, and absorbance read at 540 nm. The spectrophotometer was blanked on a sample of 160 µL standard serum index sample in 1 mL of Drabkin to account for serum colour. Readings for duplicates of the same aliquots were averaged, and are shown in Table 13.

Table 13. Results of complement stability in stored aliquots of standard serum index sample. Absorbances, calculated percentage of kodecytes lysed and calculated complement activity, using two different mathematical approaches.

Storage		A540nm (mean of duplicates)	% kodecytes lysed : formula 1 (page 55)	% active complement: formula 2 (page 55)	% active complement: formula 3 (page 55)
time	temperature				
0 day	-85°C	.166	51	>100	100
"day zero"	-20°C	.154	47	94	93
	+4°C	.151	46	92	91
1 day	+22°C	.221	>100	>100	>100
	+37°C	.122	37	74	75
	-20°C	.214	>100	>100	>100
	+4°C	.184	>100	>100	>100
2 days	+22°C	.162	50	100	98
	+37°C	.081	25	50	49
	-20°C	.174	53	>100	>100
	+4°C	.191	59	>100	>100
3 days	+22°C	.171	52	>100	>100
	+37°C	.063	19	38	38
	-20°C	.151	46	92	91
	+4°C	.161	49	98	97
7 days	+22°C	.124	38	76	75
	+37°C	0	<1	<1	<1
	-20°C	.159	49	98	96
14 days	+4°C	.153	47	94	92
	+22°C	.048	15	30	29
	-20°C	.16	49	98	96
21 days	+4°C	.154	47	94	93
	+22°C	.015	5	10	9
28 days	-20°C	.148	45	90	89
	+4°C	.153	47	94	92
	+22°C	.032	10	20	19
60 days	-20°C	.155	47	94	93
	+4°C	.099	30	60	60
	+22°C	0	<1	<1	0
90 days	-20°C	.142	43	86	86
	+4°C	.073	22	44	44
	+22°C	0	<1	<1	0
120 days	-20°C	.223	68	>100	>100
	+4°C	0	<1	<1	0
150 days	-20°C	.161	49	98	97

2.9.3 Mathematical interpretation of complement stability lysis assay data

Formula 1

A calculation was formulated to relate an absorbance reading to a percentage of kodecytes lysed; formula 1 as follows:

% kodecytes lysed = sample A540nm/0.327 x 100
where 0.327 was the absorbance of the haemolysis standard sample in which 100% of kodecytes lysed (Table 7).

Formula 1 was applied to each of the absorbance readings obtained for stored samples to relate the absorbance to the percentage of lysis, using the absorbance value from the 100% haemolysis standard as the denominator in the equation above. Results are shown in Table 13.

Formula 2

Formula 2 related the percentage of kodecytes lysed (obtained from formula 1) to the percentage of active complement in a sample, as follows:

% active complement = % kodecytes lysed/50 x 100
where 50% lysis = 100% complement activity

Formula 2 was applied to the stored sample results, and calculations are shown in Table 13.

Formula 3

An alternative way to calculate the percentage of active complement in the stored samples was to express the absorbance of the stored samples as a percentage of the "day zero" sample (assumed to have 100% complement activity). This was the approach used by Garratty in his study [145], and forms formula 3 as follows:

% active complement = sample A540nm/0.166 x 100
where 0.166 was the absorbance of the "day zero" sample (Table 13)

Formula 3 was applied, and calculations are shown in Table 13, for comparison with data obtained using Formula 2.

Formula 2, using the 100% haemolysis standard absorbance, allowed an independent validation of calculations using formula 3. As can be seen in Table 13, and comparing the two columns of figures (formula 2 and formula 3), results for complement activity varied by no more than 2%, so either method of calculation can be deemed valid. To compare this study with Garratty's study [145], formula 3 was used to publish data from this research [178]. There were several instances when a calculated percentage of cells lysed was greater than 100% due to method accuracy limitation. When this occurred, complement activity was reported as 100% (Table 13). Figure 25 shows the visual appearance of reactions from this experiment.

Results of this research are presented alongside results from Garratty [145] in Figure 26. In 1970, a level of 60% complement activity was the minimum level considered acceptable for immunohaematological studies of blood group antibodies [199], therefore the 60% complement activity level has been marked on Figure 26.

At 37°C storage, Garratty [145] reported that complement activity fell below 60% after one day (dashed pink line on Figure 26), whereas this research demonstrated levels of ≥60% for between one and two days storage at 37°C (solid red line on Figure 26).

At 22°C, Garratty [145] reported complement activity fell below 60% between one and two days (dashed green lines on Figure 26), whereas this research showed 60% of complement activity was retained for between 7 and 14 days at this temperature (solid green line on Figure 26).

At 4°C complement activity was reported by Garratty [145] to drop to 60% at 10 days (dashed blue lines on Figure 26), substantially less than the 60 days seen in this research (solid blue lines on Figure 26). The minus 20°C frozen sample did not show any loss of complement activity in its entire 150 days of storage in this research (solid black line on Figure 26), whereas Garratty [145] reported activity level fell to 60% after 60 days (dashed grey lines on Figure 26).

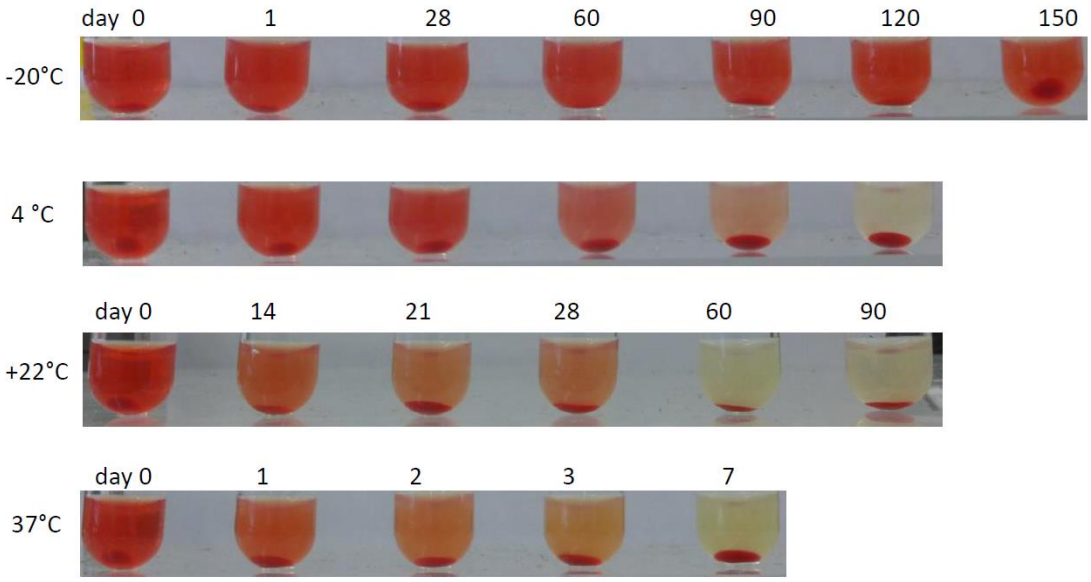


Figure 25. Appearance of range of temperature and time-stored aliquots of standard serum index sample, following testing with 100-Galα-kodecytes. A540nm readings and calculated complement activity for these samples are shown in Table 13.

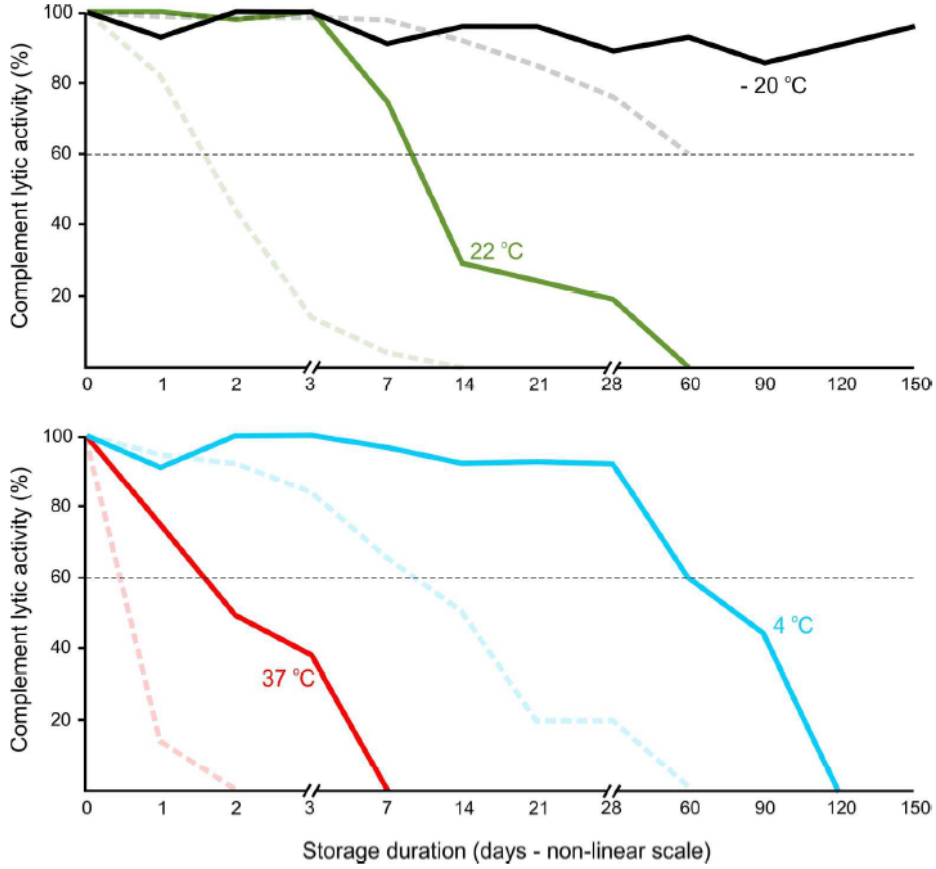


Figure 26. Comparison of two studies of complement activity in sera stored under different storage conditions, assessed in a functional lysis assay. Solid lines show results obtained from this study [178], while dashed lines show the historical results of Garratty [145], replotted onto this graph. The 60% complement activity is marked by a dashed horizontal line. Reproduced from the author's work [178].

Results for the control samples are shown in Table 14 and Figure 27. Calculations using Formula 1 (page 55) relating percentage lysis to the 100% haemolysis standard (Table 7) somewhat underestimated the amount of lysis. For example, Formula 1 calculated the 100% lysis control at 83% and the 50% lysis control lysis at 43%. The 50% control result of 43% was considered acceptable, as a ±10% error in a semi-quantitative assay. However, the result of 83% lysis for the 100% control was outside the ±10% error range. The implication of underestimating complement activity is that the claims around complement stability made in this study are conservative.

Table 14. Results of control samples* in study of complement stability.

Lysis % (Table 12)	A540nm	complement % (Formula 1)
100	0.27	83
50	0.14	43
10	0.04	12
0 A	0.00	0
0 B	0.00	0
0 C	0.00	0
0 D	0.00	0

*Description of composition of each control sample is in Table 12.

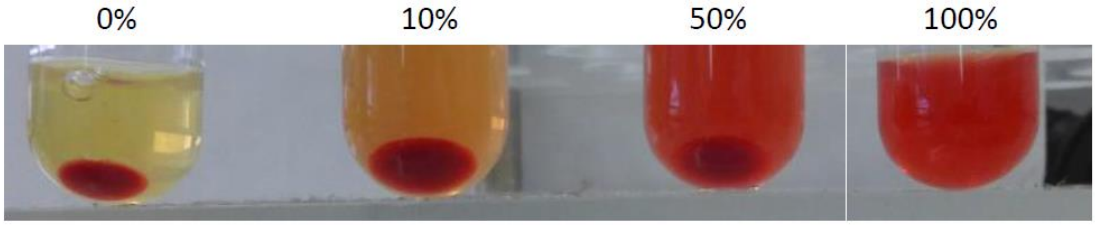


Figure 27. Control samples for study of complement stability in stored samples, showing controls of 0, 10, 50 and 100 % lysis.

2.10 Complement stability in frozen-thawed aliquots of standard serum index sample

Aliquots subjected to up to seven freeze-thaw cycles (Table 3) were tested together with the temperature and time range-stored samples, using the same methodology as that described for the lysis assay for assessing complement stability in the stored standard serum index sample (method as per section 2.9.2 Complement stability lysis assay). Results are shown in Table 15 and Figure 28.

Formula 4

Formula 4, using the same mathematical approach as Formula 3 (page 55), was devised to compare the percent of active complement in multiple frozen-thawed aliquots to the aliquot which was frozen and thawed only once.

% active complement = sample A540nm/0.193 x 100

where 0.193 = absorbance A540nm of single frozen-thawed aliquot (Table 15)

As can be seen in Table 15, there was an unexpected rise in absorbance over the seven freeze-thaw cycles, which caused complement percentages to be calculated as greater than 100% in all aliquots except the single freeze-thaw. This could be due to pre-analytical complement activation [146] (see Discussion chapter).

Table 15. Results of complement stability in aliquots of standard serum index sample subjected to up to seven freeze-thaw cycles.

# of freeze-thaw cycles	A540nm (mean of duplicates)	Lysis % (Formula 1)	Complement % (Formula 4)
1	.193	59	100
2	.213	65	>100
3	.245	75	>100
4	.228	70	>100
5	.241	74	>100
6	.262	80	>100
7	.267	82	>100

Figure 28. Appearance of frozen-thawed aliquots of the standard serum index sample, following testing with 100-Galα-kodecytes. A540nm readings and calculated complement activity for these samples is shown in Table 15.

2.11 Titration of anti-Galα

To ensure that the complement stability study on the stored standard serum index sample was measuring complement deterioration rather than antibody loss during storage, the level of anti-Galα was assessed by titration in the "day zero" sample and aliquots stored at all temperatures across various time points (Table 16).

Each serum aliquot was EDTA-treated (final EDTA concentration 120 mmol/L) to prevent lysis of Galα-kodecytes, as titres are traditionally determined using agglutination [101] rather than haemolysis reactions. Master doubling dilution series of standard serum index sample aliquots were prepared in PBS as per standard titration methods [101]. Mixtures of 200 µL of each serum dilution and 100 µL of 100-Galα-kodecytes (5% v/v in PBS) were mixed together in tubes and incubated for 60 minutes at 37°C. Tubes were spun to sediment kodecytes, and cell buttons graded for agglutination on the 0-4 scale to assess the IgM titre [101]. Negative tubes were washed four times in PBS, and AHG was added to assess the total (IgG + IgM) titre. Negative reactions were validated by the addition of IgG sensitized cells. Titres were designated by the last tube showing a positive agglutination reaction of ≥ grade 1 [101]. Results are shown in Table 16. The titre remained at two (IgM) and two or four (total antibody) throughout the storage periods, validating that the assays to assess complement stability were measuring complement rather than antibody deterioration.

Table 16. Titres of anti-Galα against 100-Galα-kodecytes in standard serum index sample aliquots at various temperature and time storage points.

		agglutination grades (0-4)					
storage		dilution factor					
temperature (°C)	time (days)	1	2	4	8	titre	Ig class
-85	0	2	1	0	0	2	IgM
				0	0	2	total
	90	2	1	0	0	2	IgM
				0	0	2	total
-20	120	2	1	0	0	2	IgM
				1	0	4	total
	150	4	2	0	0	2	IgM
				0	0	2	total
+4	60	1	1	0	0	2	IgM
				0	0	2	total
+22	60	2	2	0	0	2	IgM
				1	0	4	total
+37	7	1	1	0	0	2	IgM
				0	0	2	total

2.12 Comparison of sensitivity of assays of Garratty and Perry

This research found complement to be at least twice as stable as previously reported by Garratty at a range of storage temperatures [145]. Two of the differences between Garratty's study and this research were:

- Target RBC. This research used human RBC modified to express a controlled loading of single target antigen Galα1-3Galβ1-4GlcNAc (100-Galα-kodecytes). Garratty [145] used sheep RBC which were uncontrolled in terms of antigen loading and also expressed multiple target antigens; Fs, Galα1-3Galβ1-4GlcNAc and other unspecified antigens (Figure 9)
- One-stage versus two-stage technique. There was no presensitization of RBC in this research, as opposed to presensitization of target RBC with anti-Fs by Garratty [145]. The two-stage approach of antibody attachment followed by complement activation taken by Garratty [145] was in contrast to the one-stage technique used in this research. Here both anti-Galα and complement were

present in the serum and antibody attached and complement was activated during a one-stage incubation phase

In order to investigate these RBC-related differences as possible cause(s) for the difference in results of the two investigations of complement stability, and to address whether one approach was more sensitive than the other, testing of presensitized sheep RBC with diluted serum was undertaken, and results compared to 100-Galα-kodecytes with undiluted serum. For these experiments, the complement activity standards shown in Table 9 were tested, allowing comparison of actual known complement percentages from the standards with calculation of complement levels from the lytic assay. Ideally, the experiment would also have been conducted with the stored standard serum index sample, however there was insufficient sample remaining. These experiments on sheep RBC and kodecytes were conducted in triplicate and performed concurrently on all three testing dates to minimize inter-experimental variables.

2.12.1 Presensitization of sheep RBC with rabbit anti-sheep RBC antibody

Rabbit anti-sheep RBC stroma antibody was reconstituted in distilled water, then prepared at a final dilution of 1 in 50 in PBS. Sheep RBCs were washed three times in PBS and prepared as a 5% v/v suspension in PBS, then equal volumes of washed 5% sheep red cells and diluted rabbit anti-sheep antibody were mixed and incubated for 60 minutes at 37°C. Following incubation the sheep RBC were washed four times in PBS and prepared as 5% v/v suspensions in PBS.

To test efficacy of sensitization, sensitized sheep RBC were tested in a direct antiglobulin haemagglutination assay [101], replacing anti-human globulin with anti-rabbit globulin (Dako, Hamburg, Germany). Sensitized cells showed medium agglutination strength (2 on the 0-4 grading system [101]), whilst unsensitized cells showed no agglutination.

2.12.2 Determination of dilution factor of serum

In order to determine the dilution of serum that would produce lysis of 50% (or as close as possible to 50%) of sheep RBC, a doubling dilution series of the 100% complement activity standard was prepared and tested as per Garratty's method [145]. To 200 µL of each serum dilution was added 200 µL of a 2.5% v/v suspension of sensitized sheep RBC. The RBC and serum were incubated for 30 minutes at 37°C. Following centrifugation of

the samples, absorbance was quantified as previously described (sampling cell-free supernatant to Drabkin and measuring absorbance at A540nm). In order to account for different dilutions of serum having different colours, a series of serum blanks (160 µL of each serum dilution in 1 mL of Drabkin) was prepared and used to blank each appropriate serum dilution.

Percent lysis was calculated with Formula 5, as below.

Formula 5

% sheep RBC lysed = sample A540nm /0.594 x 100

Where 0.594 = A540nm of undiluted sample

Table 17. Dilutions of 100% complement activity standard, tested with sheep RBC to determine dilution producing lysis closest to 50%.

Dilution factor	A540nm (mean of triplicates)	% lysis (Formula 5)
1(undiluted)	.594	100
2	.547	92
4	.539	91
8	.509	86
16	.487	82
32	.403	68
64	.126	21
128	.018	3

Highlighted cells are to show that a dilution factor of 32 produced lysis of 68% of sheep RBC.

A dilution factor of 32 of the 100% complement activity standard produced lysis of 68% of the presensitized sheep cells (Table 17). This was the closest percentage to 50%, therefore a dilution factor of 32 was selected for the assay to compare sensitivity of the assays of Garratty [145] and this research [178]. Figure 29 shows the visual appearance of dilutions detailed in Table 17.

Figure 29. Sensitized sheep RBC with titrated 100% complement activity serum to determine dilution factor lysing approximately 50% of sheep RBC. Neat serum is depicted by number 1 under the photo, and each dilution factor by its reciprocal (for example 1 in 2 dilution is represented by 2). A dilution factor of 32 lysed 68% of sheep cells.

2.12.3 Lysis assay for comparison of sheep RBC and kodecytes

Suspensions of sheep RBC and 100-Galα-kodecytes were washed in PBS and prepared at 5% v/v in PBS.

A. Sheep RBC assay:

Triplicates of 200 µL of each complement activity standard (Table 9) at a dilution factor of 32, and 100 µL of sensitized sheep RBC (5% v/v in PBS) were mixed together and incubated for 30 minutes at 37°C.

B. Kodecyte assay

Triplicates of 200 µL of each complement activity standard (Table 9)(undiluted) and 100 µL of 100-Galα-kodecytes (5% v/v in PBS) were mixed together and incubated for 30 minutes at 37°C.

C. Sheep RBC versus Kodecyte assay

All lysis reactions were stopped with the addition of 1 µL of K_2EDTA at 120 mmol/L. Tubes were mixed and centrifuged to sediment cells. Cell-free supernatant (160 µL) was sampled to 1 mL of Drabkin, and absorbance read at 540 nm. The spectrophotometer was blanked on an appropriate sample of 160 µL serum in 1 mL of Drabkin to account for serum colour (undiluted serum for the kodecyte assay and serum diluted 1 in 32 for the sheep RBC assay). Readings for triplicates were averaged. Percentage lysis of each sheep RBC or kodecyte sample (and thus the percentage of complement activity where 50% lysis = 100% complement activity) was calculated from the A540nm readings,

expressing the A540nm of each sample as a percentage of the maximal lysis reaction obtained for the 100% complement standard (Formula 6 below).

Formula 6

$$\%\ \text{active complement} = \frac{\text{sample A540nm}}{\text{100\% complement activity standard A540nm}} \times 100$$

D. Assay comparison

Known complement values of the complement standards were plotted against calculated complement values (Formula 6). Results are shown in Figure 30.

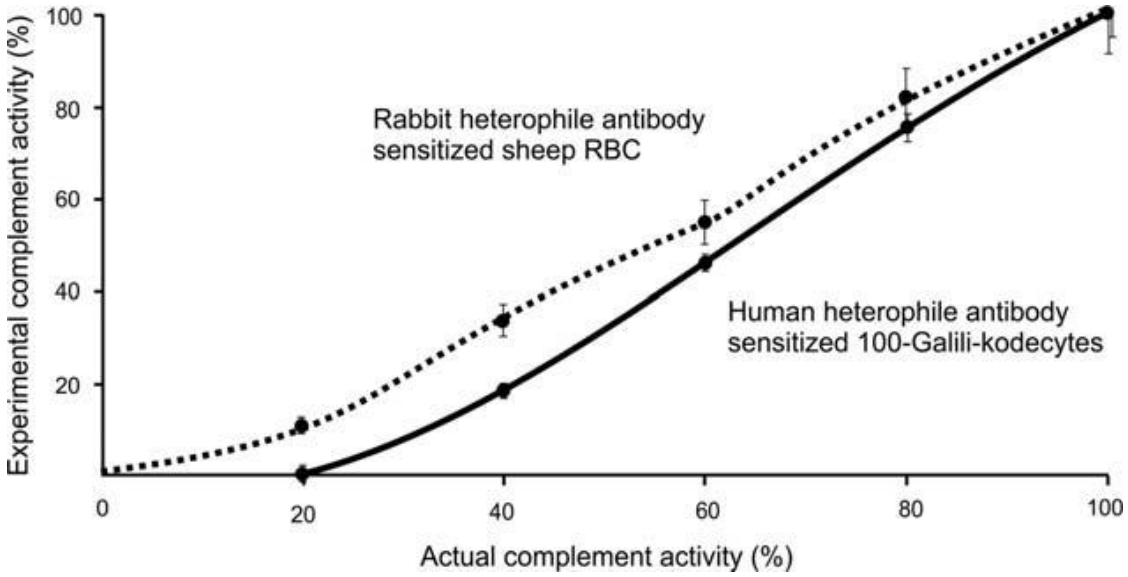


Figure 30. Comparison of two-stage sensitized sheep RBC with one-stage 100-Galα-kodecytes. The dotted black line shows the presensitized sheep RBC with serum at a dilution factor of 32, based on the method reported by Garratty [145], while the solid black line shows the 100-Galα-kodecytes against undiluted serum. Actual complement activity (complement activity standards) is plotted on the x axis, whilst experimental complement activity is on the y axis and is expressed as a percentage of the 100% complement activity standard (Formula 6). Reproduced from the author's work [178].

The correlation of the two curves shown in Figure 30 was found to be very high showing that the two assays were comparable in terms of sensitivity (r = 0.98). Both assays somewhat underestimated percentages of active complement and the Kodecyte assay showed slightly lower sensitivity than the sensitized sheep RBC assay.

2.13 Investigation of anti-complementary properties of stored serum

In their 1961 paper, Polley & Mollison [153] noted that dilutions of serums stored at minus 20°C sometimes produced more lysis in lytic assays than undiluted serum did under the same storage conditions. They proposed that this was due to a change during storage; namely the accumulation of "complementoid: a form of complement which may be adsorbed on to sensitized red cells and block the adsorption of normal complement" [153]. Undiluted sera samples would be more affected by this phenomenon, as diluted samples would have less complementoid present than undiluted samples.

Because the Kodecyte assay used undiluted sera samples, the method of Polley & Mollison [153] was reproduced to test for evidence whether anti-complementary properties of serum stored at minus 20°C could have affected results of this research. Sheep RBC, as used by Polley & Mollison were replaced by 100-Galα-kodecytes in this experiment. The "day zero" sample was regarded as a sample that would be free of anti-complementary properties, having been preserved at minus 85°C, a temperature shown to preserve complement without any effect on complement-dependent biomarker activity [146].

Aliquots of the standard serum index sample, stored at minus 20°C for 30 days and 150 days, together with the "day zero" sample were heated to 56°C for 30 minutes. Doubling dilutions (dilution factors 1 to 32) of heat-treated sera were prepared in PBS, and then aliquots from these dilution series were diluted again in an equal volume of a non-heated aliquot of the "day zero" sample to supply a standard source of complement free of any anticomplementary substances or properties. In this way, the stored aliquots would have equal amounts of complement available, but different amounts of complementoid, depending on length of storage at minus 20°C. Different dilutions also had different amounts of anti-Galα present.

In aliquots of 200 µL, sera mixtures were incubated with 100 µL of 5% v/v suspension of 100-Galα-kodecytes for 45 minutes at 37°C. At the end of the incubation, 1µL of K_2H_2EDTA at 120 mmol/L was added to all tubes to halt lysis. Tubes were briefly centrifuged. 160 µL cell-free supernatant was sampled to 1 mL Drabkin solution and absorbance read at A540nm, against a serum blank.

When tested with non-koded RBC, the “day zero” serum showed no visual haemolysis (Figure 31), and low absorbance values (Table 18), as expected. A similar problem to that encountered in the experiment to determine the concentration of FSL-Galα required to produce lysis of 50% of kodecytes occurred, namely the absorbance of the negative controls was low, but not zero.

The undiluted “day zero” serum tested with 100-Galα-kodecytes produced an absorbance reading of 0.323, which was unexpectedly high. This reading was equivalent to that of the 100% haemolysis standard reading of 0.327 (Table 7), however as this sample contained equal volumes of heat-treated and non heat-treated serum, it should have only had half the complement activity of the 100% haemolysis standard.

No evidence of anti-complementary effect was seen within the series of dilutions of sera as a product of serum storage. Within each series, absorbances declined overall with dilution of sera (trendlines in Figure 32), reflecting the lowering concentration of complement-binding anti-Galα present in higher dilutions. In addition, A540nm readings for each dilution were comparable at “day zero”, 30 and 150 days of storage (with the exception of the unexpectedly high A540nm for the undiluted “day zero” serum).

Table 18. Investigation of anti-complementary properties of serum stored at minus 20°C. Dilutions of heat-treated stored aliquots of standard serum index sample, tested with 100-Galα-kodecytes.

Serum storage time at minus 20°C	100-Galα-kodecytes	A540nm of dilutions of sera dilution factor					
		1	2	4	8	16	32
30 days		.216	.157	.138	.128	.123	.115
150 days		.268	.182	.143	.154	.108	.123
“day zero”		.323	.130	.143	.187	.123	.172
“day zero”	non-koded RBC	.103	.072	.061	.075	.068	.050

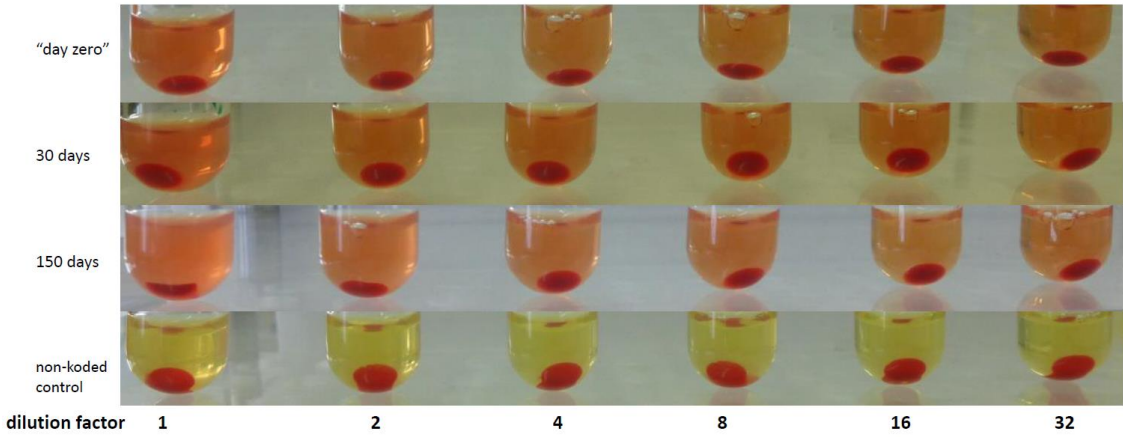


Figure 31. Test for anti-complementary activity in standard index serum sample. Dilutions (dilution factors 1 – 32) of serum at "day zero", 30 days and 150 days of storage at minus 20°C, tested in a lysis assay with 100-Galα-kodecytes, and a non-koded RBC control.

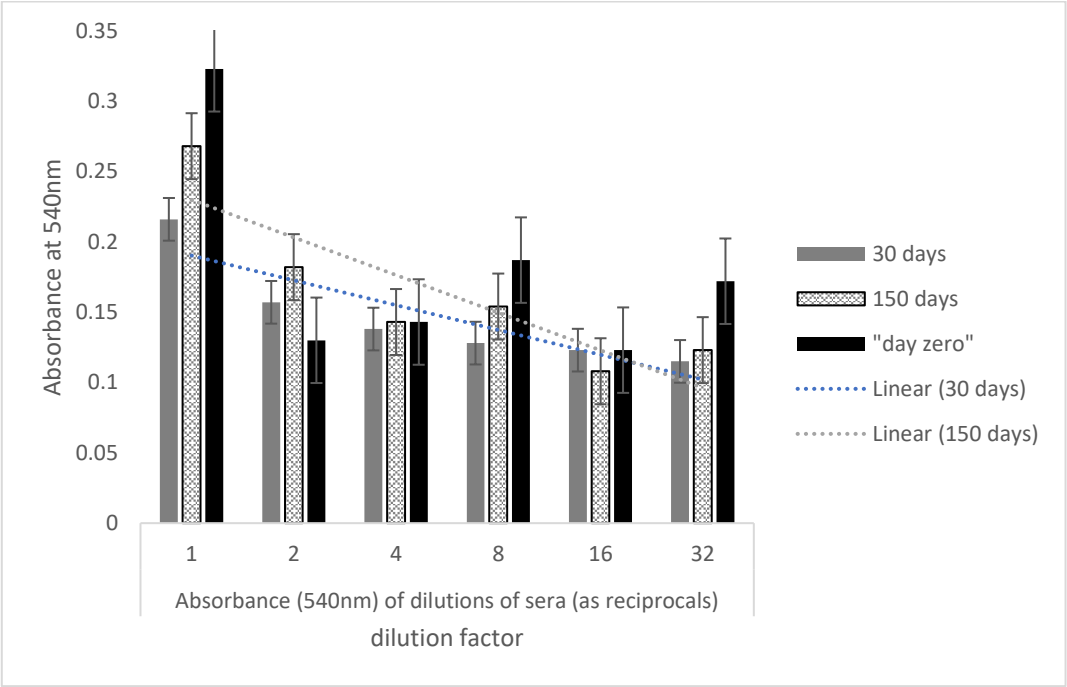


Figure 32. Absorbance (A540nm) of samples tested for anti-complementary activity in standard index serum sample. Undiluted and diluted series of "day zero" standard serum index sample, compared with aliquots of the same serum stored for 30 days and 150 days, to assess anti-complementary activity in serum stored at minus 20°C.

2.14 Lysis of kodecytes with FSL-Galα, FSL-GalNAcα and FSL-Rhaα

Three natural antibodies to glycans (anti-Galα, anti-GalNAcα and anti-Rhaα) were tested with corresponding antigen-specific kodecytes in lysis assays with sera from three healthy individuals. The three FSL used to produce these kodecytes were designed with CMG_2 spacer (Table 2) .

2.14.1 Lysis assay

Each FSL was reconstituted with Celpresol to a concentration of 1 mg/mL. Working FSL solutions were subsequently prepared at two different molarities (60 µmol/L and 20 µmol/L) as molar equivalents (Table 19).

Table 19. Comparison of concentration in µg/mL and µmol/L for FSL-Galα, FSL-GalNAcα and FSL-Rhaα.

FSL	MW	µmol/L	µg/mL
FSL-Galα(CMG_2 spacer)	2654	60	159
		20	53
FSL-GalNAcα	2695	60	162
		20	54
FSL-Rhaα	2272	60	136
		20	45

2.14.2 Preparation of kodecytes

Kodecytes were prepared by the standard method (section 2.2.1 Kodecytes) then suspended to 5% v/v in Celpresol and rested overnight. Kodecytes were washed in PBS, prepared as 5% v/v in PBS, and kodecytes numbers standardised to ±10% using the method described in section 2.5 Standardization of RBC number.

2.14.3 Lysis Controls

A negative lysis control consisted of 200 µL of 100% complement activity standard (Table 9) incubated with 100 µL of non-koded RBC. This control showed no lysis. A 100% lysis control consisted of 200 µL of 100% complement activity standard (Table 9), incubated with 100µL of 5% v/v 750-Galα-kodecytes. This control produced lysis of 100% of kodecytes.

2.14.4 Lysis Assay

A lysis assay was performed in duplicate with randomly selected sera from three healthy individuals. Three sera were used rather than a single serum, as titres of carbohydrate antibodies do vary naturally amongst individuals [32]. It was assumed that the three individuals had comparable complement levels.

Duplicates of 200 µL of each serum aliquot and 100 µL of kodecytes (5% v/v in PBS) were mixed together and incubated for 60 minutes at 37°C. Lysis reactions were stopped with the addition of 1 µL of K_2EDTA at 120 mmol/L. Tubes were mixed, centrifuged to sediment kodecytes and 160 µL of cell-free supernatant was sampled to 1 mL of Drabkin, and absorbance read at A540 nm. The spectrophotometer was blanked on a sample of 160 µL appropriate serum (specific to each of the three sera from three different individuals) in 1 mL of Drabkin to account for serum colour. Following A540nm readings, duplicate results were averaged. Absorbances were converted to a percentage of cells lysed by comparing the absorbance of each sample to the A540nm of the 100% lysis control. Results are shown in Table 20 and Figure 33.

Table 20. Percentage of kodecytes lysed by three different sera, comparing three different FSL at two different molarities.

kodecytes	Galα		GalNAcα		Rhaα	
µmol/L	60	20	60	20	60	20
individual	percentage of kodecytes lysed					
1	100	100	100	14	100	60
2	18	9	100	31	100	100
3	17	3	100	67	100	60

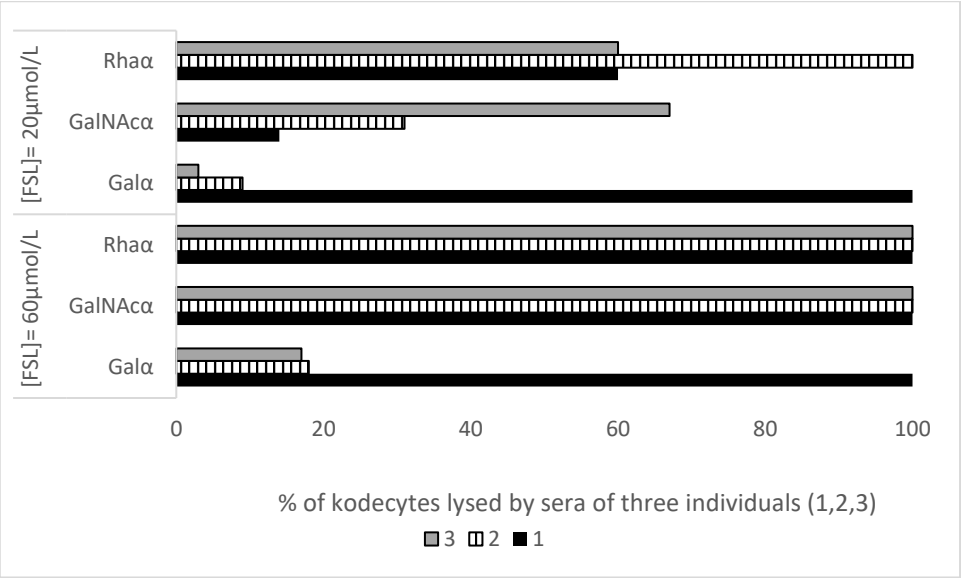


Figure 33. Gal α-, GalNAcα-, and Rhaα-kodecytes tested with sera from three individuals in a lysis assay. Kodecytes were prepared with FSL at 60 and 20 µmol/L. Percentage of kodecytes lysed by sera of each individual is plotted.

With FSL at 60 µmol/L, 100% of GalNAcα-kodecytes and Rhaα-kodecytes were lysed by sera of all three individuals. With 60-Galα-kodecytes, serum of one individual produced lysis of 100% of kodecytes, whilst sera of two individuals produced lysis of only approximately 20% of kodecytes. Kodecytes prepared with 20 µmol/L of FSL resulted in lower proportions (less than 100%) of kodecytes lysed in seven of nine data points (Table 20). The exceptions to this were:

- Individual one's serum produced lysis of 100% of 20-Galα-kodecytes
- Individual two's serum produced lysis of 100% of 20-Rhaα-kodecytes

It was presumed that individual 1 had a high level of anti-Galα, and that individual 2 had a high level of anti-Rhaα.

2.15 Complement activation on non-cellular surfaces of paper, glass and plastic

FSL-Galα (ad spacer, Table 2) was incubated with fresh human serum containing both anti-Galα and complement on media gloss paper, microscope glass slides and plastic. An enzyme immunosorbent assay (EIA), applying a secondary antibody of murine anti-human-C3d, and a tertiary antibody of alkaline phosphatase conjugated anti-mouse immunoglobulin was used to detect the presence of C3.

Method details were as follows:

2.15.1 Paper

1. FSL-Galα was prepared as 500, 400, 300 and 200 µg/mL solutions in PBS
2. A number representing each FSL concentration was printed onto media gloss paper (Spicers, Auckland, New Zealand) with an inkjet printer (Epson Stylus T21, Seiko Epson, Nagano, Japan) where the inkjet cartridge was loaded with the appropriate FSL construct, as reported by Barr [200] (Figure 34). Positive controls FSL A and FSL B at 500 µg/mL were also printed, as ABO antibodies are known to activate complement [6,42]. Printed papers were dried overnight
3. A microplate was constructed to support and contain the FSL-printed paper and subsequent EIA reactions in two wells. The microplate construction was as described by Barr [200] (Figure 34)
4. The plate was blocked for 1 hour with 2% bovine serum albumin (Gibco, Waltham, USA) in PBS at volumes of 200 µL per well, then drained and blotted
5. To both wells, 100 µL of freshly thawed group O serum was added. The plate was incubated for 30 minutes in a moist chamber at 37°C
6. The plate was washed six times with PBS
7. To one well was added 100 µL murine anti-human-C3d (Immucor, Peachtree Corners, USA), and to the second well, 100 µL murine anti-human-IgG (Immulab, Scoresby, Australia) was added. The plate was incubated for 30 minutes at 22°C
8. The plate was washed six times with PBS
9. To both wells, 100 µL of alkaline phosphatase conjugated goat anti-mouse Ig (Millipore, Burlington, USA) at a dilution of 1 in 1000 was added
10. The plate was incubated for 30 minutes at 22°C, and then washed six times with PBS
11. To each well was added 100 µL nitro-blue tetrazolium and 5-bromo-4-chloro-3'-indolyphosphate (NBT/BCIP) (Roche,Basel, Switzerland) at a dilution of 1 in 50 in Tris buffered saline (TBS)
12. The plate was left until colour developed in the control FSL-A and FSL-B constructs (approximately 30 minutes), then washed once with distilled water, and left overnight to dry

Results showed successful antibody mediated complement deposition onto paper impregnated with FSL-Galα at all concentrations tested (200-500 µg/mL). As seen in Figure 34, a purple coloured precipitate (positive EIA signal) developed with all printed FSL constructs. The reactions with anti-human-IgG were due to the reaction of FSL-Galα and IgG anti-Galα in the case of FSL-Galα, and to FSL A/B and IgG anti-A/anti-B in the case of FSL-A and FSL-B. The reactions with anti-human-C3d were specific for C3, caused by complement deposition on paper, following antibody-mediated complement activation. A negative control of FSL-biotin run at the same time on a separate microplate showed no colour development (result not shown). No reactivity was seen outside the printed area, which supported specificity of the reaction. The experiment was repeated on two further occasions, with the same results (Figure 34).

Figure 34. Fresh human serum tested with FSL-Galα in an EIA assay on paper. FSL-Galα was added to paper at concentrations of 500, 400, 300 and 200 µg/mL. The secondary antibodies were murine anti-human-C3d and anti-human-IgG (in individual wells as labelled). Antigen-antibody reactions, and antigen-antibody-complement deposition reactions were visualised with an alkaline phosphatase labelled tertiary antibody; anti-mouse immunoglobulin. Upon addition of NBT/BCIP, alkaline phosphatase precipitated the NBT/BCIP to form a purple colour. Colour development occurred with all FSL concentrations with both anti-human-C3d in the first well (showing that C3d was deposited on the paper) and anti-human-IgG in the second well (showing that IgG was present on the paper).

2.15.2 Glass

1. Microscope glass slides (Liberty, Scoresby, Australia) were cleaned with ethanol
2. FSL-Galα at 500 and 100 µg/mL was spotted (2 µl) onto slides and dried overnight. A spot (2 µl) of FSL-biotin was included as a negative control
3. The same protocol described for 2.15.1 Paper (steps 4-12) were performed on glass, with the exception that only anti-human-C3d was applied as a secondary antibody (not anti-IgG in addition)

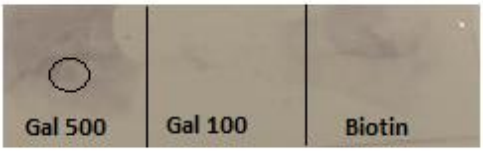


Figure 35. Fresh human serum tested with FSL-Galα in an EIA assay on glass. FSL-Galα was added to glass slides at concentrations of 500 and 100 µg/mL, and subjected to the EIA assay as described previously in Figure 34. The circle in Gal 500 area of the slide marks the spot where FSL-Galα at 500 µg/mL was originally applied.

Results with glass (Figure 35) showed a positive reaction with FSL-Galα at 500 µg/mL but not with 100 µg/mL suggesting that complement was deposited on the glass due to the interaction of FSL-Galα at 500 µg/mL and complement-activating anti-Galα. However, the positive reaction was not restricted to the original place where the FSL was spotted (marked with a black circle in Figure 35), but showed smearing. Smearing was expected, as the first wash step is well-established to spread the FSL coating [189], and FSL then immediately adheres to the glass. The negative control was valid, showing no colour development. The smearing of positive results on this surface made glass unsuitable without method modification, which was not pursued as part of this study.

2.15.3 Plastic

1. FSL-Galα at 500 and 100 µg/mL was spotted (2µl) onto the lid of a plastic 96 well plate (Greiner Bio-One, Kremsmunster, Austria) and dried. A spot (2 µl) of FSL-biotin was included as a negative control
2. The same protocol described for 2.15.1 Paper (steps 4-12) were performed on plastic, with the exception that only anti-C3d was applied as a secondary antibody (not anti-IgG in addition)

Results showed that complement could be deposited to plastic. Colour development (positive reaction) occurred with both concentrations of FSL-Galα. The positive reaction was limited to the spotted area with FSL-Galα at 500µg/mL, and mostly contained to the applied area with FSL-Galα at 100µg/mL. The negative control showed no colour development.

Figure 36. Fresh human serum tested with FSL-Galα in an EIA assay on plastic. FSL-Galα was added at concentrations of 500 and 100 µg/mL on the lid of a 96 well plate, and subjected to an EIA assay; details as per Figure 34.

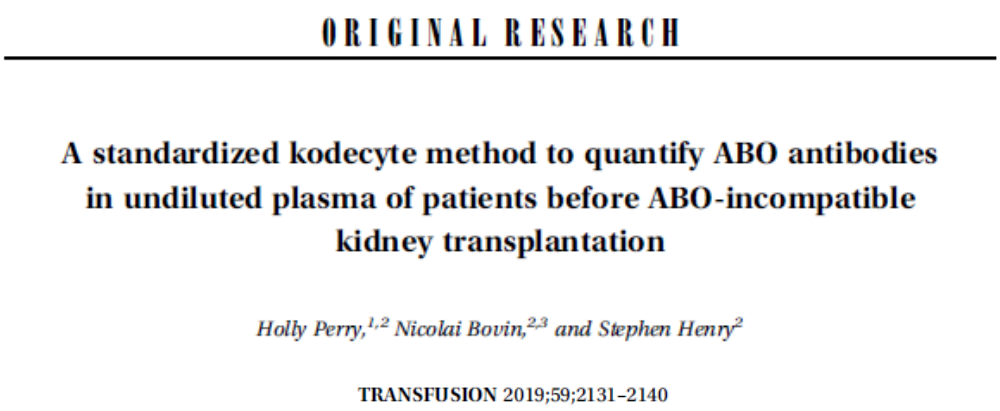
2.16 Chapter summary

In this chapter, complement deposition on kodecyte membranes, paper, glass and plastic was demonstrated with three different FSL and corresponding specific complement-activating antibodies present in undiluted fresh sera samples. Complement deposition on kodecytes led to quantifiable lysis. These novel methods were applied in a study of stored aliquots of a standard serum index sample to yield new insights into complement stability. Attempted extensions of the methodology to a solid phase assay were promising but were discontinued as they required significant optimization, and did not appear to add further information to that obtained by working with kodecytes.

Chapter Three: Methods and Results: quantification of antibodies with standardised kodecyte agglutination assays

3.1 Overview of method principles

The aim here was to evaluate optimized panels of kodecytes for quantifying antibodies of the ABO blood group system. Quantification of ABO antibodies by kodecyte assays was compared to the traditional method of antibody titration [101]. Blood group A kodecytes were prepared with FSL-A type 2 at different concentrations, to prepare a panel of kodecytes loaded with different quantities of A antigen. This kodecyte panel was tested with undiluted plasma samples to determine whether an antibody was present in relatively high, medium or low levels. The principle here is that an antibody at high levels should react with kodecytes loaded with FSL at a range of antigen densities including a low density, a medium level antibody should react with some of these kodecytes, and a low level antibody with only the kodecyte expressing the highest loading of antigen. Optimal concentrations of FSL-A for a panel of kodecytes capable of differentiating these high, medium and low levels of antibody in sera were determined, using a set of "validation sera". Blood group A-kodecytes, prepared with FSL-A at the optimised antigen dilution range, were tested with 125 plasmas (102 group O and 23 group B) from New Zealand blood donors, and 40 plasmas from five patients preparing for ABOiKT. All samples were tested by A_1 cell titration ("A_1 cell assay") and the "Kodecyte assay", and assay concordance rates were evaluated. Testing platforms of tube and CAT were assessed. Portions of this chapter have been published [177] (Figure 37) and the full publication is in Appendix A Publications.



ORIGINAL RESEARCH

A standardized kodecyte method to quantify ABO antibodies in undiluted plasma of patients before ABO-incompatible kidney transplantation

Holly Perry,[1,2] Nicolai Bovin,[2,3] and Stephen Henry[2]

TRANSFUSION 2019;59;2131–2140

Figure 37. Front-page title information of the publication arising from work reported in Chapter 3.

In addition to ABO antibodies, antibodies to potential immuno-oncotherapy xeno-antigens Galα, GalNAcα and Rhaα were quantified in a Kodecyte assay to assess their relative levels in 100 healthy group O blood donors.

3.2 Materials and Samples

3.2.1 FSL and Kodecytes

FSL were supplied by Kode Biotech Materials, Auckland, New Zealand. Kodecytes were prepared with FSL-A type 2 (Figure 12) for the ABO antibody quantification work, and FSL-Galα (CMG_2 spacer), FSL-GalNAcα and FSL-Rhaα (Figure 13b, Figure 14 and Figure 15) for the quantification of antibodies to xeno-glycan antigens.

Details of preparation and storage of kodecytes was as described in Chapter 2, section 2.2.1 Kodecytes.

3.2.2 Natural A cells

A single known A_1 phenotype RBC individual (genotype confirmed) was used throughout the titration methods. Diagnostic laboratories use a pool of A_1 cells for titration [69], however in this study it was more accurate to use a single standardized cell to compare results from A_1 cell assays with Kodecyte assays, in order to avoid the confounding variable of antigen copy number [109-111].

3.2.3 Sera/plasma

A. Validation sera and plasmas

FSL-A kodecytes were validated with several well characterized sera and plasmas during method development:

- In date monoclonal reagent anti-A sera (Epiclone, Immulab, Melbourne, Australia). This had a high titre of IgM anti-A (A_1 cell saline tube titre 1024) and no IgG anti-A content
- Expired polyclonal reagent anti-A,B sera (Copper Biomedical, West Chester, USA). Although expired in 1986, this reagent was found to be of high titre (A_1 cell saline tube titre 128, antiglobulin tube titre 2048). It had the advantages of being a polyclonal reagent from humans, containing both IgG and IgM, and was

available in large quantities. This antisera is hereafter referred to as "CB polyclonal anti-A,B"

- In-house plasma from three group O laboratory (lab) staff. Plasmas from three lab staff were selected for their different levels of anti-A/anti-A,B:
    - Lab staff 1: A_1 cell saline tube titre 32, antiglobulin tube titre 1024
    - Lab staff 2: A_1 cell saline tube titre 128, antiglobulin tube titre 512
    - Lab staff 3: A_1 cell saline tube titre 256, antiglobulin tube titre 256

B. Donor plasma samples

EDTA plasma samples from 102 group O and 23 group B donors (NZBS, Auckland, NZ) were used in the quantification of ABO antibodies.

A further 100 samples from group O blood donors (NZBS, Auckland, NZ) were used to quantify antibody levels to antigens of FSL-Galα, FSL-GalNAcα and FSL-Rhaα in a separate study.

C. ABOiKT patient samples

Five patients (four blood group O and one group B) presenting at Auckland District Health Board for ABOiKT consented to take part in this study (AUTEC #16/175). Accordingly, 40 plasma samples from these five patients were collected. Each patient presented eight samples; immediately pre (before) and immediately post (after) plasmapheresis (PP) at four separate PP events during the week prior to their scheduled transplantation date. All samples were maintained at minus 85°C and tested on the day of thawing.

3.2.4 Quality control (QC) samples

- External standard QC material (World Health Organization (WHO) reference reagent 14/300 formulated to standardize and control haemagglutination titrations for anti-A and anti-B in serum and plasma) was purchased from National Institute for Biological Standards and Control, Potters Bar, England [113]
- Internal QC sample; plasma from lab staff member 1 was validated against the external standard QC material and was used to control the A_1 cell assay and the Kodecyte assay performance

- Negative control RBC; a group O RBC without FSL but exposed to the same processing steps as the kodecytes was included to monitor agglutination reactions due to non-ABO antibody

3.3 General method considerations

- All reagents and samples were brought to 22°C before use
- In both tube and CAT methods, agglutination was graded using the 0-4 grading system [101]. In the 0-4 grading system grades 3 and 4 are strong agglutination reactions, 2 is a medium reaction, 1 is a weak reaction, and 0 denotes a negative reaction (no agglutination). w is used to denote a very weak positive reaction (less than grade 1)

3.4 Quantification of ABO antibodies

3.4.1 A_1 cell assay

Master doubling dilution series were prepared in dilution factors from 1 (undiluted) to 2048. These master series were prepared on the day of testing, in volumes sufficient to perform all methods on the same day. This practice reduces pipetting variation error, allowing comparison between methods independently of this confounding variable. To optimize accuracy as much as possible, all dilutions in the series were well mixed and prepared using a clean pipette tip for each transfer.

Saline tube

A set of twelve glass tubes was labelled with the dilution factor, including a tube for the undiluted plasma. Beginning from the highest dilution of 2048, 100 µl from the master dilution series was transferred to glass test tubes. A_1 RBC (100 µl of a 2% v/v suspension in PBS [101]) were added to all tubes. Tubes were gently mixed and incubated for 1 hour at 22°C. Tubes were briefly centrifuged to sediment RBC, and RBC were examined for agglutination with a 10x magnification eyepiece. The titre end point was recorded as the highest dilution showing agglutination of at least grade 1 [101].

Antiglobulin tube

A set of test tubes was prepared from the master dilution, in the same manner and volumes as described above for the saline tube method. To all tubes containing plasma and dilutions thereof, 100 µl of 2% v/v A_1 RBC suspended in PBS was added [101]. The tubes were gently mixed and incubated for 1 hour at 37°C. The RBC were washed four times with PBS, completely decanting the last wash. Two drops of AHG (anti-IgG, anti-C3d) (Epiclone, Immulab, Melbourne, Australia) were added to each tube, and tubes were briefly centrifuged to sediment RBC. Agglutination grading and titre result was as for the saline tube method. IgG sensitized cells were added to confirm negative reactions. Tests which did not convert to positive after addition of IgG sensitized cells were repeated.

Saline CAT

Neutral cassettes (Ortho Clinical Diagnostics, Raritan, NJ) were labelled, and 50 µL of A_1 RBC suspended 1% v/v in red cell diluent (RCD) (Ortho Clinical Diagnostics, Raritan, NJ) was added, followed by 40 µL of appropriate plasma dilution from the master titration series. Cassettes were incubated for 15 minutes at 22°C, then centrifuged in the Ortho Biovue System centrifuge (Ortho Clinical Diagnostics, Raritan, NJ) and examined and graded for agglutination using the 0-4 grading system [101].

Antiglobulin CAT

Cassettes containing AHG (anti-IgG, anti-C3d) (Ortho Clinical Diagnostics, Raritan, NJ) were labelled. To individual cassette wells, 50 µL of A_1 RBC suspended 1% v/v in Ortho RCD was added, followed by 40 µL of plasma and dilutions thereof. Cassettes were incubated for 15 minutes at 37°C in the Ortho Biovue System heat block (Ortho Clinical Diagnostics, Raritan, NJ), then centrifuged. Cassette wells were examined and graded for agglutination as for saline CAT.

3.4.2 Kodecyte assay

Saline tube

Glass tubes were labelled with the plasma and kodecyte FSL concentration identifiers. Undiluted plasma was added to all tubes in volumes of 100 µL. Kodecytes (2% v/v suspensions in PBS) were added in volumes of 100 µL. Tubes were then treated in the same way as described in the A_1 cell assay saline tube method (section 3.4.1 A_1 cell assay).

Antiglobulin tube

Undiluted plasma was added to labelled tubes in volumes of 100 µL. Kodecytes (2% v/v suspensions in PBS) were added in volumes of 100 µL. Tubes were then incubated, washed and assessed for agglutination in the presence of AHG (anti-IgG, anti-C3d) in the same way as described in the A_1 cell assay antiglobulin tube method (section 3.4.1 A_1 cell assay). As per standard practice [101], IgG sensitized cells were added to negative tubes. Tests which did not show agglutination after addition of IgG sensitized cells were repeated.

Saline CAT

Labelled neutral cassette wells were prepared with kodecytes (50µL of 1% suspensions in Ortho RCD) and undiluted plasma (40 µL) in each well. Cassettes were then centrifuged and assessed for agglutination in the same way as described in the A_1 cell assay saline CAT method (section 3.4.1 A_1 cell assay).

Antiglobulin CAT

Cassette wells containing AHG (anti-IgG, anti-C3d) were labelled with the plasma and kodecyte identifiers. Kodecytes (1% v/v suspensions in Ortho RCD) were added in volumes of 50 µL, followed by 40 µL of undiluted plasma in each well. Cassettes were then incubated, centrifuged and assessed for agglutination in the same way as described in the A_1 cell assay antiglobulin CAT method (section 3.4.1 A_1 cell assay).

3.4.3 Artificially created range of antibody levels

The validation sera/plasmas (section 3.2.3 Sera/plasma) were titrated by the saline tube and antiglobulin tube A_1 cell assays (section 3.4.1 A_1 cell assay) to establish their A_1 cell titres in an undiluted state. Dilutions of validation sera/plasmas were then individually prepared (i.e not serially diluted) in PBS to artificially create different levels of antibody. These were assigned as high, medium and low levels of antibody, with these designations guided by literature (Table 21). This range of antibody levels was created to test against the kodecytes, in order to guide the optimization of the FSL-A concentrations used to prepare a kodecyte panel for quantifying anti-A and/or anti-A,B.

Table 21. Assignment of A_1 cell titres (saline and antiglobulin) into levels of high, medium and low*.

	Saline	antiglobulin
High	≥64	≥256
Medium	32	32 - 128
Low	≤16	≤16

*High titres are those reported in literature as those capable of causing haemolysis in non-ABO matched transfusion blood products [68-70,72,74] . Low titres are those adopted by some as permissible for ABOiKT [46,49,50,61]. Medium is the range between high and low.

Table 22 shows the range of dilutions prepared for each validation sera, together with their designated antibody level from Table 21 criteria.

Table 22. Titres of validation sera/plasma, in both undiluted and diluted states. Dilutions were prepared to artificially create different levels of antibody.

serum/plasma	dilution factor	A_1 cell titre (saline tube)	saline level (Table 21)	A_1 cell titre (antiglobulin tube)	Antiglobulin level (Table 21)
Epiclone monoclonal anti-A	1 (undiluted)	1024	H	NA*	
	16	64	H		
	32	32	M		
	64	16	L		
CB polyclonal anti-A,B	1	128	H	2048	H
	2	64	H	1024	H
	4	32	M	512	H
	8	16	L	256	H
	16	8	L	128	M
	32	4	L	64	M
Lab staff 1†	1	32	M	1024	H
Lab staff 2	1	128	H	512	H
Lab staff 3	1	256	H	256	H

*NA = not applicable; this reagent contains only IgM.

† Lab staff 1 was used as the internal QC sample (see section 3.2.4 Quality control (QC) samples)

3.4.4 Kodecyte panel

Optimization

With a view to ultimately creating a panel of kodecytes to produce different reaction patterns to differentiate high, medium and low levels of anti-A and anti-A,B, the artificially created antibody levels (validation sera/plasma Table 22) were tested with FSL-A type 2 kodecytes prepared with FSL A at 35, 8, 2.5 and 0.5 µmol/L. These initial concentrations were guided by previous experience with another FSL-A construct [122]. First, three dilutions of Epiclone anti-A (Table 22) were tested in a saline tube platform. Results (Table 23) suggested that the range of FSL-A type 2 concentrations of 35, 8, 2.5 and 0.5 µmol/L was not wide enough to differentiate between different antibody levels, as the reaction pattern with the kodecytes was the same for two different dilutions of the antisera (i.e titres of 64 and 32 were both positive with 35-, 8- and 2.5-A-kodecytes). In addition, the kodecyte prepared with FSL at 0.5 µmol/L failed to react with any antibody dilution (Table 23), so was eliminated.

Table 23. Kodecyte assay (saline tube). Results of Epiclone anti-A sera with kodecyte panel.

		FSL concentration (µmol/L)-A-kodecytes				
		35	8	2.5	0.5	0†
Saline antibody titre		grades of agglutination				
Epiclone anti-A	64	4	4	2	0	0
	32	3	2	1	0	0
	16	2	2	0	0	0

Agglutination is using the 0 to 4 system [101].
† 0 = non-koded RBC

Accordingly, further kodecytes were created, with FSL-A type 2 concentrations of 80, 40, 20, 10, 5, and 2.5 µmol/L. These were tested with several dilutions of the CB polyclonal anti-A,B validation sera in a chequer board series (where different concentrations of both antigen and antibody are used to produce reaction patterns of decreasing agglutination strength as concentration of both antigen and antibody decreases). Results showed a promising pattern (Table 24). As illustrated in the green highlighted cells in Table 24, three different decreasing reaction grades (4,2,1) were produced over a decreasing range of antigen and antibody concentrations.

Table 24. Kodecyte assay (saline tube). Results of CB polyclonal anti-A,B validation sera with kodecyte panel.

		FSL concentration (µmol/L)-A-kodecytes						
		80	40	20	10	5	2.5	0
CB polyclonal anti-A,B	Saline antibody titre							
	128	4	4	3	3	3	2	0
	64	4	4	4	2	2	1	0
	32	4	4	2	2	1	1	0
	16	3	3	2	2	1	1	0
	8	2	2	1	2	1	0	0
	4	1	1	1	1	1	0	0

Agglutination is graded using the 0-4 system [101]. Green highlighted cells illustrate the chequer board pattern, with weakening reactions of 4, 2, 1 observed as antigen and antibody concentrations are lowered.

However, all kodecytes except the 2.5-A-kodecyte were reactive with all titres of the CB polyclonal anti-A,B. This was not ideal, as it would be desirable to have clear positive and clear negative reactions to differentiate reactivity patterns. An ideal assay would not solely rely on having to differentiate reaction strengths, which is problematic due to different grading ability amongst different people [120-122]. Therefore it was apparent that the kodecyte range was still not ideal. Accordingly, additional kodecytes were prepared with FSL-A at 2, 1.5 and 1 µmol/L, and added to the panel. Results with kodecytes prepared at 80, 40, 20, 10, 5, 2.5, 2, 1.5 and 1 µmol/L are shown in Table 25. More kodecytes produced negative reactions with the lower antibody titres (yellow shaded cells in Table 25), and positive reactions with the higher titres of anti-A,B (grey shaded cells in Table 25). For the lowest antigen concentration kodecyte, FSL-A type 2 in the concentration range 1 – 2.5 µmol/L looked promising.

Table 25. Kodecyte assay (saline tube). Results of CB polyclonal anti-A,B sera with extended kodecyte panel.

		FSL concentration (µmol/L)-A-kodecytes								
		80	40	20	10	5	2.5	2	1.5	1	0
CB polyclonal anti-A,B	Saline antibody titre										
	128	4	4	4	4	3	2	2	2	1	0
	64	3	3	3	2	2	1	1	1	1	0
	32	3	2	2	2	2	1	1	1	1	0
	16	2	2	2	2	1	1	1	1	1	0
	8	2	2	2	1	1	1	1	0	0	0
	4	2	2	1	1	1	0	0	0	0	0

Agglutination is graded using the 0-4 system [101]. Green highlighted cells illustrate the chequer board pattern, and grey shaded cells versus yellow shaded cells show positive versus negative reactions with kodecytes created with different FSL concentrations, with different dilutions of CB polyclonal anti-A,B.

All testing to this point had been with commercial reagent antisera. As commercial reagents contain preservatives, and possibly potentiators , testing was commenced with a human plasma (lab staff 2) to test samples more representative of a patient population. Plasma from lab staff 2 was selected because it had similar antibody levels to the CB polyclonal anti-A,B. The same range of kodecytes shown in Table 25 was re-tested with dilutions of plasma from lab staff 2 (Table 22). Results with plasma from lab staff 2 are shown in Table 26.

Table 26. Kodecyte assay (saline tube). Results of lab staff 2 plasma with extended kodecyte panel.

		FSL concentration (µmol/L)-A-kodecytes									
		80	40	20	10	5	2.5	2	1.5	1	0
Lab staff 2	Saline antibody titre										
	128	4	4	3	3	2	2	1	1	1	0
	64	4	3	3	3	2	2	1	1	1	0
	32	4	3	2	2	1	1	1	1	1	0
	16	2	2	2	1	1	1	1	1	1	0
	8	2	1	1	1	1	0	0	0	0	0
	4	1	1	1	0	0	0	0	0	0	0

Agglutination is graded using the 0-4 system [101]. Green highlighted cells illustrate the chequer board pattern, and grey shaded cells versus yellow shaded cells show positive versus negative reactions with kodecytes created with different FSL concentrations, with different dilutions of lab staff 2 plasma.

Results from lab staff 2 were somewhat different to the commercial polyclonal anti-A,B. The plasma from the staff member, although having the same saline titre as the CB polyclonal anti-A,B in the undiluted state, reacted with fewer kodecytes at titres of 8 and 4. It remains unexplained why this is so, but the possible presence of potentiators in the commercial reagent, added to optimise reactivity [4], could account for the reactivity at the same dilutions with the same kodecytes in the commercial sera, and lack of reactivity in the human plasma.

Three-kodecyte panel, saline Kodecyte assay

At this point, it was not decided how many kodecytes would be in the final panel, but it was considered desirable to investigate the lowest possible number of kodecytes which could differentiate antibody levels (as an assay with lower consumable and workload demands would be more attractive to diagnostic laboratories). With a view to using two or three of the kodecytes in Table 25 and Table 26 to differentiate high, medium and low level anti A/anti-A,B, it was decided to test three of these kodecytes with the CB

polyclonal anti-A,B, and concurrently with plasma from lab staff 2. Two kodecyte panels were prepared; one with kodecytes prepared with FSL-A type 2 at 80, 5 and 1 µmol/L, and one with kodecytes at 80, 10 and 2 µmol/L. Testing in saline CAT agglutination technology was introduced at this point, as this platform would be attractive to diagnostic laboratories.

An ideal pattern to differentiate antibody levels with three kodecytes and undiluted plasma would be:

- High level antibody: positive reactions with all three kodecytes
- Medium level antibody: positive reactions with the two kodecytes prepared with the higher concentrations of FSL, and negative reaction with the kodecyte prepared with the lowest FSL concentration
- Low level antibody: positive reaction with the kodecyte prepared with the highest FSL concentration, and negative reactions with the remaining two kodecytes

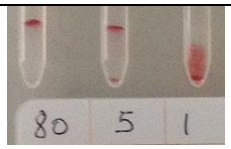
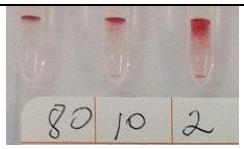
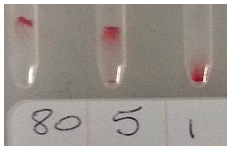
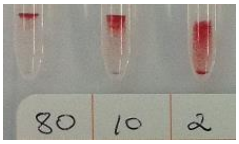
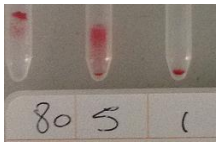
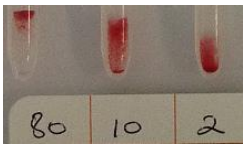
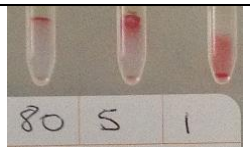
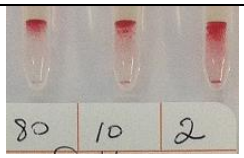
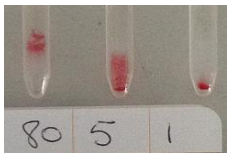
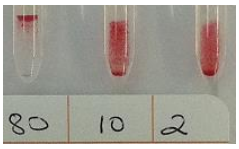
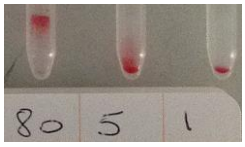
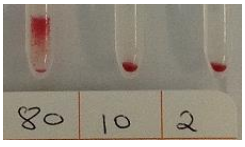
This criteria for ideal pattern differentiation is presented in tabular form in Table 27, using the kodecytes prepared with FSL-A type 2 at 80, 10 and 2 µmol/L as an example.

Table 27. Assignment of antibody level by theoretical reactions with three-kodecyte panel.

			FSL concentration (µmol/L)-A-kodecytes		
antibody level	Saline titre range	Antiglobulin titre range	80	10	2
High (H)	≥64	≥256	+	+	+
Medium (M)	32	32-128	+	+	0
Low (L)	≤16	≤16	+	0	0

According to Table 27 criteria, results appeared promising with both three-kodecyte panels. Although neither kodecyte panel satisfied all conditions set out above (Table 27) with either sera, a pattern towards this end was starting to emerge. High level antibodies agglutinated all three kodecytes (both panels). Medium level antibodies agglutinated the two higher FSL concentration kodecytes on two occasions of four (both panels). Low level antibodies agglutinated only the highest FSL concentration kodecyte on two occasions of four (both panels) (Table 28). For the purposes of this analysis, the very weak reactions (Table 28) were regarded as negative reactions.

Table 28. Kodecyte assay (saline CAT). Results of two validation sera with three-kodecyte panel.

	saline titre	antibody level (Table 27)		kodecyte panel 80, 5, 1			kodecyte panel 80, 10, 2		
CB polyclonal anti-A,B	64	H							
			grade	4	3	1	4	3	2
	32	M							
			grade	4	3	w	4	3	2
	16	L							
			grade	3	2	0	3	2	1
Lab staff 2 plasma	64	H							
				4	3	2	4	3	2
	32	M							
			grade	3	2	0	3	2	2
	16	L							
			grade	3	1	0	2	0	0

w = weak agglutination

Three-kodecyte panel, antiglobulin method

With saline results looking promising, testing proceeded to antiglobulin testing. Whereas the saline test predominantly detects IgM antibodies, the antiglobulin test is required to detect IgG antibodies [102,103]. It should be noted that when IgM antibodies are present in plasma tested in an antiglobulin test, IgM will agglutinate the RBC during the incubation phase, and so still cause a positive antiglobulin test even in the absence of IgG. It is therefore more accurate to describe the antiglobulin test for detection of total antibody (IgG plus IgM) rather than IgG alone.

First, the full range of kodecytes (80 – 1 µmol/L) were tested with the CB polyclonal anti-A,B in tube Kodecyte assay by the antiglobulin method. Results are shown in Table 29. From these results three candidate kodecytes for an antiglobulin three-kodecyte panel were those prepared with FSL at 80, 10 and 1 µmol/L. As before, for an ideal panel three kodecytes (80, 10 and 1) would react with a high level antibody, two kodecytes (80 and 10) with a medium level antibody and one kodecyte (80) with a low level antibody. This held true in this experiment.

Table 29. Kodecyte assay (antiglobulin tube). CB polyclonal anti-A,B with extended kodecyte panel.

				FSL concentration (µmol/L)-A-kodecytes										
CB polyclonal anti-A,B	dilution factor	Antiglobulin tube titre ≡	antibody level	80	40	20	10	8	5	2.5	2	1.5	1	0
	2	1024	H	4	3	3	3	4	4	4	4	2	2	0
	4	512	H	3	4	4	4	4	4	4	3	3	2	0
	8	256	H	3	4	4	4	4	4	4	3	3	2	0
	16	128	M	4	4	3	3	4	3	2	2	1	0	0
	32	64	M	4	3	2	3	3	3	4	3	2	0	0
	64	32	M	2	2	2	2	2	2	4	2	1	0	0
	128	16	L	2	2	1	0	0	0	0	0	0	0	0
	256	8	L	2	2	0	0	0	0	0	0	0	0	0
	512	4	L	1	0	0	0	0	0	0 *	0	0	0	0

* The 2.5-A-kodecyte was noted to have a weaker cell suspension strength that the other kodecytes, therefore the antigen antibody ratio was not ideal and this may account for the unexpectedly strong reactions with this kodecyte. Agglutination is graded using the 0-4 system [101]. Bordered columns show how the three candidate kodecytes (80, 10 and 1) react with high (H), medium (M) and low (L) level antibody preparations of CB polyclonal anti-A,B.

The three antiglobulin candidate kodecytes (80, 10 and 1 µmol/L) were then tested with the CB polyclonal anti-A,B and plasma from lab staff 1 in antiglobulin CAT format, as per the method described in section 3.4.2 Kodecyte assay. Results are shown in Table 30. Promising results were obtained, with three distinct patterns produced with plasma

dilutions representing antibody levels of high, medium and low in lab staff 1, although not with CB polyclonal anti-A,B, where the three kodecytes were not differentiating between high and medium level antibodies, and the low level antibody (titre 16) was reacting with two of the three kodecytes. Again this difference between CB polyclonal anti-A,B and plasma from lab staff 1 could be due to potentiators in the CB antisera.

Table 30. Kodecyte assay (antiglobulin CAT). Results of two validation sera/plasma with three-kodecyte panel.

	antiglobulin titre	antibody level (Table 21)	kodecyte panel 80,10,1		
CB polyclonal anti-A,B					
	512	H			
[FSL µmol/L]			80	10	1
grade			4	3	3
	128	M			
[FSL µmol/L]			80	10	1
grade			3	3	2
	16	L			
[FSL µmol/L]			80	10	1
grade			2	1	0
Lab staff 1 plasma					
	512	H			
[FSL µmol/L]			80	10	1
grade			3	3	2
	128	M			
[FSL µmol/L]			80	10	1
grade			3	2	0
	16	L			
[FSL µmol/L]			80	10	1
grade			2	0	0

Three-kodecyte panel, saline and antiglobulin methods

The three antiglobulin candidate kodecytes (80, 10 and 1 µmol/L) were showing promise in both saline and antiglobulin methods. Therefore, a larger number of undiluted plasma samples was tested with this three-kodecyte panel. Accordingly, 11 donor plasmas were tested by the following methods:

- Kodecyte assay in CAT; saline and antiglobulin (methods as per section 3.4.2 Kodecyte assay). Tube methods were not performed due to sample shortage
- A_1 cell assay (methods as per section 3.4.1 A_1 cell assay)
    - saline tube
    - saline CAT
    - antiglobulin tube
    - antiglobulin CAT

Results are shown in Table 31 and Table 32.

Table 31. Comparison of A_1 cell assay with three-kodecyte panel in 11 human plasmas (saline CAT).

Sample	A_1 cell titre	antibody level* (by titre)	reaction grades†			antibody level‡ (by kodecytes)	agreement§
			FSL-A (µmol/L)				
			80	10	1		
2	128	H	4	3	2	H	Y
10	128	H	4	4	4	H	Y
11	128	H	4	4	4	H	Y
4	64	H	4	4	3	H	Y
7	64	H	4	0	0	L	N
3	32	M	3	2	2	H	N
5	32	M	4	2	2	H	N
8	32	M	4	4	2	H	N
1	8	L	3	0	0	L	Y
6	16	L	w	0	0	L	Y
9	16	L	4	3	2	H	N

* assigned by titre (H.M.L: Table 21)

† grading system = 0 – 4 [101], with w indicating agglutination strength of less than 1

‡ assigned by kodecyte (H.M.L: Table 27)

§ agreement between antibody level as assigned by A_1 cell titre and kodecytes. Y (Yes) indicates agreement, N (No) indicates no agreement

A_1 cell assay and Kodecyte assay by saline showed 55% concordance (6 of 11 samples). When the antibody level was high, there was a higher rate of concordance (80%) than when the antibody level was low (67%). Medium level antibodies showed no concordance between the two methods (Table 31).

Table 32. Comparison of A_1 cell assay with three-kodecyte panel in 11 human plasma (antiglobulin CAT).

Sample	A_1 cell titre	antibody level* (by titre)	reaction grades†			antibody level‡ (by kodecytes)	agreement§
			FSL-A (µmol/L)				
			80	10	1		
9	1024	H	4	3	2	H	Y
4	256	H	2	1	1	H	Y
10	256	H	4	3	2	H	Y
2	128	M	4	4	4	H	N
5	128	M	4	4	3	H	N
11	128	M	3	2	2	H	N
8	64	M	4	3	2	H	N
6	32	M	3	0	0	L	N
7	32	M	4	2	1	H	N
1	16	L	3	2	0	M	N
3	16	L	3	2	2	H	N

* §assigned by titre (H.M.L) Table 21

† grading system = 0 – 4 [101]

‡ assigned by kodecyte (H.M.L) Table 27

§ agreement between antibody level as assigned by A cell titre and kodecytes. Y (Yes) indicates agreement, N (No) indicates no agreement

The A_1 cell assay and Kodecyte assay by antiglobulin showed 27% concordance (three samples of 11; all with high level antibody). These poor concordance results in both saline and antiglobulin in the medium and low level ranges led to the conclusion that antibody levels could not be differentiated with only these three kodecytes, and that further kodecytes prepared with FSL-A type 2 concentrations; between 80 and 10 umol/L, and between 10 and 2 umol/L should be added to the panel.

Five-kodecyte panel

The poor concordance observations with the three-kodecyte panel led to the development of a five-kodecyte panel, with kodecytes prepared with FSL-A at concentrations of 100, 20, 10, 2.5 and 1 µmol/L. The 20-A-kodecyte was added as the kodecyte prepared with FSL between 80 and 10 umol/L, and the 2.5-A-kodecyte as that prepared with FSL between 10 and 2 umol/L. Also, the 80-A-kodecyte was replaced with a 100-A-kodecyte, as some low level antibody samples were reacting very weakly with the 80-A-kodecyte (for example sample 6 in Table 31), and it was anticipated that some low level antibody samples might produce a negative reaction with the 80-A-kodecyte.

It was important to have at least one kodecyte reactive, to know that the assay was working.

Five-kodecyte panel, antiglobulin method

With the introduction of a five-kodecyte panel, emphasis was placed on validating a panel of kodecytes to differentiate levels of antibody by the antiglobulin technique. When measuring ABO antibody levels for transplantation applications, total IgG and IgM antibody is of interest [60] and therefore the antiglobulin technique is the most useful methodology. The five-kodecyte panel was therefore not fully validated for the saline technique, although it was tested with a subset of the donor plasmas in later experiments.

It was recognised that the antiglobulin antibody level of "medium" as defined by A_1 cell assay (Table 21) covered a broad range of antibody levels (with titre range from 32 to 128). Therefore new theoretical criteria to describe antibody level were developed. Table 33 shows the five-kodecyte panel theoretical reaction patterns to determine antibody level and allow comparison of antibody level as determined by A_1 cell assay and Kodecyte assay. Whilst the medium range remained at 32 to 128, two new antibody levels were also created; low-medium (8 to 32) and medium-high (64 to 256). This overlap in titre ranges (for example the titre 32 appears at the upper end of the low-medium range and the lower end of the medium range) reflects the reality that antibody titres seldom yield exactly the same answer when tested on multiple occasions, whether tested by different laboratories [113,114,116-119] or the same laboratory, although intra-laboratory variability is usually more limited [113]. Many laboratories accept a ±1 dilution difference [58], or ±2 dilution difference (unpublished observation) when reporting titres. For example, applying a ±1 dilution leeway, a reported titre of 128 can be interpreted as being in the range 64 – 256. This acknowledged inaccuracy in titration was reflected in the overlap in titre ranges (Table 33).

Table 33. Assignment of antibody level by reaction with five-kodecyte panel (antiglobulin).

Antibody level by kodecytes		A_1 cell titre	Kodecyte reactivity pattern				
			100	20	10	2.5	1.0
High	H	≥256	+	+	+	+	+
Medium High	MH	64-256	+	+	+	+	0
Medium	M	32-128	+	+	+	0	0
Low Medium	LM	8-32	+	+	0	0	0
Low	L	≤16	+	0	0	0	0

Using the theoretical criteria in Table 33, and allowing for the concept that a ±1 or ±2 dilution difference in titre is acceptable in most laboratories, a table of zones of concordance between A_1 cell assay and Kodecyte assay was created; Table 34.

Dilutions of two sera (see section 3.2.3 Sera/plasma and Table 22) were prepared in individual dilutions to produce sera with A_1 cell antiglobulin titres over the range 4 to 1024:

- X = CB polyclonal anti-A,B
- Y = internal QC sample from lab staff 1

The dilutions of these two sera were then tested, in tube only for sample X and in both tube and CAT techniques for sample Y. Testing was conducted on four occasions, but it was not possible to test every dilution on every occasion. Results are shown in Table 34 to show where results of dilutions of plasmas X and Y react. Grading on the 0-4 scale of agglutination [101] is shown in brackets after each entry. The green zone boundaries in Table 34 are defined by the criteria set in Table 33 and allow for a ±1 dilution difference in titre, whilst the zones marked in yellow allow for a ±2 dilution difference in titre. These zones defined whether a samples showed concordance between the A_1 cell and the Kodecyte assays.

For example, a sample with an A_1 cell titre of 64 which reacted with three of five kodecytes would be deemed to show concordance of antibody level as determined by the two assays. Conversely, a sample which reacts with three of five kodecytes could be said to have an A_1 cell titre in the range of 32-128.

Table 34. Criteria for determining concordance zones* between antiglobulin A_1 cell assay and five-kodecyte panel Kodecyte assay, and results of dilutions of known titre plasmas X (CB polyclonal anti-A,B) tested in tube and Y (internal QC sample) tested in tube & CAT.

	Kodecyte reactivity with dilutions of X & Y				
Kodecytes†	100	20	10	2.5	1.0
Reactions‡	+ 0 0 0 0	+ + 0 0 0	+ + + 0 0	+ + + + 0	+ + + + +
Ab level	L	LM	M	MH	H
XY titre||					
4	Y(2) ¶			Negative reactions	
8	X(2) y(2)	Y(2,1)			
16		X(2,2) y(2,2)	Y(3,2,2)		
32		Y(2,2)	Yy(3,3,3)	X(2,2,2,1)	
64			Yy(3,3,3)	X(3,3,2,1)	
128			Yy(3,3,2)	X (4,3, 3, 2) Y(3,3,2,1)	
256	Positive reactions			y(3,3,3,2)	X (4,4,4,4,2) Yy(3,3,3,2,1)
512					X (4,4,4,4,2) Yy (4,3,3,2,2)
≥1024					X (4,4,4,4,2) Y (4,4,3,2,2) y (4,4,4,3,3)

Ab = antibody
X = end point reactions of CB polyclonal anti-A,B in tube
Y = end point reactions of internal QC sample in tube, whilst y = end point reactions of internal QC sample in CAT
* Concordance zones are marked in green at the ±1 dilution level, and yellow at the ±2 dilution level
† Concentrations (100, 20, 10, 2.5, 1.0) of FSL-A (µmol/L) used to prepare kodecytes
‡ Kodecyte assay reactivity patterns shown as positive (+) or negative (0) reactions as a kodecyte panel in the order of 100, 20, 10, 2.5, 1.0
|| Antiglobulin titre of antibody of individually prepared dilutions of CB polyclonal anti-A,B (X) and internal QC sample (Y).
¶ Grades of agglutination on a 0-4 grading scale [101] shown for each reaction set.

Analysis of the theoretical reaction patterns depicted in Table 33 and the actual reactions depicted in Table 34 showed the following:

- Antibodies with an A_1 cell titre of ≥256 were expected to react with either 4 or 5 kodecytes. This proved to be the case with appropriate dilutions of samples X and Y
- Antibodies with an A_1 cell titre of 64-128 were expected to react with either 3 or 4 kodecytes. This proved to be the case with appropriate dilutions of samples X and Y
- Antibodies with an A_1 cell titre of 32 were expected to react with either 2 or 3 kodecytes. This proved to be the case with sample Y diluted to titre of 32. With sample X dilution of titre 32, this was not the case, as sample X reacted with four kodecytes on one occasion in tube technique
- Antibodies with an A_1 cell titre of ≤16 were expected to react with either 1 or 2 kodecytes. This was observed with samples X and Y in CAT on all occasions. Sample Y tested in tube did react with either 1 or 2 kodecytes on two occasions, but reacted with three kodecytes on one occasion

In summary, there were two occasions in a total of 28 tests of dilutions of samples X and Y (Table 34) which did not meet the criteria shown in Table 33. This represented a compliance rate of 93%. Grades of reactions were appropriate in both tube and CAT, with low antibody levels showing weaker reactions with the 100-A-kodecyte than high antibody levels. For example, sample Y dilution of titre 512 showed reactions in both tube and CAT with 100-A-kodecyte of grade 4, whereas sample Y dilution of titre 16 showed reactions of grade 2 with the same kodecyte (Table 34).

Therefore it was considered that the range of FSL in the five-kodecyte panel was suitable, and that sample (donor and ABOiKT patient) testing could commence (see sections 3.4.9 Analysis of donor samples; A1 cell assay and Kodecyte assay and 3.4.11 ABOiKT patient samples)

3.4.5 Consistency of kodecyte batches

Batches of the five-kodecyte panel were prepared on 15 different occasions over the course of the research (a period of approximately two years). On each of the 15 occasions, koding was quality checked after 24 hours storage at 4°C by adding 50 µL of a 1% v/v suspension prepared in Ortho RCD and 40 µL of monoclonal anti-A (Epiclone, Immulab, Melbourne, Australia) to wells of an Ortho neutral cassette. After centrifugation in the Ortho centrifuge agglutination was graded and checked for consistency against previous panels of kodecytes prepared with the same concentrations of FSL-A. All 15 batches prepared over the duration of this study gave consistent grades representative of the level of FSL-A modification (Table 35). The kodecyte prepared with FSL-A at 1 µmol/L was at the limit of detection with monoclonal anti-A in a saline CAT method, and showed a negative reaction in the quality check on 12 of 15 occasions. This was not unexpected, and this kodecyte never failed to react with the high titre internal QC sample (see section 3.2.4 Quality control (QC) samples) in the antiglobulin Kodecyte assay, proving that koding had occurred.

Table 35. Results of five-kodecyte panel prepared on 15 separate occasions, tested in saline CAT with monoclonal anti-A.

	Reaction grades* with A-kodecytes					
	FSL concentration (µmol/L)-A-kodecytes					
Batch #	100	20	10	2.5	1	0†
4	4	4	4	3	1	0
9	4	4	4	2	1	0
3	4	4	4	2	1	0
1	4	4	4	2	0	0
2	4	4	4	2	0	0
5	4	4	3	2	0	0
7	4	4	3	2	0	0
8	4	4	4	2	0	0
10	4	4	4	2	0	0
11	4	4	4	2	0	0
12	4	4	3	2	0	0
13	4	4	4	2	0	0
14	4	4	4	2	0	0
15	4	4	3	2	0	0
6	4	4	3	1	0	0

*grading = 0-4 system [101]. † = non-koded RBC

3.4.6 FSL-A Kodecyte stability

Effects of inserting FSL-A into RBC, and stability of the resulting kodecytes over the four week storage period was assessed in three ways:

A. Measurement of RBC mean cell volume (MCV) was undertaken at 24 hours and four weeks post koding of freshly collected RBC. A rise in MCV indicates RBC swelling [201]. Measurements were made on the Haematology Sysmex XT analyser (Sysmex, Kobe, Japan). Results are displayed in Table 36
B. Lysis over the four week storage period was visually observed and also measured. The five-kodecyte panel was prepared, and stored in red cell preservative; Celpresol. Each week cell-free supernatant was sampled; 160 µL into 1 mL Drabkin solution and absorbance measured at A540nm. Results are shown in Figure 38 and Figure 39
C. Kodecytes were tested by saline agglutination tube test with monoclonal anti-A at 24 hours post koding, and weekly intervals thereafter, to assess if kodecytes consistently produced the same haemagglutination grades throughout the storage period. Results are in Table 37

Table 36. Red cell MCV in A-kodecytes and non-koded cells after 24 hours and four weeks storage.

		FSL-A concentration (µmol/L) used to prepare kodecytes					
	storage	100	20	10	2.5	1	0†
MCV (fl)*	24 hour	85.6	76.7	76.3	76.4	76.4	76.4
	4 week	87.5	82.6	83.0	83.3	83.2	83.5

* Values are the means of two readings on Sysmex XT analyser

† 0 = non-koded RBC

The MCV of the kodecytes prepared with FSL at concentrations of 20, 10, 2.5 and 1 µmol/L was comparable to the non-koded RBC, both at 24 hours, and four weeks storage. However, the MCV of the 100-A-kodecyte was elevated in comparison to the kodecytes prepared with FSL at concentrations of 20, 10, 2.5 and 1 µmol/L, and the non-koded RBC, at both 24 hours post-koding ($p = 0.00001$) and at four weeks post-koding ($p = 0.00001$).

There was visible lysis in all kodecytes and also the non-koded RBC after four weeks storage, with the greatest lysis observed in the 100-A-kodecyte (Figure 38).

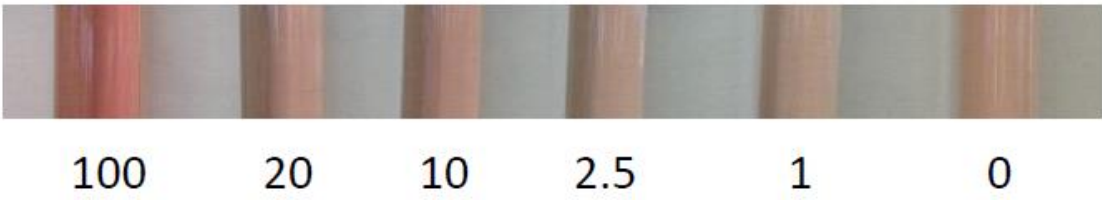


Figure 38. Appearance of supernatant of A-kodecytes prepared with FSL-A type 2 at 100, 20, 10, 2,5 and 1 µmol/L after four weeks storage in red cell preservative solution Celpresol. Non-koded cells (0) are included for comparison.

A540nm readings to quantify the lysis of kodecytes and non-koded RBC (Figure 39) confirmed the visual lysis observations, with the highest absorbances seen in the 100-A-kodecyte, and comparable absorbance readings in the 20-, 10-, 2.5-, 1-A-kodecytes and the non-koded RBC after four weeks storage.

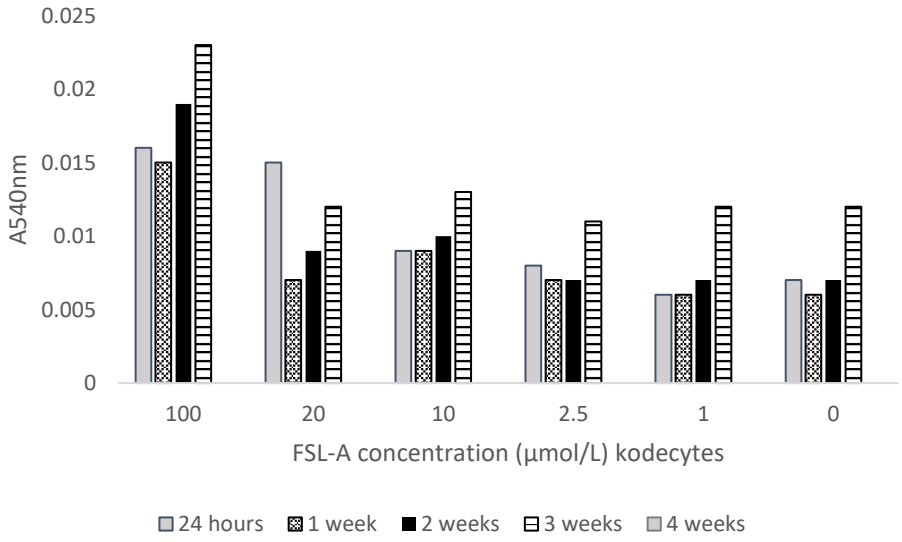


Figure 39. A540nm readings to quantify lysis of five-kodecyte panel and non-koded RBC over a four week storage period. Kodecytes were stored in red cell preservative Celpresol.

Loss of cells due to lysis was assessed by comparing spectrophotometric readings (A540nm) of an equivalent strength suspension prepared from freshly collected RBC from the same individual whose cells were used to prepare the kodecytes. Loss after four weeks storage was calculated to be 5% loss in the 100-A-kodecyte, 2% loss in the other kodecytes, and 2% loss in the non-koded RBC stored for four weeks.

Each kodecyte yielded either the same grade of agglutination throughout the storage period (100-, 20-, 10-, 1-A-kodecytes), or a maximum of 1 grade difference (2.5A-kodecytes) (Table 37).

Table 37. Agglutination grades (saline tube) with monoclonal anti-A tested with A-kodecytes and non koded RBC across 4 week storage.

	Reaction grades*					
	FSL-A concentration (µmol/L) used to prepare kodecytes					
Storage	100	20	10	2.5	1	0
24 hours	4	4	4	3	0	0
1 week	4	4	4	2	0	0
2 week	4	4	4	2	0	0
3 week	4	4	4	3	0	0
4 week	4	4	4	2	0	0

*grading = 0-4 system [101]

3.4.7 Assay precision (reproducibility)

As intra-laboratory variation is somewhat problematic in the measurement of ABO antibodies [113], precision was tested in this study for both the A_1 cell assay, and the Kodecyte assay. Precision was defined here as the ability to reproduce the same result from the same sample on multiple occasions in the same laboratory. Tube and CAT platforms were assessed. Twenty of the donor plasma samples (fourteen group O and six group B) (see section 3.2.3 Sera/plasma) were tested by the A_1 cell assay (antiglobulin tube method section 3.4.1 A_1 cell assay) on three separate occasions, with three separate preparations of dilution series to test the effect of the variable of assay error in the preparation of dilutions. On the same three occasions, the Kodecyte assay (antiglobulin tube method section 3.4.2 Kodecyte assay) was tested with the five-kodecyte panel prepared on three separate occasions to test the variable of kodecyte batch preparation. Precision was assessed by tabulating the difference in titre steps in the A_1 cell assay, against the difference in the antibody level in the Kodecyte assay. Consensus was defined where two of the three, or all three results for a single sample

agreed. Analysis is reported in Table 38. No sample showed greater than a two dilution difference in the A_1 cell assay. No sample showed greater than one kodecyte antibody level difference (Table 33) in the Kodecyte assay. As there was no variance direction (i.e. neither stronger nor weaker) for titres or kodecyte antibody levels, absolute values of change (delta) in grade or dilution from the consensus were applied. Ten Kodecyte assay antibody levels and eight A_1 cell assay titres gave the same results for all triplicates. Fifteen samples showed either 1 titre (n = 5) or 1 antibody level (n = 4) or both 1 antibody level and 1 titre variance (n = 6). Only 1 sample showed a 2 titre variance, but had identical triplicate kodecyte assay antibody levels (Table 38).

Table 38. Triplicate inter-assay deltas in 20 donor samples (tube antiglobulin).

	A_1 cell assay		
	identical	1 dilution difference	2 dilution difference
Kodecyte assay		Sample numbers	
identical	4	5	1
1 antibody level difference	4	6	0

The method was repeated in CAT on the same samples. However, due to some samples having insufficient volume remaining, only 17 of the 20 could be assayed, and only on two separate occasions. Consensus was defined in the same way as for the tube assay. Results are shown in Table 39. Again, no sample showed greater than a two dilution difference in the A_1 cell assay, nor greater than one kodecyte antibody level difference in the Kodecyte assay. In CAT, 14 sample duplicates gave identical Kodecyte assay results, and seven A_1 cell assay titres gave the same results for duplicates. Ten samples showed either 1 titre (n = 7) or one antibody level difference (n = 1) or both 1 antibody level and 1 titre variance (n = 2). Again, one sample showed a two titre variance, but had identical duplicate kodecyte assay results.

Table 39. Duplicate inter-assay deltas in 17 donor samples (CAT antiglobulin).

	A_1 cell assay		
	identical	1 dilution difference	2 dilution difference
Kodecyte assay		Sample numbers	
identical	6	7	1
1 antibody level difference	1	2	0

Both assays showed a high degree of reproducibility in both tube and CAT platforms, and it is impossible to say that either assay is more reproducible, at least in the hands of a single scientist.

3.4.8 QC sample results

The negative control (non-koded RBC) produced a negative result with all sera and plasmas tested.

The internal QC sample had an antiglobulin titre of 1024 as validated against external WHO reference reagent 14/300 [113], and was tested with every batch of donor or patient samples. It produced an A_1 cell titre in the range 512-2048, and a Kodecyte assay antibody level of high on all occasions.

3.4.9 Analysis of donor samples; A_1 cell assay and Kodecyte assay

Antiglobulin assays

Donor plasma samples (102 O and 23 B) were tested by the A_1 cell assay and the Kodecyte assay (five-kodecyte panel) by antiglobulin in both tube and CAT platforms.

Numerical analysis

Criteria shown in Table 34 were used to analyse agreement (concordance) rates between the A_1 cell assay and the Kodecyte assay. Numerical analysis (simple percentage calculations) was variously conducted to allow for either ±1 and ±2 dilution differences in A_1 cell assay (green and yellow zones respectively in Table 34). Results falling outside the yellow zone were defined as discordant.

Blood group O: A_1 cell assay compared with Kodecyte assay

All five possible kodecyte reactivity patterns were observed in the Kodecyte assay. Figure 40 illustrates the typical appearance of reactions in CAT in five different group O samples from the cohort showing different antibody levels ranging from high to low as determined by the Kodecyte assay. Similarly, samples showed a wide range of A_1 cell titres of anti-A/anti-A,B from 4 to ≥1024, and titres by platform type were comparable (Figure 41).

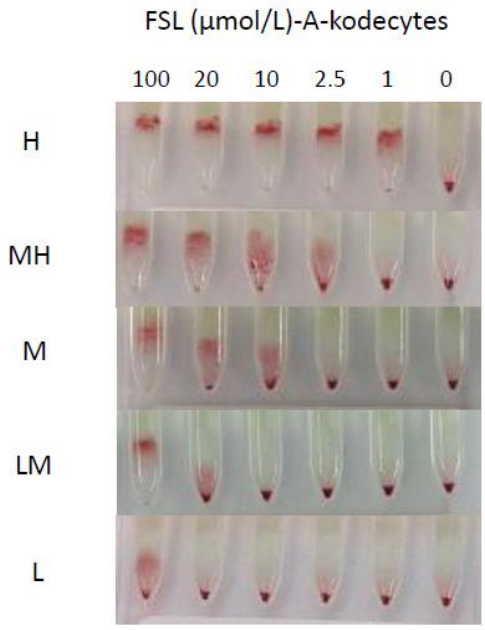


Figure 40. Five-kodecyte panel in CAT antiglobulin technique, showing five different patterns of kodecyte reactivity with antibodies from five group O blood donors of different levels; high (H), medium high (MH), medium (M), low medium (LM) and low (L).

As can be seen in Table 40 and Figure 41 almost all samples (98/102) had tube antiglobulin A_1 cell assay titres greater than 16 and Kodecyte assay antibody levels of medium or greater (100/102). When comparing agreement of antibody level as assigned by A_1 cell assay, versus Kodecyte assay in tube, the majority of samples (83/102, 81% concordance) fell within the green zone (±1 dilution factor) and (98/102, 96% concordance) fell within the yellow zone (±2 dilution factors) (Table 34 criteria). Discordant samples (outside the green or yellow zones) are shaded in pink and are labelled a-d. Selected concordant samples are labelled e-m. Samples a-m are indicated because they were subjected to further analysis (see section 3.4.10 Analysis of donor samples showing discordancy).

Table 40. Blood group O plasma tube antiglobulin reactivity, Kodecyte assay vs A_1 cell assay.

	Reactivity against 100, 20, 10, 2.5, 1.0 A-kodecytes (n = 102)				
A_1 cell titre	+ 0 0 0 0 L	+ + 0 0 0 LM	+ + + 0 0 M	+ + + + 0 MH	+ + + + + H
4	0	0	0	0	0
8	2	0	0	0	0
16	0	0	1	1(a)	0
32	0	0	4(e)	1	0
64	0	0	8(f) (ii,iii,vii,x)	4	0
128	0	0	7(i,l)	10	5(k)
256	0	0	5	7	10(g, h, j, m) (iv, v, vi)
512	0	0	1(b)	3	18 (viii, ix)
≥1024	0	0	1(c)	1(d)	13

Cells shaded in green indicate concordant results between A_1 cell assay and Kodecyte assay, allowing for a ±1 dilution difference in titre. Cells shaded in yellow indicate concordant results between A_1 cell assay and Kodecyte assay, allowing for a ±2 dilution difference in titre. Cells shaded in pink indicate results which are discordant between A_1 cell assay and Kodecyte assay. Samples labelled (a - m) and (ii – x) were subjected to further analysis. Samples (a-d) were discordant here. Samples (e-m) were concordant here, but discordant in CAT platform (Table 41). Samples (ii – x) were concordant here and in CAT (Table 41), and were selected for further analysis in parallel with discordant samples.

In CAT (Table 41) almost all samples (99/102) again had A_1 cell assay titres greater than 16 and kodecyte assay antibody levels of medium or higher. The two samples which gave kodecyte assay L grades by Tube (Table 40) were the same samples giving L grades by CAT (Table 41). Most samples (73/102, 72% concordance) fell within the green zone (±1 dilution factor) and the majority (93/102, 91% concordance) fell within the yellow zone (±2 dilution factors). In contrast to tube, in CAT platform all nine discordant samples (e, f, g-m) were in the same zone in Table 41 and showed higher Kodecyte assay reactivity than predicted by the A_1 cell assay.

Table 41. Blood group O plasma CAT antiglobulin reactivity, Kodecyte assay vs A_1 cell assay.

	Reactivity against 100, 20, 10, 2.5, 1.0 kodecytes (n = 102)				
A_1 cell titre	+ 0 0 0 0 L	+ + 0 0 0 LM	+ + + 0 0 M	+ + + + 0 MH	+ + + + + H
4	1	0	0	0	0
8	1	0	0	0	0
16	0	0	0	1 (e)	0
32	0	0	5 (x)	1 (a)	1 (f)
64	0	1	4	6 (b) (vii)	7 (g-m)
128	0	0	3 (ii,iii)	5	18 (vi)
256	0	0	0	0	20 (iv,v,viii)
512	0	0	0	0	14 (c, d) (ix)
≥1024	0	0	0	0	14

Colour shading indicates zones of concordancy or discordancy, and is as described in the footnote of Table 40. Samples labelled (a-m) and (ii-x) are the same samples labelled in Table 40 and were subjected to further analysis.

Differences in distribution of antibody level in the group O sample cohort determined on two platforms (tube and CAT) were analysed. Figure 41(A) shows the titre distribution as determined by tube versus CAT to be very similar, with a normal distribution curve Figure 41(B). Neither CAT nor tube favoured a higher antibody titre in the A_1 cell assay. However, the antibody level as determined by the Kodecyte assay showed that CAT favoured higher antibody level determination Figure 41(C), with a right-skewed distribution curve Figure 41(D).

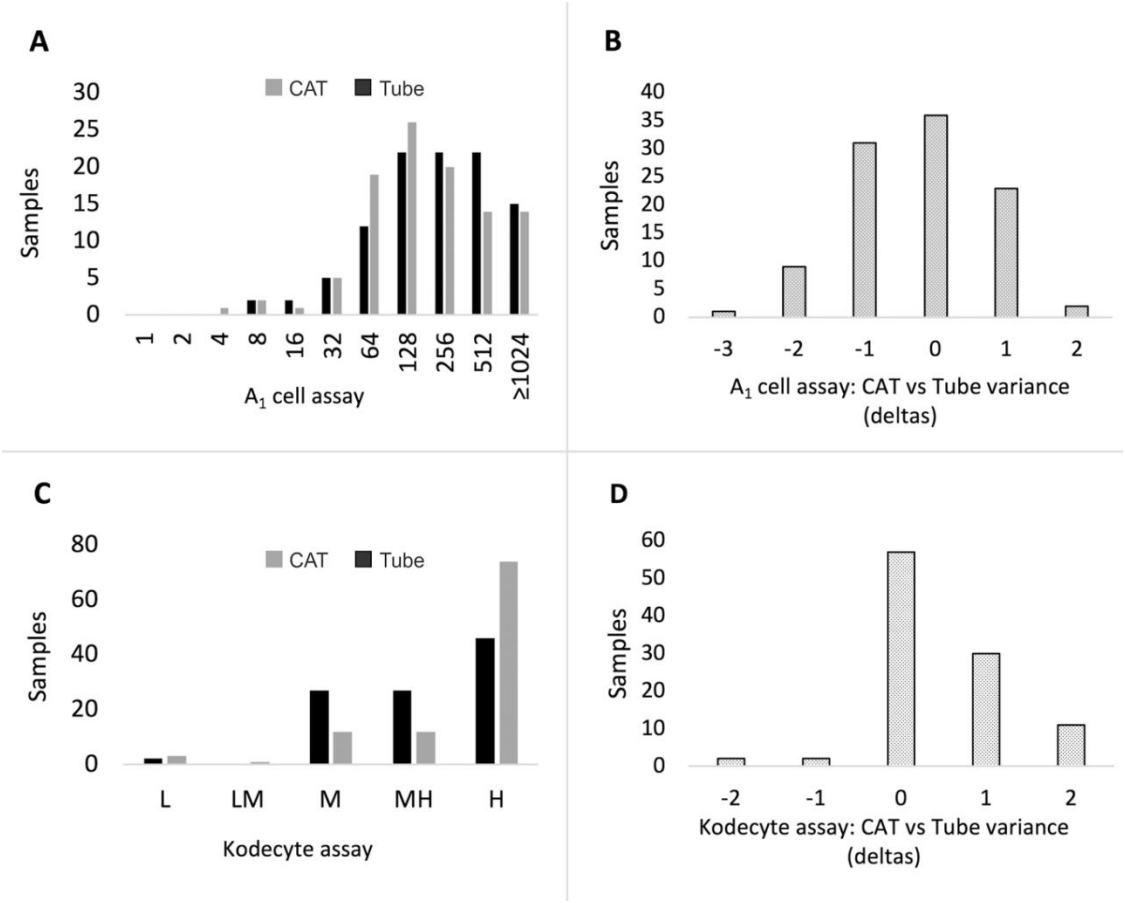


Figure 41. Analysis of group O donor samples (n = 102) in A_1 cell assay and Kodecyte assay. (A) Overall titre distribution against A_1 cells by platform type. (B) Variance (deltas) of the A_1 cell assay titres in CAT compared to Tube. (C) Overall antibody level distribution of the Kodecyte assay in CAT and Tube . (D) Variance (deltas) of Kodecyte assay antibody level in CAT compared to Tube. Reproduced from the author's work [177].

Blood group B: A_1 cell assay compared with Kodecyte assay

There was also a wide range of titres of anti-A observed in group B samples (ranging from 4 to 512), although more samples had lower titres than those observed in group O individuals in both tube and CAT platforms (Figure 42). All five possible kodecyte reactivity patterns were observed (Table 43). Approximately half of the samples (11/23)

had tube A_1 cell assay titres >16 and Kodecyte assay antibody level of medium or greater, with almost a quarter (5/23) having anti-A titres < 16 (Table 42). In tube, the majority of samples (17/23, 74% concordance) fell within the green zone (±1 dilution factor) and (22/23, 96% concordance) fell within the yellow zone (±2 dilution factors) (Table 42).

Table 42. Blood group B plasma tube antiglobulin reactivity, Kodecyte assay vs A_1 cell assay.

	Reactivity against 100, 20, 10, 2.5, 1.0 kodecytes (n = 23)				
A_1 cell titre	+ 0 0 0 0 L	+ + 0 0 0 LM	+ + + 0 0 M	+ + + + 0 MH	+ + + + + H
4	2	1	0	0	0
8	2	0	0	0	0
16	0	0	0	0	0
32	0	4(q)	2(r)	0	0
64	0	2	5 (n)(xi)	0	0
128	0	1 (p)	1	0	0
256	0	0	2	0	1
512	0	0	0	0	0
≥1024	0	0	0	0	0

Colour shading indicates zones of concordancy or discordancy, and is as described in the footnote of Table 40. Samples labelled (n, p, q and r) were subjected to further analysis. Sample p was discordant here. Sample q and r were concordant here, but discordant in CAT (Table 43). Sample xi was a concordant sample here selected for further analysis in parallel with discordant samples.

CAT analysis of group B donor samples (Table 43) found most samples had A_1 cell assay titres < 32 and Kodecyte assay antibody levels of medium or lower (13/23). Of the four samples with Kodecyte assay level L by Tube (Table 42), two were the same samples giving L grades by CAT, with the remaining two samples giving LM grades. Most samples (15/23, 65% concordance) fell within the green zone (±1 dilution factor) and the majority (21/23, 91% concordance) fell within the yellow zone (±2 dilution factors).

Table 43. Blood group B plasma CAT antiglobulin reactivity, Kodecyte assay vs A_1 cell assay.

	Reactivity against 100, 20, 10, 2.5, 1.0 kodecytes (n = 23)				
A_1 cell	+ 0 0 0 0	+ + 0 0 0	+ + + 0 0	+ + + + 0	+ + + + +
titre	L	LM	M	MH	H
4	1	2	0	0	0
8	1	2 (n)	2 (q, r)	0	0
16	0	3	2	0	0
32	0	0	1	0	0
64	0	1	3	0	0
128	0	0	3 (p)(xi)	0	0
256	0	0	0	0	1
512	0	0	0	1	0
≥1024	0	0	0	0	0

Colour shading indicates zones of concordancy or discordancy, and is as described in the footnote of Table 40. Samples labelled (n, p, q r) and (xi) are the same samples labelled in Table 42 and were subjected to further analysis.

Analysis of difference in distribution of antibody level in the group B sample cohort determined on tube and CAT platforms showed the titres determined by CAT to be lower than tube, with a left-skewed distribution curve Figure 42(B). In the Kodecyte assay however, CAT favoured a higher level determination of antibody than tube Figure 42 (D).

Samples indicated with lower case alphabet letters in Table 42 and Table 43 were subjected to further analysis to investigate possible causes of discordancy between the A_1 cell assay and the Kodecyte assay (see section 3.4.10 Analysis of donor samples showing discordancy).

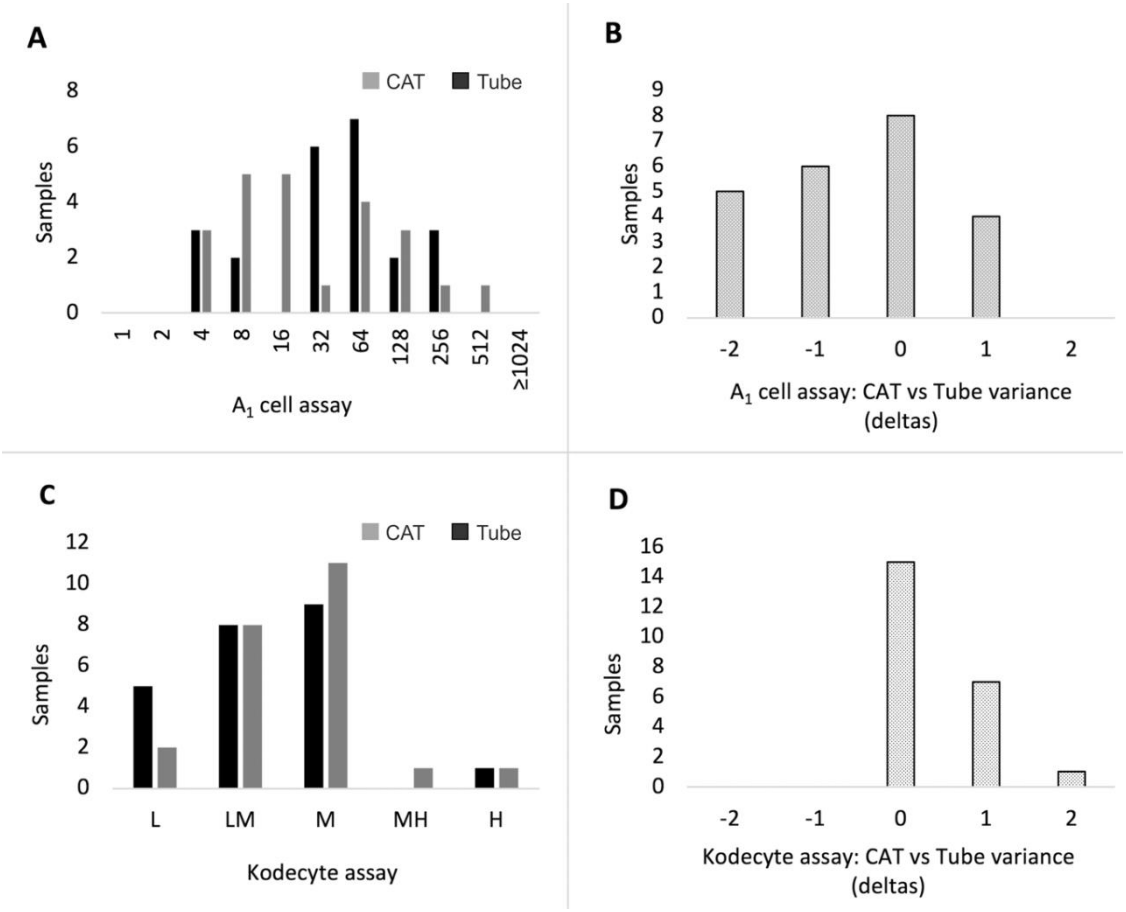


Figure 42. Analysis of Group B samples (n = 23) in A_1 cell assay and Kodecyte assay. (A) Overall titre distribution against A_1 cells by platform. (B) Variance (deltas) of A_1 cell assay titres in CAT compared to Tube. (C) Overall antibody level distribution of the Kodecyte assay in CAT and Tube. (D) Variance (deltas) of Kodecyte assay grades in CAT compared to Tube. Reproduced from the author's work [177].

Saline assays

Fifty-one of the samples (48 group O and three group B) were tested by the A_1 cell assay and the Kodecyte assay by saline tube technique (methods as per tube section 3.4 Quantification of ABO antibodies). Criteria for analysis of concordance in saline between the A_1 cell assay and the Kodecyte assay were different to the criteria used for the antiglobulin assay. Saline titres against A_1 cells in group O individuals are naturally lower than antiglobulin titres [161]. When using titre to predict group O blood donations capable of causing haemolysis in non-ABO matched transfusion blood products, saline titre cut-off limits are lower than antiglobulin titre cut-off limits [68-70,72,74]. Therefore the titre ranges were compressed from those shown in Table 33 to three ranges: L (≤16), M (32) and H (≥64) (Table 21). Table 44 was constructed to create theoretical zones of agreement between antibody levels as determined by A_1 cell assay versus Kodecyte saline assay. Green zone in Table 44 indicates agreement and yellow zone indicates agreement when allowing for a maximum of ±2 dilution differences in titre. The antibody

level as determined by Kodecyte reactivity was not changed; as there are five possible kodecyte patterns of reactivity, it was still possible to assign five different antibody levels (L, LM, M, MH, H).

Blood group O: A_1 cell assay compared with Kodecyte assay

Results for group O samples comparing A_1 cell titres, and kodecyte antibody levels are shown in Table 44. There was a concordance rate of 58% in the green zone, and 88% in the yellow zone. The samples showing discordant results (A_1 cell assay versus Kodecyte assay) were subjected to further analysis in an attempt to further understand the performance of kodecytes (see section 3.4.10 Analysis of donor samples showing discordancy). Samples (aa - af) (Table 44) were clearly discordant in the saline assay, showing antibody titres of ≤16, but Kodecyte assay grades of medium-high (aa) and high (ab,ac,ad,ae and af). Samples (aa - af) produced concordant results in the antiglobulin assays.

Table 44. Blood group O plasma tube saline reactivity, Kodecyte assay vs A_1 cell assay.

	Reactivity against 100, 20, 10, 2.5, 1.0 kodecytes (n = 48)				
A_1 cell titre	+ 0 0 0 0 L	+ + 0 0 0 LM	+ + + 0 0 M	+ + + + 0 MH	+ + + + + H
≤16	0	2 (ag,ah)	5 (ao,ap)	1 (aa)	5 (ab,ac,ad,ae,af)
32	0	0	2	5 (as)	2 (av)
≥64	0	0	2 (ar)	2 (ak,al)	22

Cells shaded in green indicate concordant results between A_1 cell assay and Kodecyte assay. Cells shaded in yellow indicate concordant results between A_1 cell assay and kodecyte assay, allowing for a maximum ±2 dilution difference in titre. Cells shaded in pink indicate results which are discordant between A_1 cell assay and kodecyte assay. Samples labelled (aa-av) include discordant samples aa – af and selected concordant samples ag, ah, ak, al, ao, ap, ar, as, av which were subjected to further analysis.

The three group B samples each had a saline tube titre of 32, and medium level antibody (reactivity with three kodecytes) assigned by the Kodecyte assay, and were therefore concordant.

3.4.10 Analysis of donor samples showing discordancy

Two hypotheses were tested in an attempt to elucidate reasons for some samples to show discordancy between A_1 cell assays and Kodecyte assays. Firstly, it was considered that high-titre, low-avidity (HTLA) antibodies may have been responsible. HTLA antibodies are those that react to a high titre, but show weak agglutination reactions not only at the end at the titre, but throughout the plasma dilution series in the antiglobulin phase [202]. In order to assess whether HTLA antibodies were present, the grades of agglutination of all tubes showing a positive reaction in the A_1 cell assay were examined in all antiglobulin discordant samples, and in a selection of concordant samples. It is normal for the titre to gradually weaken in grades of agglutination before then becoming negative. A pattern of several 1+ reactions at the end of the titre, or weak (1+ or 2+) agglutination reactions throughout the titre is more suggestive of HTLA antibodies. This HTLA pattern was not found in either the concordant or the discordant samples (results not shown), therefore it was concluded that low affinity antibody reactions were not a cause of discordancy.

Secondly, samples with discordancy between A_1 cell assays and Kodecyte assays in either tube or CAT platforms were analysed to establish their relative ratio of IgG:IgM anti-A and/or anti-A,B. Samples were subjected to Dithiothreitol (DTT) treatment to degrade IgM antibodies [203]. Equal volumes of plasma and freshly prepared 5 mmol/L DTT (Sigma Aldrich, St Louis, MO) in PBS at pH 7.4 were incubated for two hours at 37°C as per the method of Okuno & Kondelis [203]. After mixing, DTT treated samples were titrated on the same day as DTT treatment, with the DTT treated plasma assigned a dilution factor of two from the outset. Efficacy of IgM degradation by DTT treatment was assessed by performing saline room temperature titres on the DTT treated samples, and checking that this titre was either less than two, or less than the saline titre on the untreated plasma.

The following methods were used to establish IgG and IgM titres against A_1 cells and immunoglobulin G:M ratios in the samples:

- Antiglobulin Tube titre on untreated plasma = total (IgG+IgM) titre
- Antiglobulin Tube titre on DTT treated plasma = IgG titre
- Saline tube titre on untreated plasma = IgM titre, with the caveat that IgG antibodies can cause direct agglutination [204]
- Saline tube titre on DTT treated plasma acts as a control of DTT treatment. The titre should be less than two in the case of complete IgM degradation, or greater than two but less than the saline titre on untreated plasma in the case of partial IgM degradation
- G:M = relative ratio IgG:IgM. For example an IgG titre of 32, with an IgM titre of 8, has a G:M ratio of 4:1

A. Antiglobulin assay discordancies

Sufficient sample was available to analyse 3 of the 4 group O antiglobulin tube discordant samples (samples b, c and d in Table 40) and six of the nine group O antiglobulin CAT discordant samples (e, f, h, i, j and k in Table 41) for their relative IgG and IgM content. Group B discordant sample p (Table 42) was also analysed, but there was insufficient sample to analyse discordant samples q and r (Table 43). Results of G:M ratios for all samples tested are shown in Table 45.

At the same time as the discordant samples were analysed for G:M ratio, a selection of nine group O and one group B sample showing agreement in the A_1 cell assay and the Kodecyte assay were also analysed (concordant samples ii – x in Table 40 and Table 41, and sample xi in Table 42 and Table 43). Selection of concordant samples was largely influenced by sample sufficiency. Results of G:M ratios for these samples are also shown in Table 45.

Samples b, c and d with discordancies in tube all had high anti-A plus anti-A,B ratios of G:M, ranging from 8:1 to 32:1 (Table 45). In contrast, the majority (8 of 10) of tube concordant samples (samples ii, iii, iv, vii, viii, ix, x and xi) had lower G:M ratios ranging from 2:1 to 1:4 (Table 45). Discordant tube samples b, c, d showed lower Kodecyte assay antibody levels than predicted by the A_1 cell assay. Results for these 3 samples (b, c, d) were concordant by CAT (Table 41). All the CAT discordant samples (samples e, f, h, i, j

and k) had low anti-A plus anti-A,B ratios of G:M, ranging from 2:1 to 1:16 (Table 45). These samples showed higher Kodecyte assay antibody levels than predicted by the A_1 cell assay in CAT (Table 41). Results for these six samples (e, f, h, i, j and k) were concordant by Tube (Table 40).

Table 45. Analysis of samples showing discordancy/concordancy in antiglobulin assays.

		Original results*				IgG:IgM titres and ratios	
		Tube		Card		A_1 cells	
ID	ABO	A_1	Kode	A_1	Kode	IgG:IgM	G:M
Discordant samples							
c	O	1024	M	512	H	256:32	8:1
d	O	1024	MH	512	H	256:8	32:1
b	O	512	M	64	H	128:4	32:1
j	O	256	H	64	H	4:64	1:16
e	O	32	M	16	MH	2:32	1:16
i	O	128	M	64	H	32:128	1:4
k	O	128	MH	64	H	32:128	1:4
h	O	256	MH	64	H	64:32	1:2
f	O	64	M	32	H	8:16	2:1
p	B	128	LM	128	M	4:128	1:32
n	B	64	M	8	LM	2:16	1:8
Concordant samples							
ix	O	512	H	512	H	128:512	1:4
viii	O	512	H	256	H	64:64	1:1
v	O	256	H	256	H	256:16	16:1
iv	O	256	H	256	H	128:128	1:1
vi	O	256	H	128	H	128:16	8:1
iii	O	64	M	128	M	64:128	1:2
ii	O	64	M	128	M	64:64	1:1
x	O	64	M	32	M	16:16	1:1
vii	O	64	M	64	MH	16:8	2:1
xi	B	64	M	128	M	16:8	2:1

*Original results as reported in Table 40 - Table 43, with discordant results indicated by shading (with A_1 cell assay and Kodecyte assay headers abbreviated to A_1 and Kode respectively).

However, it was not only the ratio of G:M which appeared to affect results in CAT. The pattern which emerged in antiglobulin CAT was a "right shift" of Kodecyte assay antibody level, with CAT generally assigning higher Kodecyte antibody level than tube (Figure 41C and D). A_1 cell assay titres were comparable between platforms of CAT and tube (Figure 41A and B). This was true for both concordant and discordant samples, but had the end result that there are more discordant samples in CAT than in tube (Table 40 - Table 43).

From the analysis of G:M ratios, the following observations were made:

- The antiglobulin Kodecyte assay in Tube may underestimate antibody levels compared with the A_1 cell assay if the ratio of G:M is high
- The antiglobulin kodecyte assay in CAT may overestimate antibody levels compared with the A_1 cell assay if the ratio of G:M is low

B. Saline assay discordancies

Analysis of relative IgG and IgM content was also undertaken for samples showing discordant results in the saline tube assay (Table 44). Although the saline test generally detects only IgM antibodies [6,101], it has been noted that IgG anti-A and anti-B can cause agglutination of RBC in saline tests [6,204], and this could potentially cause discordancies between the saline A_1 cell assay and the Kodecyte assay.

Sufficient sample was available to analyse all six group O discordant samples (samples aa – af in Table 44) and a selection of concordant samples (ag, ah, ak, al, ao, ap, ar, as, av in Table 44) for their relative IgG and IgM content. Results are presented in Table 46. The six group O discordant samples aa - af had results in the Kodecyte assay which indicated a medium-high (MH) or high (H) level of anti-A/anti-A,B but low titre results (≤16) in the A_1 cell assay. These samples had high G:M ratios ranging from 4:1 to 16:1 (Table 46). For the concordant samples, six of ten (ag, ak, al, ao, ar and av) had low G:M ratios ranging from 1:1 to 1:8. Therefore it appeared that the saline kodecyte assay in tube may over estimate antibody levels compared with the A_1 cell assay if the ratio of G:M is high.

Table 46. Analysis of samples showing discordancy/concordancy in saline assays.

		Original results*				IgG/IgM titres and ratios	
		Saline Tube		Antiglobulin tube		A_1 cells	
ID	ABO	A_1	Kode	A_1	Kode	IgG/IgM	G:M
		Discordant samples					
aa	O	16	MH	256	H	256:16	16:1
ab	O	16	H	512	H	128:16	8:1
ac	O	16	H	256	MH	64:16	4:1
ad	O	16	H	128	M	128:16	8:1
ae	O	8	H	64	M	32:8	4:1
af	O	16	H	1024	H	256:16	16:1
		Concordant samples					
ag	O	8	LM	32	M	16:8	2:1
ah	O	8	LM	64	H	64:8	8:1
ak	O	64	MH	64	M	16:64	1:4
al	O	256	MH	256	M	32:256	1:8
ao	O	8	M	32	M	8:8	1:1
ap	O	16	M	512	H	256:16	16:1
ar	O	128	M	64	M	64:128	1:2
as	O	32	MH	256	H	256:32	8:1
av	O	32	H	128	MH	16:32	1:2

*Original results as reported in Table 44, with discordant results shaded. A_1 cell assay and Kodecyte assay headers abbreviated to A_1 and Kode respectively.

DTT treated plasma (IgG) and native plasma (IgG plus IgM), both at final dilution factor of two, were tested in a direct agglutination assay with both the five-kodecyte panel and A_1 cells to test if IgG was causing direct agglutination of kodecytes. Diluted native plasma was used to control that there was sufficient antibody at a dilution factor of two to cause agglutination with kodecytes, since A-kodecytes were optimised to react with undiluted plasma. Results are shown in Table 47. Results showed no evidence of IgG anti-A/anti-A,B causing direct agglutination of A-kodecytes. Eleven of 14 samples tested showed agglutination of kodecytes with diluted native plasma, but no agglutination of kodecytes with DTT treated plasma. None of the 14 samples showed agglutination of kodecytes with DTT treated plasma.

Table 47. Results of testing DTT-treated and native plasma by saline tube method with five-kodecyte panel and A_1 cells for assessment of IgG contribution to direct agglutination.

	DTT treated plasma (dilution factor 2)							Native plasma (dilution factor 2)						
	A-kodecytes						A_1 cells	A-kodecytes						A_1 cells
	FSL concentration (µmol/L)-A-kodecytes							FSL concentration (µmol/L)-A-kodecytes						
sample	100	20	10	2.5	1	0		100	20	10	2.5	1	0	
	Grades of agglutination													
aa	0	0	0	0	0	0	2	w	0	nt	nt	nt	0	nt
ab	0	0	0	0	0	0	1	1	1	1	1	1	0	4
ac	0	0	0	0	0	0	0	w	w	0	0	0	0	2
ad	0	0	0	0	0	0	3	2	1	w	w	w	0	4
af	0	0	0	0	0	0	1	1	w	0	0	0	0	4
ag	0	0	0	0	0	0	0	0	0	nt	nt	nt	0	nt
ah	0	0	0	0	0	0	1	0	0	0	0	0	0	2
ak	0	0	0	0	0	0	0	1	1	1	0	0	0	3
al	0	0	0	0	0	0	w	4	4	nt	nt	nt	0	nt
ao	0	0	0	0	0	0	w	0	nt	nt	nt	nt	0	nt
ap	0	0	0	0	0	0	3	1	w	0	0	0	0	4
ar	0	0	0	0	0	0	1	1	1	w	0	0	0	4
as	0	0	0	0	0	0	0	3	nt	nt	nt	nt	0	nt
av	0	0	0	0	0	0	w	2	2	1	1	1	0	4

nt = not tested, due to inufficient sample. Agglutination is graded using the 0 to 4 system [101], with w denoting agglutination weaker than grade 1.

3.4.11 ABOiKT patient samples

All forty samples from one group B and four group O patients were tested in the A_1 cell assay and the Kodecyte assay in tube and CAT platforms; antiglobulin only. These samples were also tested (A_1 cell assay) independently at NZBS. These original clinical results were unknown to the author of this study at the time of testing (i.e. samples were tested "blind" in this research), but were later made available for comparison. When comparing results of this research and results from NZBS, 19 samples were A_1 cell

titre-identical, a further 19 had a ±1 dilution difference, and two samples showed a ±2 dilution difference.

Table 34 criteria was applied to analyse agreement between the A_1 cell and Kodecyte assays in the 40 samples from the five patients. In tube agreement was 83% (33 of 40 samples in Table 48) for the ±1 dilution factor variance and 100% (40 of 40 samples in Table 48) for ±2 dilution variance. In CAT agreement was 90% (36 of 40 samples in Table 49) for the ±1 dilution factor variance and 100% (40 of 40 samples in Table 49) for ±2 dilution variance.

Table 48. ABOiKT patient plasma tube antiglobulin reactivity, Kodecyte assay vs A_1 cell assay.

	Reactivity against 100, 20, 10, 2.5, 1.0 A-kodecytes (n = 40)				
A_1 cell	+ 0 0 0 0	+ + 0 0 0	+ + + 0 0	+ + + + 0	+ + + + +
titre	L	LM	M	MH	H
≤ 1	1				
2					
4	2				
8	5				
16	1	2	2		
32	1	3	2	1	
64		2	7		
128			5	1	
256				1	2
512				1	1
≥1024					

Cells shaded in green indicate concordant results between A_1 cell assay and Kodecyte assay, allowing for a ±1 dilution difference in titre. Cells shaded in yellow indicate concordant results between A_1 cell assay and Kodecyte assay, allowing for a ±2 dilution difference in titre.

Table 49. ABOiKT patient plasma CAT antiglobulin reactivity, Kodecyte assay vs A_1 cell assay.

	Reactivity against 100, 20, 10, 2.5, 1.0 A-kodecytes (n = 40)				
A_1 cell titre	+ 0 0 0 0 L	+ + 0 0 0 LM	+ + + 0 0 M	+ + + + 0 MH	+ + + + + H
≤ 1	1				
2					
4	5				
8	1	1			
16	1		1		
32		1	6	1	
64		1	3	5	
128			2	2	
256			1	3	2
512					2
1024					1

Colour shading indicates zones of concordancy, and is as described in the footnote of Table 48.

Further analysis of patient data is shown in Figure 43. In 40 samples from five patients the overall antibody level distribution as determined by both the A_1 cell assay and the Kodecyte assay was wider in patients than in donors (Figure 43A and C). This was not unexpected, since the antibody level was reduced by PP in the patient cohort (see details in section 3.2.3 Sera/plasma C. ABOiKT patient samples).

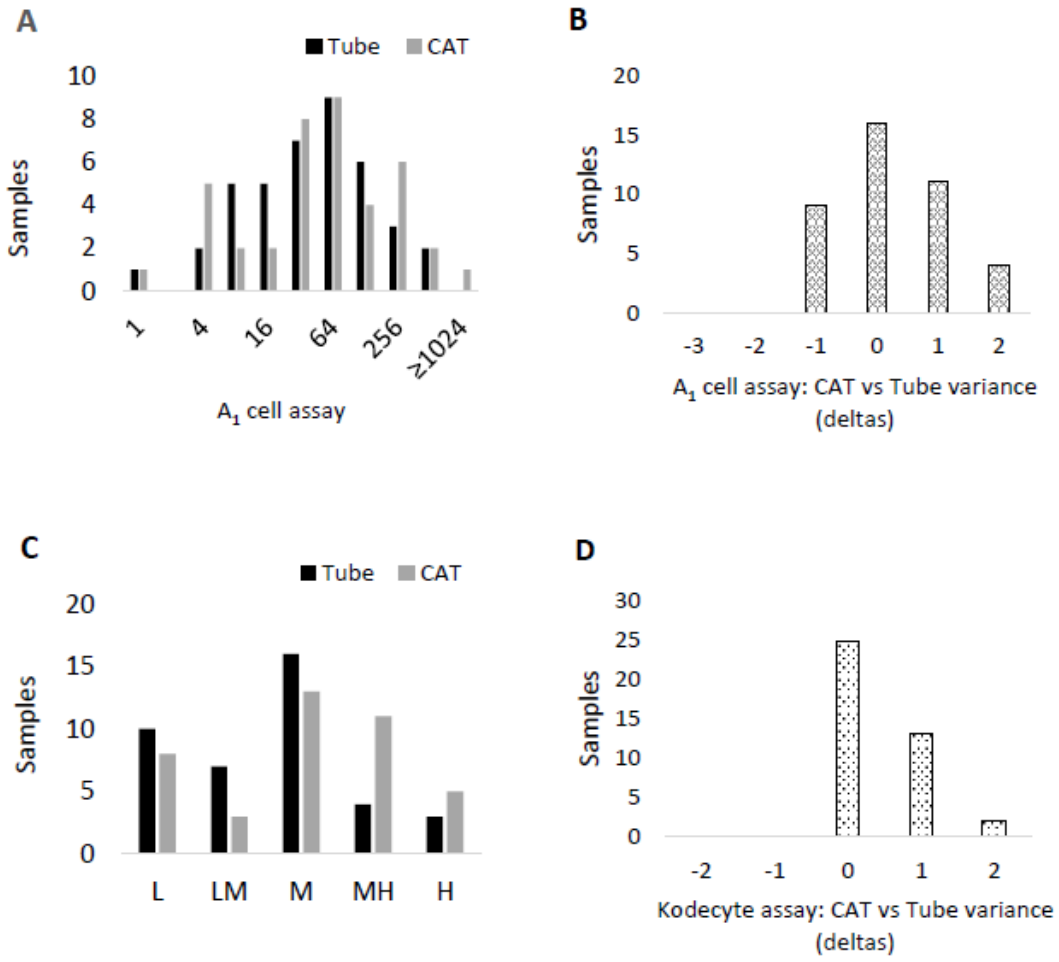


Figure 43. Analysis of patient group O and B samples (n = 40) in A_1 cell assay and Kodecyte assay. (A) Overall titre distribution against A_1 cells by platform. (B) Variance (deltas) of the A_1 cell assay titres in CAT compared to Tube. (C) Overall antibody level distribution by Kodecyte assay in CAT and Tube. (D) Variance (deltas) of Kodecyte assay antibody levels in CAT compared to Tube.

Results comparing antibody levels assigned by A_1 cell titration and Kodecyte assays in individual samples from the five patients are shown in Table 50. As expected, the level of antibody reduced as a consequence of PP events in all five patients, and this was demonstrated by both reduction in titre, and reduction in reactivity with the five-kodecyte panel (Table 50).

Table 50. Results of A_1 cell assay and Kodecyte assay in 40 samples from five patients, before and after four plasmapheresis events.

			Results pre and post PP cycles*							
			A_1 cell assay				Kodecyte assay			
			PrePP		PostPP		PrePP		PostPP	
Sample ID	ABO	Days post first PP	Tube	CAT	Tube	CAT	Tube	CAT	Tube	CAT
1a	O	0	256	256	64	64	H	H	M	MH
1b		+2	256	1024	64	64	H	H	M	MH
1c		+4	64	256	16	64	M	M	M	M
1d		+5	16	32	4	4	M	M	L	L
2a	O	0	256	512	64	64	MH	H	M	MH
2b		+1	128	512	32	32	M	H	M	MH
2c		+3	128	256	64	128	MH	MH	M	M
2d		+4	128	64	32	32	M	M	LM	M
3a	O	0	512	256	128	128	H	H	M	MH
3b		+2	512	256	32	64	MH	MH	MH	MH
3c		+4	64	128	32	64	M	MH	M	M
3d		+5	128	128	16	32	M	M	LM	LM
4a	O	0	128	256	32	32	M	MH	LM	M
4b		+2	64	64	16	32	M	MH	LM	M
4c		+4	64	32	8	16	LM	M	L	M
4d		+5	32	32	8	8	LM	M	L	LM
5a	B	0	64	64	16	8	LM	LM	L	L
5b		+1	32	16	8	4	L	L	L	L
5c		+3	8	4	4	4	L	L	L	L
5d		+4	8	4	1	<1	L	L	L	L

*PP = plasmapheresis

Internationally, different renal transplantation centres use antiglobulin titres ranging from ≤4 to ≤ 32 as the maximum allowed for transplantation to proceed [45,46,48-50,60,61]. That is patients must have an antiglobulin titre of ≤4 to ≤ 32 at the completion of their last PP session, for transplantation to proceed. A theoretical criteria of A_1 cell assay antiglobulin titre of <16 [46,56,57], and antiglobulin Kodecyte assay grade L (positive reaction with only the 100-A-kodecyte) were applied to the patients tested here (Table 50) as theoretical antibody level limits for transplantation to proceed. That is, it was considered that a patient must have a titre of <16 in the A_1 cell assay after four PP sessions to proceed. Similarly, a patient must have a Kodecyte assay result of L after four plasmapheresis sessions to proceed. When applying these criteria, all patients would have received the same decisions using the data obtained by the A_1 cell assay or the Kodecyte assay in tube platform. When using the CAT platform results, four patients (1, 2, 3 and 5) would also have received the same decisions regardless of whether the A_1 cell titration of the Kodecyte assay was used to determine the antibody level , however the decision for patient 4 would have been different, with transplantation allowed to proceed on the basis of the A_1 cell titre, but not on the basis of the Kodecyte assay. This work is summarised in Table 51. It is not known whether the five patients' transplants did proceed, as this was outside the scope of the ethics approval.

Table 51. Comparison of decisions to proceed to ABOiKT by method type. Comparison of decision based on anti-A/anti-A,B level as determined by titre in the A_1 cell assay, or antibody level in the Kodecyte assay, in five patients.

	Post-plasmapheresis (session 4) tube assays					Post-plasmapheresis (session 4) CAT assays				
Patient	A_1 cell titre	Decision*	Kodecyte assay level	Decision†	Decision consensus	A_1 cell titre	Decision*	Kodecyte assay level	Decision†	Decision consensus
1	4	Y	L	Y	✓	4	Y	L	Y	✓
2	32	N	LM	N	✓	32	N	M	N	✓
3	16	N	LM	N	✓	32	N	LM	N	✓
5	1	Y	L	Y	✓	<1	Y	L	Y	✓
4	8	Y	L	Y	✓	8	Y	LM	N	x

*Decision to transplant is based on titre of <16 in the A_1 cell assay

† Decision to transplant is based on result of L in the Kodecyte assay

Y = Yes: proceed to transplantation,N = No: do not proceed to transplantation

3.5 Quantification of antibodies to xeno-antigens Galα, GalNAcα and Rhaα

FSL-Galα (CMG_2 spacer), FSL-GalNAcα and FSL-Rhaα (Table 2), were used to prepare kodecytes at three different molarities; 40, 10 and 0.5 µmol/L (see section 2.2.1 Kodecytes for details of kodecyte preparation). The choice of FSL concentrations of 40, 10 and 0.5 µmol/L was guided by other work undertaken as part of this research [177]. The molarities represented a panel of a high, a medium and a low concentration of antigen for each FSL. Using this antigen dilution approach, undiluted plasma was tested with the nine kodecytes (three kodecytes each prepared with FSL at three molar equivalents) to determine relative antibody levels. These methods were conducted with 100 group O plasma samples from healthy blood donors (for details see section 3.2.3 Sera/plasmaB. Donor plasma samples). In tubes, 100 µL of plasma was incubated with 50 µL of a 4% kodecyte suspension in PBS for 1 hour at 37°C (saline assay to determine predominantly IgM antibody levels). Tubes were briefly spun to sediment the kodecytes and examined for agglutination with a 10x magnification eyepiece. Tubes which yielded negative reactions in the saline assay were washed four times in PBS, followed by the addition of 100 µL of AHG and brief centrifugation (antiglobulin assay to determine total IgG + IgM antibody levels). All reactions were graded on the 0-4 agglutination grading scale [101].

Relative antibody levels in individual plasmas were assigned using criteria detailed in Table 52.

Table 52. Assignment of relative antibody level by reaction with three-kodecyte Galα, GalNAcα and Rhaα panels

	FSL concentration (µmol/L)-kodecytes			relative antibody level
	40	10	0.5	
reaction of	+	+	+	High (H)
plasma with	+	+	0	Medium (M)
kodecytes	+	0	0	Low (L)

+ indicates agglutination (grades 1, 2, 3 or 4) and 0 indicates absence of agglutination.

Results showed that amongst 100 group O individuals, IgM antibody levels to GalNacα demonstrated the least variation amongst the population. A higher degree of variation in the population was seen with antibodies to Rhaα and Galα (Figure 44). The greatest number of individuals to have high IgM antibody levels occurred with antibodies to Rhaα. The greatest number of individuals to have low IgM antibody levels occurred with

antibodies to Galα. However, total IgM+IgG antibody levels as determined by antiglobulin testing were more evenly distributed amongst the specificities in the population (Figure 44).

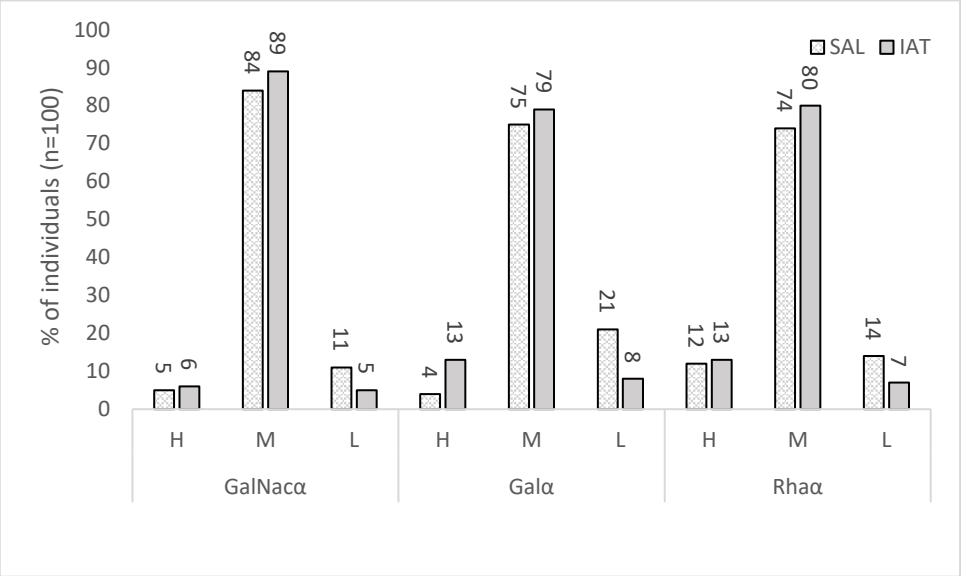


Figure 44. Distribution of antibody levels (H, M, L) to xeno-antigens GalNAcα, Galα and Rhaα, established by testing three-kodecyte panels with undiluted plasma of 100 group O blood donors by saline and IAT (antiglobulin) tube methods. Saline method results represent predominantly IgM antibodies, whilst IAT results show total (IgM+IgG) antibody levels.

3.6 Chapter summary

In this chapter, a panel of five A type 2 kodecytes was optimized to create an assay for predicting antibody level in patients presenting for ABOiKT. This panel was tested against 102 group O and 23 group B blood donors, and 40 samples from five patients undergoing antibody reduction in preparation for ABOiKT. There was a high correlation between antiglobulin antibody titre, as determined by the A_1 cell assay, and antiglobulin antibody level as determined by the Kodecyte assay. Results with kodecytes in saline assays showed lower concordance with the A_1 cell assay. Kodecytes prepared with FSL concentrations at up to 20 µmol/L were found to be stable during their storage period, whilst the kodecyte prepared with the high FSL concentration of 100 µmol/L was subject to some increased lysis over a four week storage period.

Relative antibody levels to Galα, GalNAcα and Rhaα antigens were determined to be high, medium or low using 3-kodecyte panels in a further 100 donor samples.

Chapter 4: Discussion

This research investigated the potential use of FSL Kode™ Technology constructs to develop novel assays for quantification of selected antibodies to carbohydrate antigens, and to re-visit historical findings of complement stability [145,153].

Following pilot studies, four primary glycan FSL were selected for the purposes of the research, namely:

- FSL-A type 2, functional head GalNAcα1-3[Fucα1-2]Galβ1-4GlcNAc; human blood group A antigen [12,180,181] (Figure 12)
- FSL-Galα; functional head Galα1-3Galβ1-4GlcNAc; a carbohydrate antigen found in non-human animals [182] and an important xeno-antigen in humans [89,92-95]. Both CMG_2 and adipate-spacer versions of FSL-Galα were used (Figure 13)
- FSL-GalNAcα; functional head GalNAcα1-3Galβ1-4GlcNAc; a novel antigen not found in humans or other animals (Figure 14)
- FSL-Rhaα; functional head Rhamnose; a sugar found in bacteria [24-26], fungi [184], viruses [185] and plants [186] (Figure 15)

These FSL were selected because their functional heads are synthetic equivalents of sugar sequences (carbohydrates) which are antigenic to humans [6,23,33-35,41,89,91-95,99,205]. Carbohydrate blood group systems are of importance in the clinical arenas of blood transfusion and organ transplantation [6]. Since Landsteiner discovered the ABO system in 1901 [1] it is recognised that the success of blood transfusion and organ transplantation relies in no small part on selecting blood and organs from donors compatible with patients' ABO antibody [6]. Complications of ABO incompatible blood transfusion are due in part to activation of the complement cascade following antigen-antibody binding, with ensuing lysis of transfused RBC and consequent potentially fatal sequellae [6,206]. In the organ transplantation setting, deposition of activated complement proteins, together with activation of pro-inflammatory proteins, cause organ rejection [63,207].

Both blood transfusion and organ transplantation rely on accurate and precise laboratory assays to guide clinical decisions. Assays including detection of ABO haemolysins in blood donors [72,79,83-85], auto-immunity studies [149-152] and drug reaction investigations [149,208] rely on complement activity, and therefore pre-analytical storage

of samples to maintain antibody and fresh complement activity is paramount in the accuracy of these assays. Findings of complement stability [145,153] applied to sample storage and handling guidelines were out-of-date, and informed by historical reagents and assay designs with acknowledged limitations [147,148,156,158,159].

This century it has become possible to cross the ABO barrier in solid organ transplantation of kidneys [45-59] and paediatric hearts [64-67]. However, if the antibody level is not naturally low in the organ recipient, antibody reduction and immune cell depletion [46,47,50,51,53-55,61,63] must be deployed ahead of the transplant to avoid organ loss due to antibody-mediated rejection. Laboratory quantification of ABO antibody levels informs the decision to proceed, or not, with ABO incompatible organ transplantation [45,46,48-50,56-58,60].

Identification and quantification of other natural carbohydrate antibodies in populations is being studied by several groups [33,35,98,99]. Current interest in cancer therapy includes labelling cancer cells with carbohydrate antigens [40,89,91-95] to effect antibody binding and subsequent complement-mediated tumour destruction [209,210] . Carbohydrate antibodies activate the complement cascade by the classical pathway [6,42] and higher quantities of antibody activate higher quantities of complement [211]. Therefore high-level antibodies are of most interest in identifying immuno-oncotherapy targets with which to label cancer cells for the most efficacious response. Similarly, it is important to identify individuals or populations with low level antibodies, as these people may not benefit from this particular therapy [190]. Two current candidates for immuno-oncotherapy [40,91-94] are antibodies to xeno-antigens Galα and Rhaα.

This study had five main aims, namely to:

1. Prove that a range of kodecytes are susceptible to lysis by antibody-mediated complement activation, with a range of natural antibodies to glycans
2. Revisit the topic of complement stability, using kodecytes instead of animal cells
3. Design an alternative assay to antibody titration for quantification of ABO antibodies, using undiluted plasma
4. Evaluate xeno-antigens and antibodies in human plasma
5. Investigate FSL-antibody mediated complement attachment to non-biological solid phase surfaces of paper, glass and plastic.

The rationale for the second aim to re-examine the stability of complement was formulated from literature review which showed current dogma to be non-evidence based [154], sketchy and based on well-regarded, but technologically out of date studies [145,153]. To do this, it was first necessary to prove the first aim; that a range of kodecytes were susceptible to lysis, and to show that the work with RBC targets for antibody mediated complement activation was not limited to Galα-kodecytes.

The third aim of designing new assays based on antigen dilution with A type 2 kodecytes to quantify ABO antibodies was driven by limitations in current assays. Titration is the traditional method for quantifying ABO antibodies prior to ABO incompatible organ transplantation [45-50,57,60,62,113-119,125]; and is performed by testing serial dilutions of plasma against a target RBC [101]. This method is widely acknowledged to suffer from inaccuracies and imprecision [113-119] caused by both RBC batch to batch variation [69] and antibody dilution [113].

The fourth aim to evaluate xeno-antigens and antibodies in human plasma extended the ABO antibody quantification work, again using undiluted plasma to quantify a range of antibodies to xeno-antigens important in immuno-oncotherapy [40,89,91-95]. Methods for identification and quantification of levels of a broad range of carbohydrate antibodies usually involve immunoblotting [212] or EIA assays on plates, slides or chips with either fluorescent, or surface plasmon resonance (SPR) signal detection [33,35,38,91,96,99,187,188,213]. These assays are elegant and elicit a large quantity of information but are not necessarily representative of *in vivo* interactions which occur at the cell surface and are not considered routine procedures in diagnostic laboratories.

Finally, in the fifth aim, it was of interest to see whether complement could be laid down on non-cellular surfaces in addition to RBC, as this could be a useful adjunct to current laboratory assays exploring interactions of carbohydrate antigens and antibodies.

Kode™ technology provided the tools to address the five aims of this research. The features of this technology include FSL construct water dispersibility [172,174], the ability to modify cells (including RBC) with minimal interference to cell physiology [172] optimal presentation of antigens to antibodies by virtue of spacing the antigen away from the cell surface [172] and an ability to control the amount of antigen loaded into the cell

membrane [177]. Whilst all these features make Kode™ technology very attractive for development of new assays, the ability to quantitatively control antigen loading was probably the most powerful aspect for this work as it eliminated the problems of reagent RBC batch to batch variation [69,145,148,153,156,158,159]. It also yielded an ability to use undiluted plasma with a panel of antigen-diluted kodecytes to optimise antigen-antibody ratios, thereby eliminating the problems inherent in antibody dilution [113,121,188]. Undiluted plasma was used in cell-based assays with kodecytes in the work on complement stability and in the work on antibody quantification. This was a novel approach, as all other studies [35,45,46,48,50,60,61,99,117,145,148,153] had to use plasma dilution in their testing regimes, as the cell-based antigen dilution approach is not possible without Kode™ Technology.

In the quantification of antibodies to Galα, GalNAcα and Rhaα, the Kodecyte assay differs from other assays in its ability to test molar equivalents of antigen on a human cell surface rather than a solid surface such as a glycoarray slide. Although kodecytes are modified natural cells, antigen presentation on an RBC (kodecyte) may be more representative of natural cell-based interactions with antibody and may therefore be informative to xenotransplantation or immuno-oncotherapy work.

The aims of this research were largely realised.

Firstly, it was proven that a range of kodecytes were susceptible to lysis; those with terminal antigens Galα, GalNAcα and Rhaα were all successfully lysed through antibody mediated complement activation.

Secondly, complement haemolytic activity in undiluted stored human sera samples was measured in a classical pathway functional lysis assay with 100-Galα-kodecytes. Using this approach complement was proven to be at least twice as stable as previously believed [145,153]. This research was published in 2016 [178].

Thirdly, an assay using undiluted plasma and a panel of five A-kodecytes varying in FSL loading from 100 to 1 µg/mL was tested with 125 blood donors and five patients preparing for ABO incompatible transplantation. Results from the Kodecyte assay showed high correlation with results from traditional A_1 cell titration. This research was published in 2019 [177].

The Kodecyte assay could be a valid alternative method to titration for quantification of antibody levels in patients preparing for ABOiKT in the future. However, its value still needs to be tested in larger patient cohorts, expanded to include blood group B kodecytes and potentially refined, particularly for use in CAT platforms.

Fourthly, an assay with three panels of kodecytes prepared with FSL-Galα, FSL-Rhaα and FSL-GalNAcα, and varying in FSL loading from 40 to 0.5 µmol/L was tested with undiluted plasma from 100 blood donors. This assay was able to differentiate relative high, medium and low levels of the corresponding antibodies in the population, to provide valuable information for scientists working on carbohydrate antibody immuno-oncotherapy or xenotransplantation strategies.

The fifth and final aim to deposit complement on non-cellular surfaces was only partially successful. Antibody-mediated activated complement was deposited on paper and plastic, but results with glass showed smearing, and requires significant further work, which was not pursued further.

4.1 Lysis of kodecytes with FSL-Galα, FSL-GalNAcα and FSL-Rhaα

Complement-mediated lysis was successfully demonstrated with human serum antibodies and kodecytes prepared with three different glycan FSL specificities, including Galα-kodecytes which were subsequently used in the complement stability studies. The techniques developed here could be used by other researchers to investigate complement-binding properties of antibodies to potentially any glycan of interest, in a cell-based assay using kodecytes. This may be of value in understanding responses to xeno-antigens in immuno-oncotherapy, such as Rhaα [91] and Galα [40,89,92-95].

4.2 Complement stability studies

4.2.1 Study design and rationale

The earliest studies of complement stability were undertaken in sera from various animals including pigs, rabbits, goats and guinea pigs [197,214-217]. These assays to detect complement activity were based on haemolysis of RBC, facilitated by activation of complement following antibody binding. Later, similar studies were undertaken on human sera samples [145,146,153,155,218,219]. Retention of complement activity in animal and human sera samples was considered important in several historical laboratory assays;

both where complement was used as a reagent (for example in the Wassermann reaction for syphilis testing [214]), and where complement was a serum constituent in patient samples [199]. As part of the testing undertaken before a human patient receives a blood transfusion, patient plasma or serum is screened to detect blood group antibodies which can potentially cause haemolytic transfusion reactions if donor RBC express the corresponding blood group antigen [6]. These clinically significant antibodies are of immunoglobulin classes IgG and IgM [6], and the screening test must therefore include an antiglobulin testing phase [220]. Current standards and reference texts recommend inclusion of anti-IgG and anti-C3 in polyspecific antiglobulin reagents [101,220], however automated blood grouping and screening analysers now use plasma samples rather than sera, and the ability to detect antibodies that react only in the presence of anti-C3 *in vitro*, and their clinical significance has become somewhat of a moot point. But in previous days of blood transfusion it was considered important to have active complement in patient serum samples [42,199]. Accordingly, Polley & Mollison [153] and Garratty [145] designed studies in 1961 and 1970 respectively, to establish storage conditions for patient serum samples. Both historical studies [145,153] had two arms to their design. Firstly, a functional lysis assay based on the ability of human serum (containing complement) to lyse antibody-sensitized sheep RBC was performed on human sera subjected to different storage conditions. Further details of these study designs are summarized in Table 11, Table 12 and Figure 9.

Secondly, the ability to detect complement-dependent blood group antibodies in the antiglobulin test with anti-human globulin containing anti-C3 was studied in the stored human sera samples in both historical studies [145,153]. RBC with the phenotype Le(a+b-) were incubated with human EDTA-treated serum containing a non-direct-agglutinating IgM anti-Le^a to produce RBC sensitization without agglutination or complement activation of RBC. Then the washed anti-Le^a sensitized RBC were incubated with the stored sera samples. Following washing, RBC were tested with a polyclonal rabbit antisera including anti-C3, by mixing RBC with this antisera on ceramic tiles. Reactions were observed for agglutination at one to five-minute intervals, and given a score based on how quickly agglutination occurred. In this way complement activity of sera stored at different conditions was assessed. Garratty reported that the results from his lysis assay with the antibody-sensitized sheep RBC, and the antiglobulin assay with the anti-

Le^a sensitized RBC supported the same conclusions on complement stability in stored human sera [145]. The functional lysis assays that Garratty [145] and Polley & Mollison [153] performed were adapted from well-established methods of the day to measure 50% haemolysis of an RBC population [156,158,159]. However, the alternate method using an antiglobulin assay to measure complement levels was not well established, and the ability to control the amount of antigen on a cell was not possible. In addition, the reading of agglutination reactions on ceramic tiles at timed intervals would have been difficult and subjective. The sensitivity of antigen-antibody reactions on tiles is acknowledged to be less than current testing platforms of tube, microplate and CAT [221], with weak tile reactions being difficult to interpret [4]. End-point reactions with the stored sera at the lower end of complement activity would by definition have been weak reactions. Nowadays tiles are relegated to out-of-laboratory blood grouping in a field situation to assign ABO groups, in settings where no other technology is available. A tile technique utilising high-sensitivity antisera may be appropriate in the field, but the value of its use in Garratty's [145], and Polley & Mollison's [153] studies to judge complement stability in an antiglobulin technique is now questionable.

In light of these historical method inadequacies relating to the design of the antiglobulin test, and the need to compare results from this study with historical studies, it was decided only to use the functional lysis assay for comparison in this study.

4.2.2 Lysis assay; findings of this study compared with historical studies

The findings of stability of complement stored at minus 20°C (freezer storage) were similar in this research [178], to Polley & Mollison's study [153]. Polley & Mollison reported that "serum stored (at minus 20°C) for two months gave only slightly weaker reactions than fresh serum when used as a source of complement in the lysis of sensitized sheep cells" [153]. This study also found complement in sera samples stored at minus 20°C to have full lytic ability after 150 days (5 months). Garratty [145] disagreed, reporting that storage at minus 20°C resulted in loss of complement, which declined to 60% of original levels after 60 days (Figure 26).

At 4°C storage (refrigerator storage), Polley & Mollison [153] found complement activity producing complete lysis of RBC after 60 days of sample storage. Similar to Polley & Mollison's findings, this research found complement in sera samples stored at 4°C

retained 60% of original levels after 60 days. Garratty [145], however, reported complement levels dropped to less than 60% at 10 days (Figure 26).

At 22°C storage (room temperature), Polley & Mollison [153] found no complement activity as measured in the lysis assay in samples stored for seven days. Garratty [145] found complement activity to reach 60% in samples stored between one and two days, and 0% complement in sera stored for 14 days. This research found 60% of complement to be active between seven and 14 days, with 0% activity reached only at 60 days.

At 37°C storage, Polley & Mollison [153] found complement retained the ability to lyse RBC after one day storage, but not after 7 days storage. They did not measure activity in the interval between one day and seven days. Garratty [145] reported complement levels declined to less than 60% after one day of storage, whereas this research found complement levels declined to less than 60% between one and two days of storage.

In summary, this study agreed more closely with the findings of Polley & Mollison [153] for storage temperatures of minus 20°C and 4°C, and disagreed with the "bench mark" findings of Garratty [145] at these temperatures. This study found complement to be stable for more than twice as long at these temperatures as Garratty [145] reported.

Regarding 22°C (room temperature) and 37°C storage, this research found complement to be stable for at least twice as long as both Garratty, and Polley & Mollison reported.

As shown in Figure 30, the lysis assays of Garratty [145] and this research [178] demonstrated comparable sensitivity. Therefore, a lack of assay sensitivity in Garratty's approach using dilutions of sera against sensitized sheep RBC does not explain why Garratty reported complement activity in stored human sera to be less than this study. However, the sensitivity study undertaken here was one "snapshot in time", whereas in practice other factors may have affected either or both current and historical studies.

There was no dilution of serum in this research [178], whereas serum dilution was used in the other studies [145,153]. All studies (current and historic) need to achieve an assay mid-point of lysis of 50% target RBC, in order to measure lysis of greater than, and less than 50% of the RBC population. This 50% lysis point is reported to have good sensitivity to deterioration of complement lytic activity [127,147,148] and ensures measurements are taken in a linear region of the curve [156,158] when plotting the percentage of lysis against

volumes of diluted serum complement (Figure 8). The historic studies had to use dilutions of serum to achieve lysis of 50% target RBC, because undiluted sera caused lysis of 100% of cells, due to large quantities of antibody present in the undiluted serum. Dilution was used to lower the quantity of antibody present. This research used a new approach of lowering the quantity of antigen present on the RBC surface, and optimized antigen loadings on RBC to achieve a 50% lysis point with undiluted plasma. Using undiluted plasma in this research removed the error associated with serial dilution, whereby pipetting error is compounded with each subsequent dilution in the series, and individual components in a multi-facet cascade may be differently affected; that is the level of component with the least "elasticity" to being diluted will be the weakest point in a dilution assay [6].

Furthermore, the nature of the target RBC was considerably different between this research [178] and the historical studies [145,153]. Aspects of the sheep RBC used in historical studies were diagrammatically represented in Figure 9. Both historical studies [145,153] used sheep RBC with Forssman antigen (GalNAcα3GalNAcβ3Galα4Galβ) as the probable primary target antigen. This research used Galα-kodecytes; that is human RBC koded with FSL-Galα (antigen Galα1-3Galβ1-4GlcNAc). A further difference relating to the RBC was that the historical studies presensitized the sheep RBC with rabbit or bovine anti-sheep heterophile antibody according to the method of Dacie and Lewis [159]. This heterophile antibody was a complement-activating antibody predominantly consisting of anti-Forssman but including other antibodies. This current research did not presensitize the Galα-kodecytes, as with kodecytes it is possible to control the amount of antibody binding by antigen dilution [177]. Furthermore, because only one serum pool was tested (the standard serum index sample), natural variation of anti-Galα level between human individuals [35,98,99] was not an issue. It should be noted that the experiments with kodecytes relied on the intrinsic level of anti-Galα in the standard serum index sample, and therefore this method is probably not suitable for testing different individuals, who would have different levels of anti-Galα [35,98,99], as shown in the work presented here in section 3.5 Quantification of antibodies to xeno-antigens Galα, GalNAcα and Rhaα.

The different RBC antigen targets; Forssman antigen GalNAcα3GalNAcβ3Galα4Galβ on the sheep RBC in Garratty's assay and Galα1-3Galβ1-4GlcNAc on the kodecytes in this

research should not have resulted in assay variance unless the two corresponding antibodies activated complement with different efficiency rates. There was no evidence of this when the two assays were compared for sensitivity with the known complement standards (Figure 30). The presensitization versus no presensitization could potentially have produced different amounts of complement activation, as one method may have bound more antibody than the other. However, there was no evidence that this was the case when comparing the assays for sensitivity (Figure 30).

Thirdly, batch variation of target RBC was eliminated with the use of kodecytes, as the loading of antigen Galα1-3Galβ1-4GlcNAc was controlled, being added as a measured amount of FSL construct solution to fresh human RBC. When using sheep RBC, batch variation has been reported as being problematic, both in terms of significant variance of antigen loading due to natural genetic variation between sheep, and red cell fragility of stored sheep RBC which can lead to mechanical haemolysis [145,148,153,156,158,159].

Fourthly, results of 12 sera were averaged in Garratty's assay whereas this research utilised one pool of two donors (standard serum index sample). Both studies (this research and Garratty's [145]) made the assumption that results were not affected by variation in complement physiological activity or levels in different individuals, and any effect this may have had was minimised by pooling results (Garratty), or pooling sera samples (this research). It would have been best practice to prepare the standards for the sensitivity assay from the standard serum index sample to ensure that there was no difference in complement level between the individual used to prepare the complement standards and the standard serum index sample, however this was not possible due to insufficient sample remaining. However, the sensitivity assay did have validity, as the same set of complement standards were used to reproduce the historical assay [145] and to test the sensitivity of the assay reported in this research [178].

Finally, in this study a 60 minute incubation period was shown to be optimal to reach end point maximal lysis of 100-Galα-kodecytes. However both Garratty [145] and Polley & Mollison [153] used a 30 minute incubation time in their studies of complement stability. In order to compare this work with historical studies, it was decided to also apply a 30 minute incubation for the kodecyte studies. This assay difference did not affect the results, as complement stability was calculated by expressing the absorbance of a stored

serum aliquot as a percentage of the absorbance of the "day zero" standard serum index sample, with absorbance readings taken in the same experiment.

In light of the method of Garratty [145] having comparable sensitivity with this study, it remains unknown why this study revealed complement to be more stable than reported in the historical assays [145,153]. The explanation may be method error of historical studies; specifically the potentially confounding influence of multiple antigen-antibody interactions (Figure 9), widely acknowledged [145,148,153,156,158,159] sheep RBC batch variation, and errors of serum dilution [153,156]. It would also be of interest to study stored diluted serum, in parallel with fresh diluted serum to assess whether low level but critical analytes deteriorate on storage (for example metal ions required for complement activation [131]). The serum used in the assay to compare sensitivity of the assay of Garratty [145] with this research used fresh serum, and therefore did not assess the combined effect of serum dilution and storage of serum on assay sensitivity.

4.2.3 Assay challenges

Constraints on this study included a lack of published or available method detail in Garratty's paper [145], and an inability to source reagents of the day for comparison with historical methods. For example, Garratty referred to "Forssman antibody" as the antibody used to presensitize sheep RBC. The source of that antibody was not stated, but can be presumed to be anti-species antibody from a non-ovine animal [222]. The antibody chosen for this study to replicate Garratty's work was anti-sheep RBC stroma antibody produced in rabbits. Whilst rabbit anti-sheep RBC undoubtedly contains anti-Forssman [222], it is not a pure source of that antibody.

In the method to assess complement stability in multiple frozen-thawed aliquots of the standard serum index thaw sample, there was a steady rise in absorbance as the number of freeze-thaw cycles increased (Table 15). This was unexpected, as any deterioration in complement level in samples frozen and thawed multiple times should have resulted in a lower absorbance reading when compared to the aliquot which was frozen and thawed only once. Similarly, if the level of complement was constant, absorbance readings should have stayed the same, or fluctuated in both the upward and downward direction, in a magnitude reflecting assay error. The fact that the absorbance steadily rose might be evidence of complement activation. In their study on complement

stability and effects of different pre-analytical conditions, Yang and colleagues [146] included work on effects of freezing and thawing. Using an immuno-assay to quantify individual complement proteins, they reported that complement protein levels increased upon freezing and thawing, and suggested that this was due to complement activation, although this was observed only in samples thawed at 37°C, and not in samples thawed at room temperature and tested within 60 minutes. It appears that samples in this thesis research may have been subject to some pre-analytical complement activation, and therefore claims around complement stability in samples thawed and frozen more than once should be interpreted with caution. This same caveat does not apply to the results in samples stored at different storage temperatures, as samples were thawed only once before testing.

Stability data on Galα-kodecytes suggested that FSL-Galα at higher concentrations produced damage to the RBC membrane in kodecyte preparations stored for more than one week (Figure 23), a finding which was also borne out with stability studies on A type 2 kodecytes prepared with 100 µmol/L of FSL (see later in discussion).

Although higher FSL concentration 100-Galα-kodecytes were used in the study of complement stability, results were not affected by the finding of mechanical damage to kodecytes on storage, as 100-Galα-kodecytes used here were freshly prepared on the day before the assay was performed.

The stability data on Galα-kodecytes was conducted with only the qualitative haemolysis scale. In hindsight, it would have been more accurate to take spectrophotometric measurements, using the methodology developed later for the A type 2 kodecyte stability study. Unfortunately, it was not possible to repeat the work using the spectrophotometric measurements, due to a shortage of FSL-Galα (adipate spacer).

4.3 ABO antibody quantification studies

4.3.1 Study design and rationale

ABO antibody levels inform ABO incompatible transplantation practice, because higher antibody levels in patients must be reduced to lower levels deemed safe for transplantation without risk of acute organ rejection [45-51,60,62,64-66]. Similarly, ABO

antibody levels guide selection of donors of group O blood products for transfusion to non-O patients. Although group O donations are generally regarded as "universal donations" (suitable for transfusion to patients of any blood group), this relates to antigen status, and not antibody, and therefore those with higher levels of ABO antibodies must be identified in order to avoid risk of ABO incompatible transfusion reactions in non-O patients [6,68-77].

Titration is widely used to quantify ABO antibody levels. However, there is a high level of variation in titration results between laboratories, which applies equally to estimation of titres for organ transplantation or blood transfusion purposes [57,69,113,114,116-119]. This variation is caused by inherent deficiencies in the titration approach (see later discussion) which is further compounded by inter-laboratory differences in methodology [57,68,69,113-119,123,125,166] and reagents [69]. Therefore there is a need for a standardised method of ABO antibody titration, and several countries are working on standardizing their approach to ABO antibody quantification by titration with the use of standard protocols, external surveys and reference reagents [113,114]. However, further compounding the problem is a poor relationship between titre and clinical outcome in the transfusion setting [73-76,80-82].

In this research, an alternative approach to titration for ABO antibody quantification was developed; namely the Kodecyte assay. The Kodecyte assay was based on the observation that if an antibody is present at low concentration, there must be a correspondingly high level of antigen for RBC expressing that antigen to be agglutinated, and vice versa [113,126,223]. In traditional titration methods using natural A_1 cells, it is not possible to vary the antigen density, therefore antibody quantification methods rely on plasma dilution to determine antibody levels. This results in the problems described in chapter 1.

The Kodecyte assay for quantification of ABO antibodies had two key differences to titration methods, one relating to the plasma (antibody source) and the other relating to the RBC (antigen source):

- Antibody source: There was no plasma dilution in the Kodecyte assay. The amount of antibody in each sample was kept constant throughout the assay, as were the other proteins in the plasma. This is in direct contrast to titration,

where the amount of antibody, and all other proteins decrease with each plasma dilution

- Antigen source: In contrast to titration, natural group A RBC were not used in the Kodecyte assay. Natural RBC have many variations of the same antigen (Table 1) expressed in different amounts in different individuals [109-111], and pooling of cells is used to mitigate this issue in titration [69]. In contrast the five kodecytes (100-, 20-, 10-, 2.5- and 1-A-kodecytes) have only a single chain type (type 2), and are loaded with the A antigen in precise concentrations.

Therefore for these reasons the Kodecyte assay is a significantly different approach to titration. In titration, the lowest amount of antibody that can effect agglutination of RBC expressing a target antigen is detected. This is achieved by intentionally and sequentially lowering the amount of antibody by diluting the plasma until the end-point of the assay is reached (where the reaction fails). The amount of antigen is unable to be intentionally varied in this approach (although in practice antigen variation does exist due to natural variation [109-111]). In the Kodecyte assay, the lowest amount of antigen that can effect agglutination with a constant amount of antibody is detected, based on the theory that more antibody is required to detect less antigen [113,126,223]. This is achieved by intentionally and sequentially lowering the amount of antigen on a series (panel) of manufactured kodecytes.

It is relatively easy to understand how the conventional approach of diluting the antibody to reach an end point of a titre works in practice. It is known that approximately 150 antibody molecules bound to a single RBC are necessary to agglutinate natural RBC in an antiglobulin test [224]. Below this antibody number, the assay reaches a point where there are too few antibodies present in the plasma to collide with, and bind to the RBC antigens, and “assay failure” occurs (no agglutination). It is not known how many antigens are required for agglutination to occur, and consequently it is less easy to understand the reaction dynamics of the Kodecyte assay. In the Kodecyte assay a panel of kodecytes was created with five different concentrations of FSL. The amount of antibody in an undiluted patient plasma was kept constant, and the number of antigens was lowered across the panel of kodecytes.

Plasmas containing high level antibodies were able to cause agglutination of kodecytes with a low loading of A antigen (for example the 1-A-kodecyte created with 1 µmol/L of FSL), whereas plasmas with low level antibodies were unable to agglutinate this 1-A-kodecyte; creating the "assay failure" and thus an end point to the Kodecyte assay. In the plasmas with the high-level antibody, it can be reasoned that there was sufficient antibody to collide with the limited number of antigens; that is there was a higher number of collisions and subsequent cross-linking of kodecytes to cause agglutination. Whereas in the plasmas with the low-level antibody, a limited number of antibodies available to collide with a limited number of antigens resulted in less collisions and no agglutination.

There are two parallels for this phenomenon observed with undiluted plasma and natural cells. Firstly; the dosage effect is observed with some (but not all) examples of antibodies of the Duffy, Kidd, Rh and MNS blood group systems [6]. Dosage occurs when a single plasma expressing a low level of antibody is able to agglutinate an RBC expressing the corresponding antigen in high quantity as the result of the individual being homozygous for the gene controlling expression of the antigen, but is not able to agglutinate an RBC expressing the corresponding antigen in lower quantity as the result of the individual being heterzygous for the gene [142]. Like the Kodecyte assay, the antigen number on the natural RBC is the variable in this scenario, whilst the antibody level remains constant. Evidence suggests that the antibody showing dosage is the antibody with low levels [142], whereas the antibody of the same specificity not showing dosage presumably has a higher level of immunoglobulin.

In the second parallel scenario, it is known that a single anti-A antiserum is able to agglutinate RBC of subgroups of A with high numbers of the A antigen (for example A_1 and A_2) but not able to agglutinate (or only weakly agglutinate) RBC of subgroups with low numbers of the A antigen (for example A_x) [6]. However, if increasing amounts of antibody are added to the A_x cells, it is possible to produce agglutination where there was none, or to strengthen the grade of agglutination [223].

In all these scenarios (kodecytes created with low concentrations of FSL-A, weak subgroups of A, and RBC from heterozygotes of the blood group systems mentioned above), the number of antigen-antibody collisions is insufficient when both the antigen

and the antibody are present in lower concentrations, compared with a higher number of antigen-antibody collisions when the antibody is present in higher concentration.

Another important factor which must be considered when discussing reaction kinetics is antibody and antigen affinity (the degree to which one substance binds to another; dependent on the physical "goodness of fit" between the antigen and the antibody). High affinity antigen-antibody pairs have a very good fit, low affinity pairs a poorer fit. Human polyclonal antibodies fall across a spectrum of high and low affinity, and everything in between [126], as antibodies produced in response to a newly-encountered antigen cross-react with a variety of epitopes (low affinity). Later in the immune response the clones of B lymphocytes which produce antibody with single specificity (high affinity) undergo antibody proliferation in the process known as affinity maturation [225,226]. A polyclonal antibody is also heterogeneous in composition; for example although we call the antibody that agglutinates A cells "anti-A" it is actually a mixture of paratopes that react with different epitopes of the A antigen. The affinity and specificity of ABO antibodies is known to be variable between and within human plasmas [41,98,168,227], with the majority of low or moderate affinity [12].

Klein and Anstee point out in the seminal text Mollison's Blood Transfusion in Clinical Medicine (page 326) that " the (titration) method is unsound in principle as it estimates only the amount of antibody bound to red cells, not the amount of antibody in the serum. At the endpoint of the titration, agglutination is caused by the relatively small number of antibody molecules in the serum with the highest affinity, and therefore the proportion of such molecules in the serum has a considerable influence on its titre [6] ".

Finally, the Kodecyte assay approach of using undiluted plasma avoided the errors associated with preparation of dilution series, where any pipetting error is compounded throughout the series, and low level, critical analytes (for example serum albumin) may not be available in sufficient quantities at the higher dilutions [6].

4.3.2 Assay considerations

A. Choice of ABO blood group system kodecytes

Only blood group A kodecytes were analysed in this study, to keep the trial manageable. At least in Caucasians, O patients are more likely to receive A kidneys than B kidneys [46],

due to the frequency of ABO groups in Caucasians [6]. In a study of data from 1,420 ABO incompatible organs transplanted in several countries [46], patients with anti-A who received a kidney expressing the A antigen accounted for 68% of the transplants. However, kidneys from B donors are transplanted to non-B patients [46], and on the basis of the successful trial with A kodecytes, there is now a need to test a panel of B kodecytes.

B. Kodecyte stability

Twenty four hours after koding, the mean cell volume (MCV) indicated no swelling of red cells in kodecytes prepared with FSL-A at 20, 10, 2.5 and 1 µmol/L, and the MCV of these kodecytes was indistinguishable from the non-koded cell used to prepare the kodecytes (Table 36). However, the 100-A-kodecyte had a significantly higher MCV (Table 36), which indicated some cell swelling, presumably due to membrane expansion or damage from loading a lot of lipid into the membrane. After four weeks storage, the MCV supported the same conclusion; that FSL concentrations of 20, 10, 2.5 and 1µmol/L produced no evidence of cell swelling but some evidence of cell swelling in the 100-A-kodecyte. The MCV was higher, in all stored cells (all FSL concentration kodecytes and the non-koded control RBC) than in freshly collected non-koded and koded cells (Table 36). This was due to red cell storage lesion [228,229], and not to the insertion of FSL in the 20-, 10-, 2.5- and 1-A-kodecytes.

Across four weeks storage in red cell preservative, lysis of A-kodecytes prepared with 20 µmol/L and below was negligible (2%), and comparable with the non-koded RBC. Therefore it may be concluded that the small amount of observed haemolysis in these kodecytes was due to the red cell storage lesion observed with all stored RBC [228,229], rather than the koding process. However, the 100-A-kodecyte did show an increase in lysis over the four week storage period, with 5% loss of RBC. In addition to the spectrophotometric analysis, the increased lysis in the 100-A-kodecyte was evident with the naked eye. Despite some lysis, agglutination grades of kodecytes with monoclonal anti-A remained consistent across the four week storage period (Table 37). There is probably a need to evaluate other RBC preservative solutions other than Celpresol, which may perform better to preserve RBC and kodecytes.

4.3.3 Assay challenges

Precision and accuracy are considered equally important when assessing a possible new laboratory assay [230]. To assess assay precision (reproducibility) twenty donor plasma samples were tested by both assays on three separate occasions. Both the A_1 cell assay and the Kodecyte assay showed a high degree of precision and the assays were indistinguishable in that regard.

Accuracy of the Kodecyte assay was assessed by comparing the antibody level assigned by that assay to the antibody level assigned by the reference A_1 cell assay, using the criteria shown in Table 34. Where the same antibody level was assigned, the Kodecyte assay was considered concordant with the A_1 cell assay. Where a different antibody level was assigned, Kodecyte assay was considered discordant with the A_1 cell assay. Allowance was made for variation in the reference A_1 cell assay at a maximum of ±2 tube variation. Across 125 donor samples, and 40 samples from five patients, concordance rates ranged from 96% - 100% in the antiglobulin tube method, and from 91% – 100 % in the antiglobulin CAT method. Therefore the antiglobulin assays showed a high level of concordance, and reflected well on the Kodecyte assay as an alternative technique to the A_1 cell assay for ABO antibody quantification [177]. However, the discordant samples (those giving a different antibody level according to assay type) required further investigation.

In the antiglobulin tests, seven group O discordant samples showed high or medium-high anti-A/anti-A,B levels in the Kodecyte assay, and low or medium titres in the A_1 cell assay (in the range 16 -64) (samples e-j in Table 45). These discrepancies were only observed in the CAT platform; in tube, three group O discordant samples (b, c and d in Table 45) demonstrated medium or medium-high levels in the Kodecyte assay, and high titres of 1024 in the A_1 cell assay. Thus in CAT, either the Kodecyte assay over-estimated the antibody level, or the A_1 cell assay under-estimated the antibody level. it is recognised that CAT produces weaker reactions between ABO antibodies in human plasma and natural cells than the same samples tested in tubes in standard blood grouping tests [231].

Two group B samples were also investigated for discordant results. One sample (p in Table 45) showed the same pattern as the three group O samples b, c and d; that is it

demonstrated a higher antibody level by the A_1 cell assay than the Kodecyte assay. The other group B sample (n in Table 45) showed a platform discordancy; that is antibody level was concordant between the A_1 cell assay and the Kodecyte assay in both the tube and in CAT, but tube level was medium by both assays, and CAT level was low or low-medium by both assays. This may be another example of CAT producing weaker reactions than tubes with ABO antibodies [231], regardless of whether group natural A RBC, or kodecyte are used.

There was no explanation for the three group O discordant samples (b, c and d in Table 45) which showed medium or medium-high levels in the tube Kodecyte assay, and high titres of 1024 in the tube A_1 cell assay. Consideration was given to whether the Kodecyte assay failed to detect anti-A directed against chain types other than A type 2 in these samples. Anti-A can be produced to each of chain types 1, 2, 3 and 4 (Table 1), depending on an individual's genotype and phenotype. Natural A_1 RBC express the A antigen on types 2, 3 and 4 chains, and also type 1 chains if the individual is a secretor [12] . In contrast, A type 2 kodecytes only express the A antigen on type 2 chains, and antibody against other chain types will not be detected [200]. Chain type specificity of antibody was not investigated in this study, however, it seems unlikely that this was a cause of discordancy, given the fact that A type 2 is the dominant chain type [9,12].

Other variables were also considered as potentially influential in causing assay discordancies. The possible presence of high-titre, low-avidity (HTLA) antibodies was considered. HTLA antibodies notably occur in the Chido/Rodgers and Knops blood group systems [202]. Although HTLA antibodies are not known to be associated with the ABO system, the possibility that the discordant samples were due to the effects of low avidity antibodies was considered. In her review of HTLA antibodies, Rolih [202] states "the poor reaction characteristics of HTLA antibodies may reflect, not the avidity characteristics of the antibodies, but the restricted numbers of antigen sites per red cell with which to bind". As kodecytes have deliberately restricted numbers of antigen sites [232], it was of interest to investigate whether the discordant samples showed many weak reactions either in the titre (A_1 cell assay), or in the reactions with kodecytes (Kodecyte assay). No evidence was found of HTLA antibodies in either discordant or concordant samples, so this was able to be ruled out as a cause of discordancy in the A_1 cell assay.

A further variable to be considered as a possible cause of discordancy between the A_1 cell assay and the Kodecyte assay was the IgG versus the IgM content of antibodies. Different ratios and levels of IgG and IgM show variations in individual affinity [164] which can contribute to stronger or weaker reactions with ABO antigens [169] . Competition between IgG and IgM in tests for carbohydrate antibody levels in solid phase assays has also been observed [188,233], with dilution of plasma exacerbating this effect [188]. It is as yet unknown how differences in levels of IgG and IgM, and their individual and collective antibody affinity might contribute to reactions with kodecytes, and this is now part of an ongoing research process. Samples b, c and d in Table 45, where the Kodecyte assay under-estimated the antibody level compared to the A_1 cell assay in tube, had between eight and 32 times more IgG than IgM. In contrast, six of seven samples e-j in Table 45, where the Kodecyte assay under-estimated the antibody level compared to the A_1 cell assay in CAT had between two and 16 times less IgG than IgM content. Therefore the majority of discordant samples did have differences in ratios of IgG:IgM compared to concordant samples, with a high IgG content appearing to favour antibody detection in the A_1 cell assay in tube, but a low IgG content appearing to favour antibody detection in the Kodecyte assay in CAT. Because A type 2 kodecytes present antigen differently to natural A cells by virtue of spacing the antigen further from the red cell membrane [172], and because there is a smaller number of A antigens present on kodecytes than on natural A_1 cells [232], it was reasonable to question whether plasma with different quantites/ratios of IgG and IgM anti-A and anti-A,B might react differently with kodecytes and natural A cells. However, it was difficult to tease out the combined variables of antigen presentation, antigen numbers, and testing platform here, and the discordancies seen in samples b, c and d cannot be explained at this point. Extended studies using purified IgG, IgM and mixtures of both are required to resolve these issues. This was beyond the scope of this work.

Saline assays were only conducted in tube on 51 of the donor samples and showed a lower rate of concordance than IAT assays, with 88% of samples concordant between the A_1 cell assay and the Kodecyte assay at the ±2 dilution level in the A_1 cell assay. This was perhaps not surprising as all the optimisation of the Kodecyte assay was for antiglobulin technique. A panel using different concentrations of FSL-A would be needed to establish a panel of kodecytes suitable for comparing A_1 cell titres with Kodecyte

assay antibody levels by saline techniques. Optimisation of a saline panel was not pursued because ABO antibody level determination is used in applications which require the measurement of total immunoglobulin [58,60], not merely IgM as is predominantly determined in saline techniques. For the same reasons, saline testing was not conducted in CAT. The Kodecyte assay over-estimated the antibody level compared to the A_1 cell assay in 5 of 51 samples (12%). These discordant samples were examined for IgG and IgM content, and all had between four and 16 times more IgG than IgM (Table 46) . This was the same pattern as seven discordant IAT samples, however, in those samples this pattern was observed in CAT, not in tube. Because the Kodecyte assay appeared to over-estimate antibody level in samples aa - af (Table 44), and in light of the different G:M ratios in discordant and concordant samples (Table 46) it was questioned whether IgG might be causing direct agglutination of kodecytes. As stated earlier, IgG anti-A and anti-B can agglutinate RBC in saline tests, and this has been attributed to the protruding presentation of A and B antigens on the cell surface in comparison with some other antigens; for example the Rh system antigen D [204]. As Kode™ Technology further increases the distance that the A antigen protrudes from the A-kodecyte surface by virtue of the spacer [172], it seemed reasonable to question whether IgG anti-A/anti-A,B caused direct agglutination of kodecytes. However, testing showed no direct agglutination of kodecytes with IgG (Table 47), so the hypothesis that IgG was causing direct agglutination of kodecytes was disproven. Further investigation is required to shed light on the cause(s) of the discordancies in the saline assays, but was beyond the scope of this thesis.

4.3.4 Inter-laboratory testing variation

All 40 patient samples tested in the A_1 cell assay were also independently tested for IAT tube titre in an A_1 cell assay by NZBS. These results were unknown to the author of this research at the time of testing but were later made available for comparison. Thirty eight samples were titre-identical or within one dilution difference when comparing results of this study and results from the independent laboratory. Two samples had a two dilution difference. Contrary to the literature [113-119] this is a very small amount of inter-laboratory variation.

4.3.5 CAT versus tube

In the A_1 cell assay, approximately 90% of titre results of 102 group O donors and 40 patient samples were within ±1 dilution, when comparing testing platform of tube or CAT (Figure 41B and Figure 42B). However, in 23 group B donors higher titres were assigned in tube than in CAT, with CAT yielding 2 dilutions lower titres in approximately 20% of samples (Figure 42B). Others have also found titres to be higher in either CAT or tube platforms, with no consensus as to which yields higher titres.[69,115,116,123-125]

In the Kodecyte assay, higher antibody levels were assigned in CAT than in tube in all sample cohorts (Groups O (Figure 41D) and (Figure 42D) donors and patients (Figure 43D)). The different diluents in which kodecytes were suspended (PBS in tube and red cell diluent RCD in CAT) may have contributed to the higher antibody level assignment in CAT. RCD is a low ionic strength saline (LISS) diluent to which purine and a nucleoside have been added [234] to maintain antigen stability [235]. Whereas the sodium chloride concentration in PBS is approximately 170 mmol/L, in LISS solutions it is 30 mmol/L, with osmolality favourably maintained for the RBC by the addition of glycine [236], or purine in the case of Ortho RCD. The main function of LISS is to shorten the incubation time in antibody detection [236]. Löw and Messeter demonstrated no difference in detection of anti-A with A cells suspended in either saline or LISS [236]. However, there are reports of differences in detection rates of antibodies and their comparative reaction strengths when tested with the same RBC suspended in LISS versus non-LISS diluents [234,237]. The effects of suspending kodecytes in LISS needs to be further investigated, and whilst the Kodecyte assay is performing with a high level of accuracy and precision in tube, the Kodecyte assay in CAT requires further optimisation.

4.3.6. Which assay is more correct; A_1 cell assay or Kodecyte assay?

Although the A_1 cell assay is accepted as the current gold-standard method of ABO antibody quantification for measurement in patients preparing for ABO incompatible organ transplantation [113], its inaccuracies and challenges are widely acknowledged [64,113-119,125]. Whilst most samples showed agreement between the A_1 cell assay and the Kodecyte assay in this research, there was a small proportion of discordant samples. In samples yielding discordancies between the A_1 cell and Kodecyte assays, it was impossible to know which of the two assays yielded a more accurate representation of

antibody level, or how this might affect clinical outcome. Other assays exist to quantify ABO antibodies, using methodologies including spectrophotometry [41], EIA assays [164,166] and flow cytometry [119,166,238]. It is difficult to compare assays with different reporting units (titre in the A_1 cell assay, antibody level in the Kodecyte assay, absorbance in the spectrophotometry and EIA assays, and mean fluoresence intensity in flow cytometry). Furthermore, without an independently validated standard of known antibody concentration tested by each method, it is not possible to know which of the assays should be regarded as the reference or most correct assay. Ultimately the only result that is important is the one that best correlates with clinical outcomes. There is now a need to further understand the mechanisms involved in antigen-antibody interactions. However, the methodology is complex and requires affinity purified and characterized antibodies [41] as well as defined antigens.

4.3.7 Further work : ABO antibody quantification

Although unable to resolve the impact of IgG:IgM ratios on the observed results of ABO antibody levels, data suggests that this factor, together with dilution, and type of cell used (A_1 cells or kodecytes) influence these assays to cause the observed differences in results.

Further work is now required to fully address the impact of IgG and IgM content of plasmas, and the effect of this immunoglobulin class competition [188,233] on serological tests with kodecytes. Separation of IgG and IgM forms of anti-A, anti-B and anti-A,B from group O plasma by differential adsorption and elution, and size exclusion chromatography, is planned for studies with kodecytes and natural RBC in different testing platforms. It is conceivable that flow cytometry could be added as a third method [119,166,238].

There is a also need for comparison of the haemolysin test with ABO antibody quantification [72,79]. This work has demonstrated that the Kodecyte assay can identify individuals with high levels of ABO antibodies. However, the assay as presented here was not tuned for the detection of high level ABO antibodies with potential to cause haemolysis in non-ABO matched transfusion recipients. With further tuning, the Kodecyte assay could prove useful as an alternative or adjunct to either the haemolysin assay, or antibody titration for this purpose. Furthermore, it could be applicable in

determining ABO antibody levels in ABO-incompatible pediatric heart transplantation [64-67], the monitoring of ABO-mismatched bone marrow transplants [170], production of intravenous immunoglobulin [82], detecting blood donors with high-titer ABO antibodies [69,70,77], and predicting and monitoring ABO hemolytic disease of the fetus newborn [171].

4.4 Quantification of antibodies to xeno-antigens Galα, GalNAcα and Rhaα

Although antibodies against xeno-glycan antigens terminating with the saccharides Galα, GalNAcα and Rhaα are ubiquitous amongst humans, levels do vary between individuals [35,98,99]. Concentration of antibody is important in determining biological activity in immune responses [190,191]. As Galα and Rhaα are under investigation as target antigens for immuno-oncotherapy [40,89,91-95], and patients' naturally occurring anti-Galα and anti-Rhaα form the basis of the immune response, it is important to understand population variation in relative levels of these antibodies. Current methods of quantifying antibodies to carbohydrate antigens predominantly use solid-phase assays requiring specialized laboratory equipment and procedures [33,35,38,91,96,97,99,187,188]. In this thesis, a cell-based assay using Kode™ Technology to determine relative antibody levels in 100 healthy individuals was developed.

4.4.1 Study design and rationale

The assays were based on the same principles as the ABO antibody quantification Kodecyte assay; that is undiluted plasma was tested against a panel of kodecytes prepared with different concentrations of appropriate FSL, and a level of high, medium or low antibody is assigned depending on the reactively profile with the kodecyte panels (Table 52). The number of kodecytes in the panel was reduced to three in comparison to the FSL-A-kodecyte panel of five kodecytes; to identify relative high, medium and low levels of antibody. As three kodecyte types were prepared with three different antigen specificities, and comparison of antibody levels was made between specific antibodies, it was important to use FSL at equimolar concentrations to create the kodecytes. The choice of actual FSL concentrations to prepare the three kodecytes of each specificity was informed by the work done to develop the ABO antibody quantification Kodecyte assay [177]. Had different FSL concentrations been used, the distribution of high, medium and low assigned antibody levels might have been different. Therefore levels are only

relative between specificities, and should not be used as absolute values. Nevertheless, the work was designed to be able to identify individuals with low level antibody, as these individuals are clinically important. Similar to the work on ABO antibody quantification with kodecytes, standard serological methods were used. In contrast to the solid phase assays [33,35,38,91,96,97,99,187,188], the kodecyte-based assay did not require any specialised laboratory equipment. Although only tubes were used here as a testing platform, there is no reason why these assays could not be conducted in CAT [177].

4.4.2 Assay findings

This assay identified that 5% to 8% of healthy individuals had relatively low levels of antibody (Figure 44). This work could be important for researchers in immuno-oncotherapy, in choosing antigen targets corresponding with high or medium level antibodies in the highest possible number of the population, or conversely identifying individuals with low levels who might exhibit a poor immune response [190].

4.5 Complement activation on non-cellular surfaces

The majority of work described in Chapter 2 involved complement deposition onto the surface of kodecytes; that is on the RBC membrane, thus proving that interactions between glycan FSLs and their corresponding specific antibody in fresh human serum activated complement to lyse RBC. It was of further interest to investigate whether complement could be deposited onto different surfaces, as cell-based assays may not be relevant for all researchers using FSL. Complement was successfully deposited on paper, plastic and glass (although with smearing present on glass). This opens up a possibility, not further explored in this thesis, to develop solid phase diagnostic assays to evaluate complement-binding properties of glycan antibodies.

Conclusion

This work proved that kodecytes can be lysed through activation of complement in the classical pathway; utilising the interaction of carbohydrate antigens loaded into the cell membranes as FSL with antibodies in human serum. The assays were successful with three different functional heads and two different spacer types. Extension of this work to a complement stability study re-defined storage times for serum containing active complement. In a novel assay where xeno-antigen Galα was attached to human RBC (kodecytes), and naturally occurring anti-Galα brought about complement-mediated lysis of the kodecytes, complement activity was assessed in aliquots of an undiluted serum stored under different conditions. This kodecyte approach eliminated the assay deficiencies and confounding variables associated with the CH_{50} assay of complement activity; namely serum dilution, use of variable sheep RBC expressing multiple xeno-antigens, and presence of multiple complement-binding antibodies in human serum. The kodecyte assay proved complement to be at least twice as stable during storage as previously believed. This has positive implications for laboratories with limited access to minus 80°C freezers and could extend the shelf life of complement-containing products and samples for laboratories.

Furthermore, complement was successfully deposited on solid surfaces with Kode™ Technology. This could be of interest to researchers using solid phase assays to investigate properties of natural antibodies to carbohydrate antigens.

Finally, assays to quantify antibodies to a range of carbohydrate antigens were developed with undiluted plasma and antigen dilution panels of kodecytes on standard immunohaematology testing platforms. This eliminated problems associated with traditional antibody dilution, and also standardized the target antigen-bearing cell. This new approach to antibody quantification could prove useful in a variety of clinical applications, including monitoring antibody levels in transfusion and transplantation settings, and assessing antibody level to predict immuno-oncotherapy responses in oncology treatment.

Antibody complement–mediated hemolytic studies with kodecytes reveal that human complement utilized in the classical pathway is more stable than generally accepted

Holly Perry,[1,2] Nicolai Bovin,[2,3] and Stephen Henry[2]

BACKGROUND: Complement has significant status in the field of transfusion medicine. The accepted stability profile of complement is based on historical studies of diluted human serum hemolyzing rabbit heterophile antibody–sensitized sheep red blood cells (RBCs). Contemporary tools are available to reevaluate these historical observations using human heterophile antibodies, undiluted serum, and antigen-modified human RBCs.

STUDY DESIGN AND METHODS: Human RBCs were made into "animal-like" kodecytes with heterophile Galα3Galβ4GlcNAcβ function-spacer-lipid constructs. These α-Gal-kodecytes were prepared with an antigen dilution capable of consistently producing 50% antibody-mediated hemolysis against human α1-3galactose heterophile antibodies and undiluted standardized serum. Standardized human serum aliquots from a two-donor pool stored at −85, −20, 4, 22, and 37°C for durations of up to 150 days were evaluated for loss of hemolytic activity. Where practical methodologic procedures were aligned with historical studies.

RESULTS: Comparison of the historical assay with the α-Gal-kodecyte assay against complement activity standards showed concordance. However, in most scenarios complement was found to be more than twice as stable as generally accepted. At least 60% of complement hemolytic activity was observed in serum stored at 22°C for 1 week or 2 months at 4°C. No loss of hemolytic activity was observed after 5 months' storage at temperatures below −20°C.

CONCLUSIONS: An alternative method using undiluted serum and modified human RBCs observed that classical-pathway complement hemolytic activity in stored human serum is at least twice as stable as previously accepted.

Acute intravascular hemolysis is usually caused by the actions of a family of proteins collectively known as complement, and the resultant sequelae of their activation can have fatal consequences.[1] Despite the importance of complement in immunohematology, most laboratory methods avoid seeing its hemolytic effects due to the use of plasma, where the chelating properties of anticoagulants also inhibit the complement cascade.[2] However, there are several immunohematologic assays including the detection of ABO hemolysins in blood donors,[1,3] autoimmunity studies,[4-6] transfusion and drug reaction investigations,[7] and complement deficiency,[8,9] where involvement of the human complement cascade is still measured. In vitro deposition of complement on red blood cells (RBCs) can also be used to enhance the immunological reactions of some antibodies, particularly those within the Kidd, Kell, and Lewis blood group systems.[1,10-12] Additionally, nonhuman

ABBREVIATION: FSL = function-spacer-lipid Kode Technology construct.

From the [1]School of Science, Faculty of Health and Environmental Sciences, and the; the [2]Centre for Kode Technology Innovation, School Engineering, Computer and Mathematical Sciences, Faculty of Design and Creative Technologies, Auckland University of Technology, Auckland, New Zealand; and the [3]Shemyakin Institute of Bioorganic Chemistry, Moscow, Russian Federation.

Address reprint requests to: Stephen Henry, Centre for Kode Technology Innovation, School of Engineering, Faculty of Design and Creative Technologies, Auckland University of Technology, Private Bag 92006, Auckland 1142, New Zealand; e-mail kiwi@aut.ac.nz.

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complement is still central to many assays, including human leukocyte antigen typing[13] and cytotoxicity assays.[14]

The stability or thermal lability of complement from human and other animal sera, and thus its storage conditions, has always been considered an important factor for in vitro diagnostics. Early studies that guided storage of complement were performed on serum from guinea pigs, rabbits, pigs, and goats.[15-19] As early as 1910 scientists showed complement-mediated hemolysis of RBCs with animal serum stored frozen for up to 3 months.[15,16] The first studies of human complement stability were undertaken in the early part of the 20th century[20,21] and these studies showed variable results for human sera stored at refrigerator and room temperatures. Recent studies[2,22,23] have shown human complement in serum to be stable for several years at temperatures below −70°C, but there have been no studies on the storage loss of hemolytic activity at other temperatures since 1970.[10,12] The 1961 study of Polley and Mollison[10] showed undiluted serum stored at −20°C for 2 months still induced classical-pathway complement-mediated hemolysis. Garratty's 1970 study[12] quantified, complement activity and found that serum stored at −20°C started to lose hemolytic activity at 1 to 2 weeks of storage, with levels of complement down to 60% at 2 months, and was stable up to 3 months at −90°C. In 1969, Delaney and Garratty[24] recommended serum used as a source of complement be stored no longer than 2 weeks at −20°C to retain full activity. The current accepted position is that serum may be stored for up to 2 months at −20°C for immunohematology tests.[1]

In nonimmunohematology serology the stability of complement appears also to be poorly understood,[22,25] with Lachmann stating that it "is, perhaps, surprising that there is relatively little literature on the topic of how serum should be prepared in order to perform functional complement assays . . . it is apparent that good practice in this area is not always followed, even by complementologists, let alone by companies developing diagnostic tests."[25] The topic of complement stability was recently partially revisited by Yang and colleagues,[22] who found complement to be stable at very low storage temperatures (−80°C), but did not assess higher temperature ranges. Others have examined the storage of complement in serum and find evidence of complement activation (deterioration) during storage, but did not undertake any functional (lytic) assays.[23]

Garratty's benchmark study[12] and the earlier studies of Polley and Mollison[10] (Table 1) both tested diluted human serum for loss of hemolytic activity in the classical complement pathway against sheep RBCs sensitized with nonhuman heterophile antibodies. Our study also used heterophile antibody-sensitized indicator cells, although we used human heterophile antibodies directed against human RBCs made "animal-like"

TABLE 1. Comparison of method between studies of Polley and Mollison[10] (1961), Garratty[12] (1970), and this study

	Test serum samples			Hemolysis assay							
		Storage		Anti-heterophile–sensitized indicator RBC (iRBC)				Serum		Measurement	
Authors	Source*	Range (°C)	Time (days)	Heterophile antibody	iRBC type	Sensitization†	iRBC conc	Dilution	Ratio iRBC	iRBC lysis	Method
Polley and Mollison (1961)[10]	1	−50 to +37	1 to 60	Bovine anti-sheep RBC membrane (anti-Forssman)§	Sheep RBCs	Two-stage	Unknown	Undiluted, 1:4, 1:64	Unknown	>10%‡	Visual comparison
Garratty (1970)[12]	12	−90 to +37	1 to 90	"Amboceptor" anti-sheep RBC membrane (anti-Forssman)§	Sheep RBCs	Two-stage	2.5%	Not stated (1:16–1:32)¶	1:1	50%	Spectrophotometry (results averaged)‖
This paper	2 (pool)	−85 to +37	1 to 150	Undiluted human serum anti-α1-3 galactose	Human 100- αGal - kodecytes	One-stage	5%	Undiluted	2:1	50%	Spectrophotometry

*Source relates to the number of individual samples tested or used in a serum pool (pool).
†Heterophile antibody sensitization of the indicator RBCs occurred either before the hemolysis assay (two-stage) or during (one-stage).
‡This value was not reported, but our observations indicate that 10% was probably the minimum that would have been visually clearly observable.
§The exact identity of this reagent was not reported but was inferred from references cited by the authors.
¶This value was not reported, but our observations and published data indicate that this was probably in the range of 1:16 to 1:32.
‖The results obtained from all 12 individual samples were averaged to give the result.

with heterophile Galα3Galβ4GlcNAcβ function-spacer-lipid (FSL) constructs (αGal-kodecytes)[26,27] (Table 1). Glycans with terminal Galα3Galβ4GlcNAcβ are found in non-primate mammals, and the corresponding complement-activating antibody is naturally occurring in humans.[28,29]

As antigen levels on kodecytes are controllable (i.e., antigen dilutions), kodecytes can be created to give reproducible and specific serologic reactions against undiluted reagents or serum.[26,27,30] In addition to reevaluating the storage stability of complement in the classical complement pathway, we also evaluated the sensitivity of the heterophile antibody-sensitized sheep RBC hemolysis assay and the αGal-kodecyte hemolysis assay against complement activity standards.

MATERIALS AND METHODS

αGal-kodecytes

FSL construct FSL-Galα3Galβ4GlcNAcβ (Galα1-3Galβ1-4GlcNAcβ1-adipate-1,2-di-O-oleoyl-*sn*-glycero-3-phosphoethanolamine)[26] was obtained from Kode Biotech Materials. Kodecytes were prepared as described previously[27] over the range of 10 to 750 μg/mL FSL, with 100 μg/mL selected as the FSL dilution to prepare αGal-kodecytes that would give 50% hemolysis against the standardized serum. Preparation of kodecytes involved incubating 1 vol of FSL-Galα3Galβ4GlcNAcβ with 1 vol of group O RBCs for 2 hours at 37°C.[27] After incubation, the resultant αGal-kodecytes were washed with phosphate-buffered saline (PBS) and stored as a 5% cell suspension in cell preservative solution overnight at 4°C. Terminology usage for describing kodecytes (i.e., kodecytes prepared with 100 μg/mL FSL-Galα3Galβ4GlcNAcβ are termed 100-αGal-kodecytes) and FSL constructs are as reported elsewhere.[26]

Standardized serum

Pooled serum from two group AB blood donors was obtained from the New Zealand Blood Service as the source of anti-α1-3 galactose and fresh complement. Whole blood was collected into dry donation bags (no anticoagulant) and allowed to clot for 2 hours at 37°C. Serum was separated, pooled, and frozen at −30°C within 6 hours of collection. Thawed serum was then divided into individual 1-mL aliquots for each storage time (0, 1, 2, 3, 7, 14, 21, 28, 60, 90, 120, and 150 days) and temperature (−85, −20, 4, 22, and 37°C).

Aliquoted samples maintained at −85°C and thawed for immediate use were considered the same as fresh (Day 0), and the placement of samples from various time points and storage temperatures into −85°C was considered as effectively stopping storage deterioration of that sample.[22] Therefore, a sample indicated as "−20°C storage for 7 days" has actually been stored at −20°C for 7 days and then −85°C until analysis in parallel with all other samples about 150 days later.

To ensure that the process of freezing and tha wing did not impact on the analyses, standardized serum aliquots were subjected to up to 7 weekly −85°C freeze-22°C thaw cycles. The index sample remained at −85°C while at weekly intervals decreasing numbers of samples were thawed (30 min) and refrozen so that by the end of the period there was a sample representing each point for between zero to seven freeze-thaw cycles. Analysis of these samples in parallel revealed no differences (results not shown), indicating that there was no detectable impact on freeze-thawing relevant to the assays reported in this article.

Complement activity standards

Mixtures of heat-inactivated serum (56°C for 30 min[15,31]) were mixed with fresh serum from the same source to make serum standards containing 0, 20, 40, 60, and 80% of normal complement activity.

Hemolysis standards

A standard curve accurately representing percentage increments of hemolysis in serum was created for the validation of assay results. Five hundred microliters of a 5% suspension of 750-αGal-kodecytes was split between pairs of tubes (lysis and nonlysis tubes). In the lysis tubes was added an increasing volume (50-μL increments) of kodecytes from 50 to 500 μL, while in the paired nonlysis tube was added the decreasing volume of kodecytes that would bring the combined volume of each pair to 500 μL. Into the lysis tube was added two parts of fresh standardized serum and into the nonlysis tube was added two parts of 0.5% EDTA-treated standardized serum (with the combined serum volume between the matching pairs being 1.0 mL). Tubes were then incubated for 1 hour at 37°C to allow the lysis set to 100% hemolyze. The matching lysis and nonlysis pairs were then combined, to give a range of samples with 10% increments of hemolysis from 10% to 100% with identical serum concentrations. After being mixed, the samples were centrifuged and the supernatant was sampled for spectrophotometeric analysis. This standard curve was used to validate the 50% lysis reaction (equivalent to 100% of complement activity) for each stored serum hemolysis assay, with variation of less than 10% observed.

Complement stability analysis

Samples from each time and temperature point were recovered from storage at −85°C, thawed at room temperature, and tested within 1 hour on the same day with the same kodecytes. For the stability studies, 200-μL duplicates of each serum sample were incubated with 100 μL of a 5% 100-αGal-kodecyte suspension that had been washed

free of cell preservative solution and suspended in PBS. Hemolysis reactions were allowed to progress for 30 minutes at 37°C and were then stopped with the addition of 1 µL of 120 mmol/L EDTA. Tubes were centrifuged to sediment unlysed kodecytes, and the supernatant was sampled and measured spectrophotometrically. Gram staining[32] of all long-term samples including the 37°C sample confirmed they were not microbially contaminated.

Heterophile anti-sheep–sensitized RBCs

The 1970 method reported by Garratty[12] was reproduced by sensitizing fresh sheep (ovine) RBCs (South Pacific Sera Ltd) with heterophile antibodies[33] (anti-sheep RBC stroma antibody produced in rabbit, Cat. S1389, Sigma-Aldrich).

Spectrophometric measurement of hemoglobin

Hemoglobin in supernatants of the hemolysis assays was measured with the cyanmethemoglobin technique.[4] A total of 160 µL of cell-free supernatant was diluted in 1 mL of Drabkin solution and allowed to rest for 30 minutes, and absorbance was measured at 540 nm against a serum sample blank. The sample blank consisted of cell-free supernatant of a negative (no lysis) control tested in parallel.

RESULTS

αGal-kodecyte hemolysis

αGal-kodecytes made with human RBCs and dilutions of FSL-Galα3Galβ4GlcNAcβ were created with a range in degree of hemolysis from 100% (with 750-αGal-kodecytes) through to 20% (40-αGal-kodecytes) against the standardized serum (data not shown). It was established that the 100-αGal-kodecytes would reproducibly maximally lyse 50% against the standardized serum in the hemolytic assay, as reported to be required for optimal recognition in loss of complement activity against fresh serum.[9,33,34] For clarity, maximal 50% lysis in the hemolysis assay is equivalent to the sample tested having 100 ± 10% of hemolytic activity. The level of complement hemolytic activity reported for each sample was then expressed as a percentage of the Day 0 fresh sample reaction, as per the methodology of Garratty.[12] The 100-αGal-kodecyte assay was tuned to sensitivity for the standardized serum sample and use of other serum samples with higher or lower levels of α1-3 galactose antibody than in the standardized serum sample may result in a shift in the hemolysis curve (results not shown).

To ensure that the results obtained were due to deterioration of complement, rather than anti-α1-3 galactose deterioration in the standardized serum, anti-α1-3 galactose activity in selected stored samples (0, 28, 90, and 150 days at −85, −20, 4, 22, and 37°C) was tested and found unchanged with respect to antibody titer (IgM and IgG) as

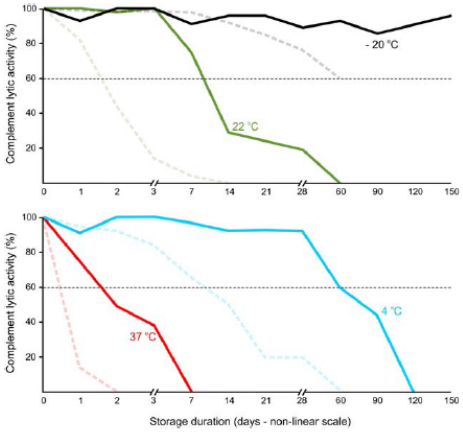

Fig. 1. Storage time and thermal stability of complement hemolytic activity in serum. Solid lines show results obtained from this study, while dashed lines show the historical results of Garratty[12] replotted onto this graph. (Top) Complement lytic activity in serum stored at −20°C (black lines), while green lines represent stability at 22°C. (Bottom) Results for 37 and 4°C storage.

determined in direct and indirect agglutination assays (results not shown). Note that a loss or reduction in anti-α1-3 galactose activity would decrease the apparent stability of complement activity. Additionally no increase (as a consequence of storage enhancement of antibody and/or complement activity) in hemolytic assay optical densities above maximal 50% was observed.

Results from the 100-αGal-kodecytes are summarized in Fig. 1 (solid lines) and plotted alongside results extracted from the original report by Garratty[12] (dashed lines). The level of 60% activity was adopted as the endpoint for storage, as complement levels below this value are considered inadequate for immunohematologic purposes in detection of blood group antibodies by either lysis or with anti-complement immunoglobulins.[1,12] After 37°C storage (dashed red lines) for less than a day, serum was originally reported to have less than 60% activity while kodecytes (solid red lines) show this level of activity at about 2 days. At 22°C (dashed green lines) serum was originally reported to have reduced to 60% activity at 2 days while kodecytes (solid green lines) still show this level of activity at about 8 to 10 days. At 4°C (dashed blue lines) serum was originally reported to have 60% activity at approximately 8 to 10 days, which is substantially less than the 60 days seen with kodecytes (solid blues lines). The possibility that results observed for the 4 to 37°C stored samples were in part due to bacterial contamination was excluded on the basis of negative Gram stain results. The −20°C frozen sample did

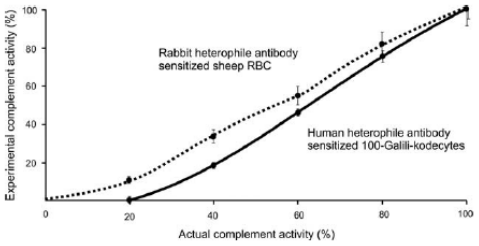

Fig. 2. Comparison of the rabbit heterophile antibody–sensitized sheep RBC hemolysis assay against the human heterophile antibody–sensitized 100-αGal-kodecyte hemolysis assay. The dotted black line represents the heterophile sheep assay using 1:32 diluted serum based on the method reported by Garratty[12] while the solid black line represents the 100-αGal-kodecyte assay against undiluted serum. Actual complement activity (x-axis) represents human serum samples artificially created by mixing complement-inactivated (heat-treated) serum with fresh serum to create complement activity standards with decreasing levels of active complement. Experimental complement activity (y-axis) was that determined experimentally for each assay and expressed as a percentage of the maximal hemolysis reaction obtained for the fresh (100%) complement sample.

not show any apparent deterioration in its hemolytic capacity against kodecytes after 150 days storage (black solid line) in contrast to the historical data, which show only 60% activity present after 60 days (gray dashed lines).

Heterophile anti-sheep RBC assay

The sensitivity of sheep RBCs sensitized with rabbit heterophile antibodies based on the method reported in Garratty[12] was evaluated against standardized complement activity standards (Fig. 2). Unfortunately the samples from the stability trial were no longer available for comparative testing. Assay results (experimental) with the serum dilution 1:32 (where 100% complement activity produced 50% hemolysis) was sensitive to complement deterioration (actual) and very similar to the results obtained with undiluted serum in the 100-αGal-kodecyte assay ($r^2 = 0.98$). The kodecyte assay tended to underestimate actual activity and was unable to differentiate 20% activity from no activity due to the background from using undiluted serum. Overall both assays were similarly sensitive to recognizing early deterioration of complement activity.

DISCUSSION

The complement cascade has a significant status in the field of transfusion medicine. Not only is it directly responsible for acute intravascular hemolysis, it can also leave signatures on the RBC surface diagnostic of immune events such as autoimmune disease and transfusion- and drug-related reactions.[6,11]

Historical studies[10,12] for the detection of blood group antibody–mediated hemolysis[35] established storage guidelines for human serum retaining more than 60% complement hemolytic activity.[12] These methods[10,12] used rabbit heterophile antibody–sensitized sheep RBCs as indicator cells for testing against dilutions of serum and the variables they established are still generally accepted today.[1]

With Kode technology, which allows for "antigen dilutions" in cells[27] rather than the need for serum dilution, we revisited the classical-pathway stability of human complement in a two-donor serum pool by a different but related approach. Where practical our experimental design was aligned with historical analyses[12,33,35] and the 50% hemolysis point was retained because it is reported to have good sensitivity to deterioration of complement hemolytic activity and allows method linearity.[8,9,33-36]

Similar to the earlier studies[10,12,33,35] we also used heterophile antibody–coated RBCs on the indicator cell; however, our approach to creating these indicator RBCs was significantly different (Table 1). Rather than using a sheep RBC as the source of heterophile antigen we modified a human RBC with heterophile antigens[28] (e.g., 100-αGal-kodecytes). Our target heterophile antigen was also different, with natural Forssman antigen (GalNAcα3GalNAcβ3Galα4Galβ-R), being the primary target on the sheep RBCs[37] and synthetic Galα3Galβ4GlcNAcβ antigen being the only target on the kodecytes. Although the heterophile antigen targets between the different assays were different there is no reason to expect that these different specificities would result in assay variance. Potentially any heterophile antigen to which complement activating antibodies are present in human serum[38] including virally related peptides[39] could be used.

Unlike the historical methods that can test different serum samples the 100-αGal-kodecyte assay was specifically tuned to produce 50% hemolysis against the standardized serum sample. If a different serum sample was used then the kodecyte assay might need to be tuned to that sample, as variance in level and ratio of IgM and IgG anti-α1-3 galactose, and genetic variations in complement activity[40] in different samples could affect the degree of hemolysis obtained. The results from our assay were the average of duplicates of a two-serum pool, while those of Garratty[12] were the average of 12 individuals (individual results not shown), with the author acknowledging variance between individuals. As our assay was tuned specificially to the levels of antibody and complement in the two-serum pool, there is no relevance to variations between individuals.

Thus in summary our assay was based on artifically attaching a low-level heterophile Galα3Galβ4GlcNAcβ antigen onto human RBCs and reacting them with human anti-α1-3 galactose and complement present in undiluted serum. The 50% hemolysis point was optimized by

limiting the amount of antibodies bound (by limiting the number of antigens) to the indicator cell. In contrast the historical method[10,12,33-35] was based on using high-level heterophile antigens on sheep RBCs presensitized with nonhuman heterophile antibodies and testing these against human serum dilutions. Their 50% hemolysis point was optimized by limiting (by serum dilution) the amount of complement in the serum that would react with an excess of antibody already bound to the indicator cells. Despite these significantly different approaches to method, comparison of sensitivity for the different assays to detect complement deterioration was suprisingly concordant (Fig. 2). However, the results obtained in the two different stability assays are discordant (Fig. 1).

In concordance with historical reports we were able to show that complement hemolytic activity decreases during storage and that the rate of loss in activity is accelerated at higher temperatures.[10,12,17] It is not known if the loss of hemolytic activity observed was due to activation-consumption of complement factors[23] and/or simply due to the deterioration of selected proteins.

In contrast with the reports of Garratty[12] and less so with others,[10] the rate of loss of complement hemolytic activity in our samples was much less than previously reported. Results show that at least 60% complement hemolytic activity is still available in serum stored at 22°C for 1 week, 4°C for 2 months, and at least 5 months at −20°C and below. Likewise other authors have shown that complement activity is well preserved at very low temperatures (e.g., −80°C).[22,23]

It is not clear why such major differences between the two different studies should exist, particularly as they appear to have relatively identical sensitivity curves for complement deterioration (Fig. 2). If either assay was less sensitive then it would be expected to result in longer stability times for the samples, as detecting complement deterioration is measured by a loss in activity. The historical results of Garratty[12] show significantly decreased stability compared with the kodecytes, and there is no evidence for a loss in sensitivity of the kodecyte assay. Therefore, the marked differences observed must be due to other factors. The most likely candidates are batch-to-batch variations in reagents and serum dilution. It was recognized by the historical studies[10,12,33-35] that batch-to-batch variation of sheep RBCs and/or anti-sheep heterophile reagents both cause significant variance in this assay. There is also no evidence that dilution of the serum is responsible for a loss in sensitivity of the assay (Fig. 2), at least against fresh serum. However, it was not established (due to long-term storage samples not being available) if serum undergoes some change during storage that will affect the hemolysis assay only when serum dilutions are used, for example, if critical components such as cofactor metal ions become less available. Although this remains to be resolved, it is of note that Polley and Mollison[10] show significant variance between dilutions of the same sample and it is well recognized that the dilution of serum is fraught with inherent error.[31,34,41]

In summary, although more than 60% of hemolytic activity was still present longer than previously accepted in stored samples, it is pertinent that serum samples are constantly undergoing substantial changes during storage.[23] As a consequence we would still recommend that for best preservation of complement hemolytic activity human serum samples be stored at temperatures of −80°C or lower.

CONFLICT OF INTEREST

SH and NB are employees and stockholders in Kode Biotech, the patent owner of Kode biosurface engineering technology. HP has disclosed no conflicts of interest.

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A standardized kodecyte method to quantify ABO antibodies in undiluted plasma of patients before ABO-incompatible kidney transplantation

Holly Perry,[1,2] *Nicolai Bovin,*[2,3] *and Stephen Henry*[2]

BACKGROUND: The ABO transplantation barrier can be breached if antibody is reduced to low levels. Current serologic methods involve testing natural RBCs against dilutions of plasma to determine antibody levels, but these methods are poorly standardized and inherently error prone with consequent large inter- and intra laboratory variation. We evaluated the feasibility of using antigen-standardized kodecytes and undiluted plasma as an alternative method for antibody measurement in patients preparing for ABO-incompatible kidney transplantation.

STUDY DESIGN AND METHODS: A panel of five kodecytes, bearing defined levels of synthetic blood group A type 2 antigen was developed (kodecyte assay) to show reaction patterns against undiluted plasma that were indicative of anti-A and anti-A,B levels. This panel was evaluated against the contemporary method of testing dilutions of plasma against A_1 cells to determine titer (A_1 cell assay) in both column agglutination and tube techniques. Evaluation samples included reference standards, 102 group O plus 23 group B donors, and 40 pre- and post-plasmapheresis samples from five prospective ABO-incompatible kidney transplant patients.

RESULTS: Comparisons between the kodecyte and A_1 cell assays found greater than 90% correlation for all samples. Tube and column agglutination technology platform differences were observed with A_1 cells and kodecytes. Discordant samples were generally found to have high ratios of IgG:IgM or vice versa.

CONCLUSIONS: The kodecyte assay is a simple method that requires no sample dilution, and an optimized two-cell kodecyte panel is potentially capable of informing ABO-incompatible kidney transplantation decisions based on antibody levels.

Historically, it was not considered advisable to transplant across the ABO blood group barrier because organs such as kidneys have ABO antigens.[1] As ABO antibodies can activate the complement cascade,[2] they have the potential to cause hyperacute organ rejection and graft loss in ABO-incompatible kidney transplantation (ABOiKT).[3,4] However, due to a shortage of kidneys for transplantation, particularly for group O recipients, crossing the ABO barrier has been actively pursued. ABOiKT is practiced in many countries,[5–7] with living donor-recipient pairs representing all ABO-incompatible donor combinations.[5] To achieve this successfully, it is necessary to reduce the level of ABO antibodies in the recipient, using strategies including nonselective plasmapheresis (PP),[5,8,9] or specific antibody reduction with adsorption columns,[5,10–13] and/or B-cell depletion with specific monoclonal antibodies such as rituximab.[5,6,8,14] If the ABO-mismatched transplanted organ is not rejected within a few weeks, it will become ABO-accepted through a fortuitous phenomenon known as accomodation.[15,16]

Although titration is universally used as the current standard method for measurement of antibody,[17] it is widely

ABBREVIATIONS: ABOiKT = ABO incompatible kidney transplantation; AHG = antihuman globulin; CAT = column agglutination technology; PP = plasmapheresis.

From the [1]School of Science, Faculty of Health and Environmental Sciences; [2]Centre for Kode Technology Innovation, School of Engineering, Computer and Mathematical Sciences, Faculty of Design and Creative Technologies, Auckland University of Technology, Auckland, New Zealand; and [3]Shemyakin Institute of Bioorganic Chemistry, Moscow, Russian Federation.

Address reprint requests to: Stephen Henry, Centre for Kode Technology Innovation, School of Engineering, Faculty of Design and Creative Technologies, Auckland University of Technology, Private Bag 92006, Auckland 1142, New Zealand; e-mail: kiwi@aut.ac.nz

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acknowledged to be prone to inter- and intralaboratory variation.[18–22] This large range in variation potentially restrains and jeopardizes the value in measurement of antibody titer for predicting ABOiKT eligibility and safety.[22–25]

Kodecytes are cells modified with function-spacer-lipid constructs to express exact and known quantities of chemically synthesized small molecules on their surface.[26–29] From studies with monoclonal ABO antibodies it is well established that there is an inverse correlation between the level of antibody and the ability to detect antigen.[19,30] That is, higher concentrations of antibody are needed to detect decreasing concentrations of antigen, and vice versa. Based on these observations, it was considered possible that measurement of ABO antibody levels could be achieved by testing a single unknown antibody concentration (undiluted plasma) against a range of kodecytes prepared with precise antigen concentrations.

The aim of this study was to evaluate the potential of an "antigen dilution" approach to quantifying ABO antibodies, and to compare this to the traditional method of plasma dilution and testing against A_1 cells, with a specific focus on application in ABOiKT.

MATERIALS AND METHODS

Terminology, grading, and IgG:IgM ratios

Terminology used to describe Kode Technology is as reported elsewhere.[31] All titers are expressed as a reciprocal of the dilution giving the endpoint reaction. The assay using A_1 cells and dilutions of plasma is referred to as the A_1 cell assay, while the kodecyte assay using a kodecyte panel and undiluted plasma is referred to as the kodecyte assay. Kodecyte assay results are reported as low, medium, and high grades (L/M/H grades; Table 1 and Table S1, available as supporting information in the online version of this paper). Tube refers to serology in glass test tubes, while column agglutination technology (CAT) refers to serology in the Ortho BioVue column agglutination technology system (Ortho-Clinical Diagnostics). Immunoglobulin isotype ratios are reported in the format of IgG:IgM titers ratios (e.g., 8:1). It should be noted these ratios are not truly quantitative comparisons but are instead indicative/surrogate ratios derived from antiglobulin titers of dithiothreitol-treated plasma (IgG content)[32] versus saline agglutination titers of untreated plasma (IgM content), with the caveat that IgG can also contribute to direct ABO agglutination.[33]

A type 2 kodecytes

Dilutions of function-spacer-lipid construct A Type 2 (GALNa3[Fa2]GALb4GLCNb)-SC2-L1, Kode Biotech Materials) in RBC preservative solution (Celpresol, Immulab) were used to prepare kodecytes.[31,34] Stability was assessed by measuring mean cell volume and mean cell hemoglobin concentration at 1 and 21 days and grades of agglutination at weekly intervals, with no changes observed (results not shown).

Natural A cells

To ensure RBC antigen standardization for this study, aliquots of fresh RBCs stored at 4°C in RBC preservative for a maximum of 2 weeks, from a single known A_1 phenotype (confirmed by genotype) individual were used in titration methods.

Polyclonal control reagents

A historical human polyclonal blood grouping anti-A,B reagent (Lot 004, Copper Biomedical; expired 1986) was used as a source of high-level IgG and IgM antibodies (sample X, A_1 cell antiglobulin tube titer 2048 and IgG:IgM ratio 2:1). Individually prepared dilutions of this reagent were prepared to provide samples of defined high-, medium-, and low-level antibody for the purpose of method development and validation. Additionally, one group O fresh plasma sample was used as a control (sample Y, A_1 cell antiglobulin tube titer 1024 and IgG:IgM ratio 16:1). The World Health Organization reference reagent 14/300 (National Institute for Biological Standards and Control)[19] was used to validate the 1024 titer of control sample Y used in all titration assays.

Donor and ABOiKT patient samples

Plasma samples from 102 group O and 23 group B blood donors were provided by the New Zealand Blood Service. Samples for research use were taken immediately before and immediately after PP from four group O patients and one group B patient presenting for ABOiKT (ethics approval 16/175). All samples were maintained at –85°C and tested on the day of thawing. All donor and patient samples were nonreactive against the O cell used to make the kodecyte panels.

Titrations and serology

Antiglobulin 37°C titers were determined for all samples, and saline 22°C direct agglutination titers were performed on 48 donor samples. Master serial twofold dilutions of samples from 2 to 2048 were prepared in phosphate-buffered saline on the day of testing in 400µL volumes. Undiluted samples, dilutions, and reagent RBCs were dispensed into test tubes according to the American Association of Blood Banks method[17] (tube) or into CAT cassettes according to the

TABLE 1. Assignment of kodecyte assay grades

			Kodecyte reactions†				
LMH grade		Tube titer*	100	20	10	2.5	1.0
High	H	≥256	+	+	+	+	+
Medium High	MH	64–256	+	+	+	+	0
Medium	M	32–128	+	+	+	0	0
Low Medium	LM	8–32	+	+	0	0	0
Low	L	<16	+	0	0	0	0

* Tube titer: reciprocal of the dilution representing the titration endpoint reaction as determined in tube by the A_1 cell assay.
† Numbers 100, 20, 10, 2.5, 1.0 represent µmol/L concentrations of function-spacer-lipid-A Type 2 used to make the kodecytes with reactivity patterns shown as positive (+) or negative (0).

manufacturer's method (CAT). Variance within the A_1 cell and kodecyte assays was established as acceptable (Table S2, available as supporting information in the online version of this paper). Samples with discordant results between A_1 cell and kodecyte assays were further analyzed after IgM degradation treatment with 5 mmol/L of dithiothreitol (Sigma Aldrich).[32]

RESULTS

Donor sample comparison of A_1 cell assay with kodecyte assay

Saline reactivity

Saline results were determined in 48 group O donor samples (Table S3, available as supporting information in the online version of this paper). Overall, the kodecyte assay reported higher direct agglutinating antibody levels than did the A_1 cell assay. As the antiglobulin method also detects direct agglutination reactions, this additional method was discontinued.

Group O plasma ABO antibodies

Overall correlations of the data from the 102 group O plasma samples found that antihuman globulin (AHG) titer distributions by the A_1 cell assay in the tube and CAT (anti-IgG and anti-C3d cassettes, Ortho Clinical Diagnostics) techniques were similar (Fig. 1A). Results show that 98 of 102 sample (96%) by tube and 99 of 102 (97%) by CAT had titers greater than 16 against A_1 cells. Similarly, results show that 59 of 102 sample (58%) had titers greater than 128 against A_1 cells by tube, while 48 of the same 102 samples (47%) had titers greater than 128 against A_1 cells by CAT (Tables 2 and 3). A comparison of the variance of CAT results compared with tube for group O samples against A_1 cells showed that 88% (90/102) of results were within ±1 dilution (Fig. 1B). The kodecyte assay in CAT tended on average to show higher levels of antibody than the kodecyte assay in tubes (74 cf 46 H grade) (Figs. 1C and D).

Blood group O A_1 cell assay versus kodecyte assay

Individual sample results obtained in the A_1 cell assay of 102 group O samples were compared with the results obtained by testing the same samples in the kodecyte assay by the tube technique. As can be seen in Table 2, almost all samples (98 of 102) had A_1 cell assay titers greater than 16 and kodecyte assay grades of medium or greater (100 of 102). The majority of samples (83 of 102, 81% concordance) fell within Zone 1 (±1 dilution factor) and 98 of 102 (96% concordance) fell within Zone 2 (±2 dilution factors). Sufficient sample was available to analyze three of the four tube discordant samples (identified in all tables with lowercase letters enclosed in brackets) for their relative IgG and IgM activity ratios (Table 4). Of note was that three samples (b, c, and d) were in the same zone in Table 2 and showed lower kodecyte assay grades than predicted by the A_1 cell assay, and they all had high anti-A ratios of IgG to IgM (Table 4). Results for these three samples were concordant by CAT (Table 3).

The same samples were tested by CAT AHG (Table 3). Again almost all samples (99 of 102) had A_1 cell assay titers greater than 16 and kodecyte assay grades medium or higher. The two samples that gave kodecyte assay L grades

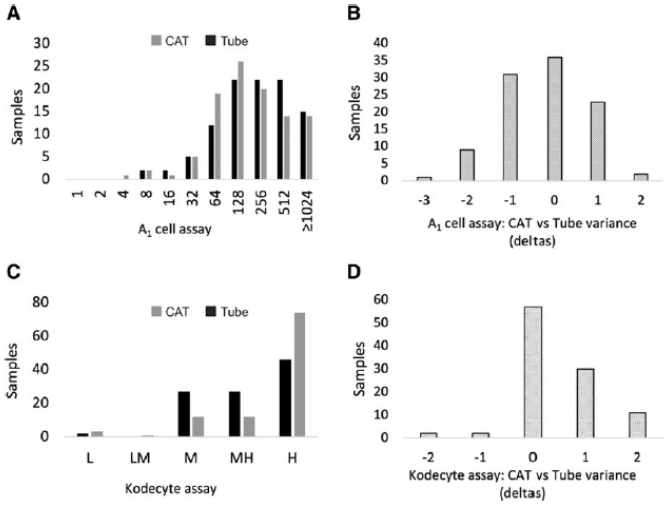

Fig. 1. Group O samples (n = 102). (A) Overall titer distribution against A_1 cells by platform. (B) Variance (deltas) of the A_1 cell assay titers in AHG CAT compared to antiglobulin tube. (C) Overall grade distribution of the kodecyte assay in CAT and tube. (D) Variance (deltas) of kodecyte assay grades in CAT compared to tube.

TABLE 2. Blood group O plasma antiglobulin tube reactivity, kodecyte assay versus A_1 cell assay

	Reactivity against 100, 20, 10, 2.5, 1.0 kodecytes (n = 102)				
A_1 cell	+0000	++000	+++00	++++0	+++++
Tube titer	L	LM	M	MH	H
4	0	0	0	0	0
8	2	0	0	0	0
16	0	0	1	1 (a)*	0
32	0	0	4 (e)	1	0
64	0	0	8 (f)	4	0
128	0	0	7 (i, l)	10	5 (k)
256	0	0	5	7	10 (g, h, j, m)
512	0	0	1 (b)	3	18
≥1024	0	0	1 (c)	1 (d)	13

* All samples (both concordant and discordant) marked with a lowercase alphanumeric character in brackets (a-m) were subjected to further serological analysis (Table 4). Criteria for zones of discordancy are as per Table S1 (supporting information in the online version of this paper) with the Zone 3 discordant results highlighted in pink.

by tube (Table 2) were the same samples giving L grades by CAT (Table 3). Most samples (73 of 102; 72% concordance) fell within Zone 1 (±1 dilution factor), and the majority (93 of 102; 91% concordance) fell within Zone 2 (±2 dilution factors). In contrast to tube, this time all nine discordant samples (e, f, g-m) were adjacent to each other in Table 3 and showed higher kodecyte assay reactivity than predicted by the A_1 cell assay. Sufficient sample was available to analyze six of these nine discordant samples (b, e, h-k) for their relative IgG:IgM ratio (Table 4). Note that for group O samples, antibody activity against A_1 cells is from both anti-A and anti-A,B (anti-A/A,B), while activity against B cells is from both anti-B and anti-A,B (anti-B/A,B). In contrast to the tube discordances, the CAT sample discordances predominantly had low IgG:IgM anti-A/A,B ratios suggesting that a high IgG:IgM ratio favors stronger results by tube (Table 4). However, although there was no clear pattern, it was notable that samples j and e had high anti-A/A,B IgM ratios (IgG:IgM ratio 1:16), sample i had a high anti-B/A,B IgG ratio (IgG:IgM ratio 16:1), and sample k was contrasting with a high anti-B/A,B IgM ratio (IgG:IgM ratio 1:16). Not supporting this concept were the three discordant samples (g, h, f), which did not show any obvious IgG:IgM ratio differences, and two of nine concordant samples (samples v, vi) had high IgG:IgM ratios (Table S4, available as supporting information in the online version of this paper). Insufficient serum was available to investigate the single discordant sample (sample a) which had a low A_1 cell assay titer yet a relatively high MH kodecyte assay grade (Table 2). To eliminate the possibility that the discordances were due to the effects of low-affinity antibodies, titration patterns and reaction scores were examined. Other than the single sample (c), which was discordant only by tube (Table 2), no other samples showed a pattern suggestive of low-affinity antibody reactions, and no differences were observed between concordant and discordant results (results not shown).

Group B plasma antibodies

Overall correlations of the data from the 23 group B plasma samples found that antiglobulin titer distributions by the A_1 cell assay in the tube and CAT techniques were different (Fig. 2A) and also substantially different from group O samples (Fig. 1A). Results show that 18 of 23 samples (78%) had titers greater than 16 against A_1 cells by tube, while 10 of

TABLE 3. Blood group O plasma CAT AHG reactivity, kodecyte assay versus A_1 cell assay

	Reactivity against 100, 20, 10, 2.5, 1.0 kodecytes (n = 102)*				
A_1 cell	+0000	++000	+++00	++++0	+++++
CAT titer	L	LM	M	MH	H
4	1	0	0	0	0
8	1	0	0	0	0
16	0	0	0	1 (e)	0
32	0	0	5	1 (a)	1 (f)
64	0	1	4	6 (b)	7 (g-m)
128	0	0	3	5	18
256	0	0	0	0	20
512	0	0	0	0	14 (c, d)
≥1024	0	0	0	0	14

* Table layout is according to footnotes in Table 2.
AHG = antihuman globulin; CAT = column agglutination technology.

TABLE 4. Further analysis of samples showing discordancy in assays

		Original results*				IgG/IgM titers and ratios†			
		Tube		CAT		A_1 cells		B cells	
ID	ABO	A_1	Kode	A_1	Kode	IgG/IgM	G:M	IgG/IgM	G:M
c	O	1024	M	512	H	256:32	8:1	16:16	1:1
d	O	1024	MH	512	H	256:8	32:1	16:16	1:1
b	O	512	M	64	MH	128:4	32:1	8:32	1:4
j	O	256	H	64	H	4:64	1:16	32:128	1:4
e	O	32	M	16	MH	2:32	1:16	16:16	1:1
i	O	128	M	64	H	32:128	1:4	64:4	16:1
k	O	128	H	64	H	32:128	1:4	1:16	1:16
h	O	256	H	64	H	64:32	1:2	64:64	1:1
f	O	64	M	32	H	8:16	2:1	32:16	2:1
p	B	128	LM	128	M	4:128	1:32		
n	B	64	M	8	LM	2:16	1:8		

* Original results as reported in Tables 2, 3, S5 & S6 with discordant results indicated by shading (with A_1 cell assay and kodecyte assay headers abbreviated to A_1 and Kode, respectively). Sample data has been sorted first by blood group, then by tube-CAT original result patterns, then by IgG/IgM ratios. Where no result is reported there was insufficient sample for testing.
† IgG titers are post DTT treatment, IgM titer is direct agglutination and G:M is the ratio of IgG:IgM with high ratios indicated by shading.
CAT = column agglutination technology.

the same 23 samples (43%) had titers greater than 16 against A_1 cells by CAT. Similarly, results show that 3 of 23 samples (13%) had titers greater than 128 against A_1 cells by tube, while 2 of the same 23 samples (9%) had titers greater than 128 against A_1 cells by CAT. A comparison of the variance of CAT results compared with tube for group B samples against A_1 cells showed that 78% (18 of 23) of results were within ±1 dilution (Fig. 2B), although there was a trend toward the CAT scoring lower than tube. However, the kodecyte assay in CAT tended to marginally show higher levels of antibody than the kodecyte assay in tube (Fig. 2C and D), with four samples grading as L by tube and only two of these samples grading as L by CAT.

Blood group B A_1 cell assay versus kodecyte assay

Individual sample results obtained in the A_1 cell assay of 23 group B samples were compared with the results obtained by testing the same samples in the kodecyte assay by tube. As can be seen in Table S5 (available as supporting information in the online version of this paper), about one-half of the samples (11 of 23) had tube A_1 cell assay titers greater than 16 and kodecyte assay grades of medium or greater, with almost one-quarter (5 of 23) having anti-A titers less than 16, which is in contrast to group O plasma, where the majority had high titers (Table 2). The majority of samples (17 of 23; 74% concordance) fell within Zone 1 (±1 dilution factor) and 22 of 23 (96% concordance) fell within Zone 2 (±2 dilution factors).

CAT analysis on the same day (Table S6, available as supporting information in the online version of this paper) found most samples had A_1 cell assay titers less than 32 and kodecyte assay grades medium or lower (13 of 23), which is in stark contrast to group O samples (Table 3). Of the four samples with L kodecyte assay grades by tube (Table S5), two were the same samples giving L grades by CAT (Table S6), with the remaining two samples giving LM grades. Most samples (15 of 23; 65% concordance) fell within Zone 1 (±1 dilution factor), and the majority (21 of 23; 91% concordance) fell within Zone 2 (±2 dilution factors).

Analysis of the one discordant sample (sample p), which gave a lower kodecyte assay grade than predicted by the A_1 cell assay, had a high IgM anti-A ratio (IgG:IgM ratio 1:32) (Table 4). This discordant result is in contrast to the group O samples with lower kodecyte assay grades in tube, which had a high IgG ratio. One group B sample (sample n), although concordant within both kodecyte and A_1 assays (Tables S5 and S6), was discordant between A_1 assays by tube (titer, 64), and CAT (titer, 8), although it was concordant with kodecyte grades in these platforms (Table 4). This sample had an IgG:IgM ratio of 1:8. Insufficient sample was available to analyze the relative IgG and IgM content of the two samples (samples q, r) discordant with low A_1 cell assay titers by CAT but with relatively high M grades in the kodeycte assay.

ABOiKT patients

Analysis of the results from five patients presenting for ABOiKT, sampled pre- and post-PP at each cycle showed, as expected, the level of antibody reduced as a consequence of PP (Table 5). Correlations of the 20 pre-PP and 20 post-PP samples between platforms were good (Table 5) with only three from one individual (1b and 1c pre-PP and 1c post-PP) of the A_1 cell assay results showing more than one dilution titer variation between antiglobulin tube and CAT. Similarly for the kodecyte assay, between platforms, only one sample (sample 4c post-PP) showed more than one grade variation. Variance between the A_1 cell and kodecyte assays within the same platform was 86% for the ±1 dilution factor variance and 100% for ±2 dilution variance, according to Table S1 criteria.

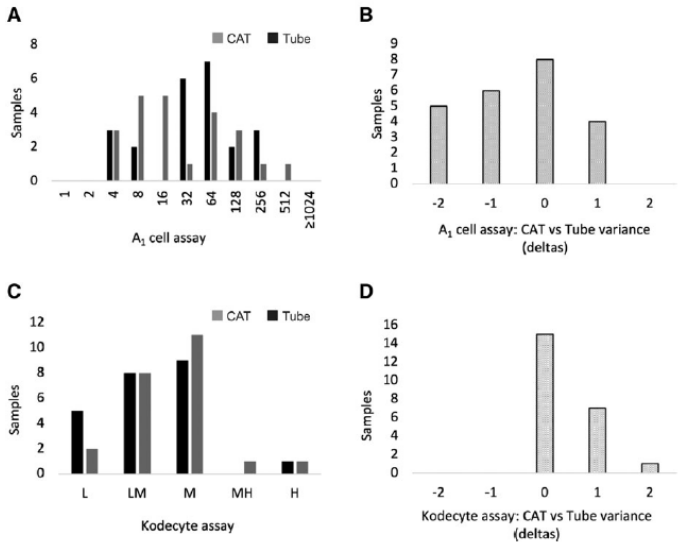

Fig. 2. Group B samples (n = 23). (A) Overall titer distribution against A_1 cells by platform. (B) Variance (deltas) of A_1 cell assay titers in AHG CAT compared to antiglobulin tube. (C) Overall grade distribution of the kodecyte assay in CAT and tube. (D) Variance (deltas) of kodecyte assay grades in CAT compared to tube.

TABLE 5. Results from clinical samples presenting for ABOiKT plasmapheresis

			AHG results pre- and post-PP cycles*							
			A_1 cell assay				Kodecyte assay			
			PrePP		PostPP		PrePP		PostPP	
Sample ID	ABO	Days post first PP	Tube	CAT	Tube	CAT	Tube	CAT	Tube	CAT
1a	O	0	256	256	64	64	H	H	M	MH
1b		+2	256	1024	64	64	H	H	M	MH
1c		+4	64	256	16	64	M	M	M	M
1d		+5	16	32	4	4	M	M	L	L
2a	O	0	256	512	64	64	MH	H	M	MH
2b		+1	128	512	32	32	M	H	M	MH
2c		+3	128	256	64	128	MH	MH	M	M
2d		+4	128	64	32	32	M	M	LM	M
3a	O	0	512	256	128	128	H	H	M	MH
3b		+2	512	256	32	64	MH	MH	MH	MH
3c		+4	64	128	32	64	M	MH	M	M
3d		+5	128	128	16	32	M	M	LM	LM
4a	O	0	128	256	32	32	M	MH	LM	M
4b		+2	64	64	16	32	M	MH	LM	M
4c		+4	64	32	8	16	LM	M	L	M
4d		+5	32	32	8	8	LM	M	L	LM
5a	B	0	64	64	16	8	LM	LM	L	L
5b		+1	32	16	8	4	L	L	L	L
5c		+3	8	4	4	4	L	L	L	L
5d		+4	8	4	1	<1	L	L	L	L

* Shading indicates go criteria (A_1 cell assay titer <16 or kodecyte grade L). Tube titer results correlated with the independently obtained clinical testing laboratory results (not shown).
AHG = antihuman globulin; CAT = column agglutination technology; PP = plasmapheresis.

If using the criteria of a kodecyte L grade or A_1 cell titer of less than 16 as the decision basis (i.e., go/no-go) for transplant and comparing the post-PP results in tube (Table 5), we observe the same go/no-go decisions for all patients. However, if applying the same go/no-go criteria to CAT, we observe that only four of five would have the same go/no-go decision. Patient 4 showed higher post-PP antibody levels with kodecytes (kodecyte grade LM cf A_1 titer, 8) and would result in a no-go decision with kodecytes and a go decision with A_1 cells.

DISCUSSION

The importance of reducing ABO antibodies to low levels is an established prerequisite for ABOiKT.[5,8–12,14] As seen in our results and also well established in the literature,[25,35–37] natural levels of ABO antibodies, especially for group O individuals, predominantly exceed ABOiKT transplant criteria with titers greater than 16. It is therefore necessary that procedures[8] be undertaken to reduce ABO antibody levels until deemed low. Unfortunately, the accuracy and precision of measuring antibody levels by current practices of sample dilution and testing against natural RBCs is inherently poorly standardized and subject to many variables that impact upon the results obtained, both intra- and interlaboratory.[20] Thorpe et al.[19] found an 8- to 64-fold variation in reported titers per preparation and method across laboratories, although intralaboratory variability was generally good, with over 90% of replicate titers within a twofold range. The antigen concentration variable is significantly mitigated by use of commercial A_1 and B reagent RBCs prepared from pooled donations; however, these pools may range from 2 to 12 donors depending on manufacturer batch sizes. There is clearly still a need for better methodology for determination of ABO antibody levels.[18,19,25,38–40]

We attempted to investigate whether serologic determination of antibody levels can be done without the need for plasma dilutions. This was based on observations that the quantity of ABO antibodies in serologic reagents[30] directly influences their ability to detect low levels of ABO antigen (i.e., in weak subgroups). With the ability to make standardized RBCs bearing precise low levels of ABO antigens using Kode Technology,[26] we were able to explore the potential of defined single A antigen–bearing RBCs, and then tuned them to react with specific levels of antibody in undiluted plasma (i.e., RBCs with synthetic low antigen dilutions vs. undiluted plasma; kodecyte assay). This is in marked contrast to current methodology that uses naturally high-level polymorphic A antigen present on unmodified A cells to react with dilutions of plasma (i.e., RBCs with natural high-level antigen vs. plasma dilutions; A_1 cell assay).

Human blood samples are not uniform in their antibody composition,[24,41] with different samples and blood types having different ratios and levels of IgG and IgM, each of which may show variations in avidity and affinity, and each of which may show further variances in specificity.[39,40,42,43] Consistent with results cited elsewhere[35,37,40] we observed significant ranges in antibody activity between individual samples. Of particular relevance to the ABO system are antibody specificity variances that can detect the same or similar antigens. For example, two major forms of anti-A exist: anti-A and anti-A,B (the latter also being able to detect the B antigen). These two different A antigen reactive antibodies copresent in group O individuals,[2] are well established to show large affinity and titer differences and will compete with each other, which may impact on the results derived by different methodologies, and even impact on clinical significance.[37,43]

The level of ABO antibody is predominantly determined with plasma dilutions tested by either manual serology in tubes, or automated or semiautomated in cassettes (CAT)[25,36,44] with both observing IgM direct agglutination and using antiglobulin reagents to detect IgG. We observed apparent differences in determination of antibody levels in both donors and patients, depending on the technology used.

This kodecyte assay evaluated only A type 2 kodecytes as reagent RBCs capable of detecting anti-A and anti-A,B and therefore is relevant only for A to O or A/AB to B ABOiKT. However, as ABOiKT are performed for all ABO-incompatible donor combinations,[5] at some stage, if the kodecyte assay was found to be clinically relevant, an equivalent B kodecyte panel would be required. Furthermore, only A type 2 kodecytes were used, and it is possible that kodecytes made with different A antigens, such as A Type 3, may be more clinically relevant for blood group O and B ABOiKT.[24] However, the A Type 2 antigen used here is capable of measuring total levels of anti-A and anti-A,B[42,43] and this is probably all that is required, as the reduction of antibody by plasmapheresis is nonselective, and monitoring a single antibody specificity would serve as a surrogate for all antibodies. In contrast, selective reduction with affinity columns[5,10–13] would require measurement of the specific antibody removed.

As total antibody titers are used to inform ABOiKT eligibility,[23] and this can be derived from the antiglobulin technique alone, our saline assays were discontinued after testing 48 donor samples, and not performed on patient samples.

The kodecyte assay was evaluated with antiglobulin in both tube and CAT platforms and contrasted with the results obtained with the A_1 cell assay. Overall, the correlation between the kodecyte assay and the A_1 cell assay in tube was relatively high, with 96% concordance (Zone 2; ±2 dilution factors) for both group O and group B samples (Tables 2 and S5). The correlation between the kodecyte assay and the A_1 cell assay in CAT was lower than in tube, but still 91% concordant (Zone 2; ±2 dilution factors) for both group O and group B samples (Tables 3 and S6). Results suggested that the kodecyte assay in tube may underestimate antibody levels compared with the A_1 cell assay if the ratio of IgG:IgM is high. The opposite explanation is equally viable, where the

A_1 cell assays may overestimate antibody levels if the ratio of IgG:IgM is high.

In view of the kodecyte assay reporting higher direct agglutination antibody levels (Table S3) and in an attempt to reveal the cause of the discordances, we evaluated the IgG and IgM content of discordant and concordant samples. Results suggest that A_1 cells and kodecytes may show different reaction sensitivities to large variations in IgG:IgM ratios. However, as IgG ABO antibodies can also cause direct agglutination,[33] they may be skewing the true IgG:IgM ratios, and IgM antibodies have also been shown to impact on the reactions of IgG in other testing platforms.[39,40,45–47] Although unable to resolve the impact of IgG:IgM ratios and relative contributions of anti-A,B and anti-A to the observed result, data are suggestive that these factors together with the effects of dilution and type of cell used (e.g., A_1 cells or kodecytes) are all factors that influence these assays and cause the observed differences in results.

A definitive ABOiKT pretransplant antibody titer has not been defined,[23] and due to significant variances between laboratories,[19] this is probably not possible; consequently, different centers use criteria ranging from 4 or less to 32 or less.[5,7,23,48]

Erring on the side of caution, we used less than 16 as the criterion for the antiglobulin A_1 cell assay[14,49] and adopted the kodecyte grade L (a positive reaction with only the 100-kodecytes and negative or very weak with the 20-kodecytes) by antiglobulin as a theoretical go/no-go for the transplant criterion. If applying this L grade kodecyte go/no-go criterion to the five ABOiKT patients (Table 5), all patients would have received the same go/no-go decisions by tube; however, by CAT one patient (sample 4) would have exceeded the kodecytes go threshold, which indicated a higher level of antibody than did the A_1 cell assay. Although we used a panel consisting of five kodecytes, this panel could potentially be reduced down to two kodecytes (100 and 20) for ABOiKT use. Samples with titer less than 16 would be positive with only the 100-kodecyte, while samples with titer 16 or greater would be positive with both the 100- and 20-kodecytes.

It is not known which assay (kodecyte or A_1 cell assay) has the best correlation with clinical outcome, and this requires clinical trials. The kodecyte method proposed here is also potentially applicable for determining ABO antibody levels in ABO-incompatible pediatric heart transplantation,[50] production of intravenous immunoglobulin,[51] detecting blood donors with high-titer ABO antibodies,[37,52] production of platelet preparations,[25] monitoring ABO-mismatch bone marrow transplantation,[53] and predicting and monitoring hemolytic disease of the newborn.[54] By eliminating the step of sample dilution and using a standardized reagent RBC, the kodecyte assay has the potential to eliminate the compounding errors of plasma dilution, simplify methodology, make it more suitable for automation, improve standardization between laboratories, and potentially produce a more clinically relevant result.

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CONFLICT OF INTEREST

SH and NB are employees and stockholders in Kode Biotech, the patent owner of Kode biosurface engineering technology. HP has disclosed no conflict of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Table S1. Criteria for determining concordance zones.
Table S2. Assay reproducibility.
Table S3. Blood group O plasma saline reactivity.
Table S4. Analysis of further samples showing concordance in assays.
Table S5. Blood group B plasma antiglobulin tube reactivity, kodecyte assay versus A_1 cell assay.
Table S6. Blood group B plasma CAT AHG reactivity, kodecyte assay versus A_1 cell assay.

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