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NEW APHERESIS TECHNIQUES IN TRANSPLANTATION

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ABSTRACT

The shortage of organs for transplantation is a global problem and therefore it is of outmost importance to increase the number of donations and improve long term graft survival. As an effort to increase kidney donations, living kidney donors are being increasingly used and both patient and graft survival is superior after living donation compared with kidney transplantations using organs from deceased donors.

If ABO incompatible kidney transplantations can be done, the pool of living kidney donors can theoretically be increased with 30-40%. Until recently ABO incompatible kidney transplantations could only be performed after a reinforced immunosuppressive protocol combined with splenectomy. These reinforced immunosuppressive protocols have had very limited use due to the significant side effects with increased morbidity as well as mortality. Therefore, in 2001 we introduced a new protocol that allows ABO incompatible kidney transplantation without splenectomy. The protocol includes rituximab to deplete B lymphocytes and to prevent rebound of antibodies post transplant, combined with a new antigen-specific apheresis technique for preoperative depletion of circulating anti-A/B antibodies.

The new antigen-specific filter has been evaluated in 15 patients with the conclusion that this new technique is safe and that anti-A/B antibodies were efficiently depleted. The clinical outcome for the 15 patients was equal compared with ABO compatible kidney transplantation. The implementation of the protocol in other transplant centers revealed significant differences regarding preoperative anti-A/B titer levels using the semi-quantitative hemagglutination technique. Collaborative analysis showed that the differences were method-related due to the high variability of the tube hemagglutination technique. We showed that by standardization of the simple gel microcolumn hemagglutination technique, titer results can be reproducible enough to allow inter-center comparison of clinical results.

Between September 2001 and September 2008, 45 patients have received ABO incompatible kidney transplantations at Karolinska University Hospital Huddinge with the new protocol. The first 20 kidney recipients were compared with a group of ABO compatible living donor kidney recipients. Mean follow-up was 3 years and the study showed that clinical outcome is similar after ABO incompatible kidney transplantation with this protocol compared with ABO compatible kidney transplantation, both short-term and long-term.

The short term graft survival after kidney transplantation has improved much over the last decades but long term graft survival is still poor. In a pilot study, seven consecutive patients with biopsy-proven ongoing acute cellular rejections, which did not respond to conventional anti rejection treatment, were successfully treated with extracorporeal photopheresis (ECP), an immunomodulatory apheresis treatment. In a case control study with a 3-year follow up we aimed to investigate if prophylactic ECP can prevent rejection and improve clinical outcome after kidney transplantation. Because our patient groups are small, we regard the study mainly as a pilot study of safety. Event-free survival time was not different in the two groups and no ECP-related side effects were noted. An adaptive immune response with a tolerogenic shift was induced during ECP treatment with a significant increase of $CD4^+CD25^{hi}FoxP3^+$ regulatory T cells.

These two approaches to combat the organ shortage a) will increase the number of living kidney donations and b) shows promise as a tolerogenic immunomodulatory therapy.

LIST OF PUBLICATIONS

I.   Tydén G, **Kumlien G**, Fehrman I. Successful ABO-incompatible kidney transplantations without splenectomy using antigen-specific immunoadsorption and rituximab. **Transplantation 2003;76:730-731.**

II.   **Kumlien G**, Ullström L, Losvall A, Persson LG, Tydén G. Clinical experience with a new apheresis filter that specifically depletes ABO blood group antibodies. **Transfusion 2006;46:1568-1575.**

III.   **Kumlien G**, Wilpert J, Säfwenberg J, Tydén G. Comparing the tube and gel techniques for ABO antibody titration, as performed in three European centers. **Transplantation 2007;84:S17-S19.**

IV.   Genberg H, **Kumlien G**, Wennberg L, Berg U, Tydén G. ABO-incompatible kidney transplantation using antigen-specific immunoadsorption and rituximab: a 3-year follow-up. **Transplantation 2008;85:1745-1754**.

V.   **Kumlien G**, Genberg H, Shanwell A, Tydén G. Photopheresis for the treatment of refractory renal graft rejection. **Transplantation 2005;79:123-125.**

VI.   **Kumlien G**, Genberg H, Lundahl J, Winqvist O, Tydén G. Photopheresis as prevention for acute rejection after kidney transplantation – a case control study with a 3-year follow-up. **Manuscript.**

CONTENTS

LIST OF ABBREVIATIONS

ABOc	ABO compatible
ABOi	ABO incompatible
ACE	Angiotensin converting enzyme
APC	Allophycocyanin
APC-Cy7	Allophycocyanin-Cyanine 7 tandem conjugate
ATG	Antithymocyte globulin
BKV	BK virus, polyomavirus type BK
CD	Cluster of differentiation
CMV	Cytomegalovirus
CTCL	Cutaneous T Cell Lymphoma
DARC	Duffy antigen receptor for chemokines
DD	Diseased donor
ECP	Extracorporeal photopheresis
ECV	Extracorporeal volume
EVA	Ethylvinylacetate
FACS	Fluorescence activated cell sorter
FITC	Fluorescein isothiocyanate
GFR	Glomerular filtration rate
GVHD	Graft versus host disease
HDN	Hemolytic disease of the newborn
HLA	Human leucocyte antigen
HSV	Herpes simplex virus
IA	Immunoadsorption
IAT	Indirect Antiglobulin Technique
LD	Living donor
LISS	Low Ionic Strength Saline
mAb	Monoclonal antibody
MMF	Mycophenolate mofetil
OKT-3	Muromonab-CD3, anti-CD3 monoclonal antibody
Pac Blue	Pacific blue
PE	Phycoerythrine
PEG	Polyethyleneglycol
PerCP	Peridinin-chlorophyll-protein complex
PUVA	Psoralen + UVA
RBC	Red blod cell
SPR	Surface plasmon resonance
TPE	Therapeutic plasma exchange
UVA	Ultraviolet irradiation type A

1 BACKGROUND

The ideal treatment for end-stage renal failure is kidney transplantation but unfortunately a severe shortage of organs for transplantation has significantly limited this practice. According to transplant data for 2003 from the United States 13,765 kidney transplants were performed this year. During the same year 24,493 patients were added to the kidney transplant waiting list that at the end of the year included 57,211 patients (*1*). Thus, the number of patients on the waiting list for kidney transplantation increases much faster than the number of actual transplants performed. At the end of 2006 there were 88,877 patients on the US kidney waiting list and 4,456 patients on the list had died during the last year (*2*).

In Sweden, a total of 11, 638 kidney transplantations have been performed between the start in 1964 and until September 30th 2008. Between 300 and 400 kidney transplantations are performed annually in Sweden. In 2007, a total of 379 kidney transplantations were performed and of those, 123 were performed using kidneys from living donors (*3*). By the end of October 2008, 457 patients were on the national Swedish kidney transplantation waiting list.

Efforts have been made to expand the kidney donor pool by increasingly using living donors. Both patient and graft survival is superior after living donor (LD) kidney transplantation compared with diseased donor (DD) kidney transplantation. The 1-year and 5-year graft survival rates after LD and DD kidney transplantation have been reported to be 94,3% compared to 88,7% and 78,6% compared to 65,7% (*1*). Data from Karolinska University Hospital Huddinge also shows better results after LD kidney transplantation *(Table 1).* Therefore, it is beneficial to increase the use of living kidney donors. It is also a major challenge in the field of kidney transplantation over the next decades to improve long-term graft survival.

This thesis has two parts. In Paper I-IV, a new protocol for major ABO incompatible kidney transplantation is introduced and evaluated. In Paper V and VI, extracorporeal photopheresis, an immunomodulatory apheresis therapy, is evaluated as treatment and prevention for acute rejection after kidney transplantation.

Table 1: Data from Karolinska University Hospital Huddinge

Long-term outcomes superior with living donor transplantation:		
	10-year survival	
	Graft (%)	Patient (%)
Living donor	70	85
Deceased donor	40	55

2 BLOOD GROUP SYSTEMS

The term blood group is a common denomination for protein, glycoprotein or glycolipid antigens expressed on the erythrocyte surface membrane, often as well as in other tissues of the human body. Immunization against these antigens can cause adverse reactions to transfusion and hemolytic disease of the newborn (HDN) as well as be a barrier against transplantation.

There are currently 29 known blood group systems and they have all recently, some twenty years after the exploration of the leucocyte surface antigens (CD), been characterized but their function is still, with a few exceptions, unknown. One example of a blood group system with a somewhat known function is the Duffy glycoprotein, also known as the Duffy Antigen Receptor for Chemokines (DARC). Duffy is also used as a receptor by the *Plasmodium vivax* merozoites, and this has led to a genetic selection with occurrence of Duffy negative phenotypes (Fy a-b-) in Africa. These individuals are resistant to infection with vivax type malaria and since they are also otherwise healthy, there are obviously back-up systems for this chemokine receptor. Other types of environmental pressure has led to different frequencies of blood group phenotypes in different populations.

Except for the 29 blood group systems there are presently some 19 other known antigens expressed on RBC surface membranes. They are mostly expressed in very high or very low percentages of the population. Immature RBC´s express HLA-antigens and some HLA-antigens are expressed also on mature RBC´s. Among these are HLA-B7 (Bg^a), HLA-B17 (Bg^b)and HLA-A28 (Bg^c). The expression of HLA-antigens is highly variable and many individuals do not express HLA-antigens on their RBC´s.

2.1 THE ABO SYSTEM

In 1900, Karl Landsteiner showed that people could be divided into three groups, called A, B and C (later O), on the basis of whether their erythrocytes agglutinated when mixed with sera from other people (*4*). A forth group (AB) was soon found. By 1910, the ABO blood groups had been shown to be inherited and in the 1950´s they were shown to represent oligosaccharide chains on glycoproteins and glycolipids. In 1990, the gene encoding the enzymes responsible for synthesis of the ABO antigens was cloned (*5-8*). According to the rule of Landsteiner an individual has antibodies with specificity against the antigens of the ABO system that the individual does not express. In this respect ABO is unique among blood group polymorphisms. *(Table 2).*

Table 2. Rule of Landsteiner

ABO blood group	Antigen on RBC´s	Antibodies in serum
O	none	anti-A,B
A	A	anti-B
B	B	anti-A
AB	A and B	none

The approximate ABO frequencies among Caucasians are A 45%, O 40%, B 10% and AB 5%. Populations with a group O phenotype greater than 60% are found in native people of the Americas and in parts of Africa and Australia but not in most of Europe or Asia. The frequency of A is quite high (40-60%) in Europe, especially in Scandinavia and parts of Central Europe. Type A_2 is dominant with >50% in Swedish Lapps, whereas it is almost non-existing in Japanese people.

The ABO polymorphism is expressed in most animals and in most human tissues with a few exceptions (9). ABO antigens are not expressed on leucocytes. Bone, skin, cornea and heart valves can be transplanted without ABO matching (10).

The A and B blood groups are inherited in a co-dominant manner, while type O is recessive. The genes encoding the A and B genotype are located on chromosome 9. The gene products are the enzymes glycosyltransferase for the A gene and galactosyltransferase for the B gene. These enzymes add the terminal monosaccharide to the precursor oligosaccharide (11). Individuals expressing both enzymes belong to the AB blood group.

The A and B trisaccharides may be attached to several different core oligosaccharide chains. In humans, at least five different core chains carry the terminal A or B antigen epitopes (12). Type 4 chains are abundant in kidney, but are only detected in small quantities on RBC´s. Type 2 chains dominate on RBC´s as on vascular endothelium throughout the body. Type 3 and 4 chains express A antigen on A_1 cells but not on A_2 cells which may account for the qualitative differences between A_1 and A_2 subtypes.

Type 1-4 chains (9).
Type 1: Galβ1-3GlcNAcβ1-R
Type 2: Galβ1-4GlcNAcβ1-R
Type 3: Galβ1-3GalNAcα1-R
Type 4: Galβ1-3GalNAcβ1-R

2.2 A_1, A_2

The A phenotype can be subdivided into A_1, A_2, A_3 and so on. Only A_1 and A_2 is of clinical significance. A_1 and A_1B have stronger antigen expression than A_2 and A_2B respectively. Estimated number of antigen sites are, A_1: 8-12 x 10^5, A_2: 1-4 x 10^5, A_1B: 5-9 x 10^5, A_2B: 1 x 10^5 (12). In addition to the quantitative difference, there is also a qualitative difference between A_1 and A_2. About 2% of A_2 and 25% of A_2B individuals produce an antibody, called anti-A_1, that reacts with A_1 and A_1B cells, but nor with A_2 or A_2B cells. The usual serological interpretation is that both A_1 and A_2 cells have A antigen, but A_1 cells have an additional antigen called A_1, absent from A_2 cells (13).

2.3 ABO BLOOD GROUP ANTIBODIES

Anti-A and anti-B are almost always present in adult individuals when the corresponding antigen is absent. Exceptions are individuals with weak A or B antigens, chimerism after transplantation, hypogammaglobulinemia or haematological malignancies. Small children start producing ABO antibodies around the age of three months and reach adult levels between 5 and 10 years of age. The levels of ABO antibodies have been thought to decline with old age but this dogma has been questioned in later studies (14). Anti-A and anti-B antibodies may be of IgM, IgG or

IgA type but some sera may contain all three immunoglobulin classes. Anti-A/B antibodies of so called non-stimulated individuals are predominantly IgM, but IgA and IgG may be present. After stimulation, such as after pregnancy, transfusion or transplantation, titres may increase with increased level of IgG and/or IgA. ABO antibodies of IgG class may be of all subclasses (*15*) but are predominantly IgG1 and IgG2. Most anti-A/B antibodies have specificity against epitopes within the terminal trisaccharide of A or B antigen (*16*), but they may also be directed against epitopes in the core chain of the ABO antigen structure (*17, 18*).

Although ABO antibodies are often referred to as "naturally occurring", they probably appear as a result of immunization by A and B substances in the environment. One hypothesis is that the structures of the A and B antigens cross-react with substances in food and on bacteria, such as the bacteria in the normal gut flora. However, the fact that virus particles have been shown to express the same A or B antigen as the cells they multiplied in, and also to be neutralized by the corresponding antibody, may be of at least the same importance (*19, 20*).

2.4   SECRETOR STATUS

Individuals who express the secretor gene (genotype SeSe or Sese) secrete soluble A/B antigen into the circulation. About 80% of the Caucasian population express the Se gene whereas 20% are so called non-secretors (genotype sese) and have no or little soluble A/B antigen. The soluble A/B antigens may serve as a defence against infection since many micro organisms bind these antigens to their surface where they may function as blocking and/or neutralizing agents. This theory is supported by the fact that non-secretor individuals are more susceptible to various infections, including infections caused by *Streptococcus pneumonie, Haemophilus influenzae and Candida albicans (21)* . The secretor genotype is related to the Lewis phenotype. Individuals with Le(a-b+) phenotype are ABH secretors, Le(a+b-) individuals are so called non-secretors with only very small amounts of soluble ABH antigen whereas Le(a-b-) individuals must be genotyped to determine the secretor gene expression (*12, 22*). Of Le(a-b-) individuals, 80% are ABH secretors (*23*).

2.5   EXPRESSION OF ABH ANTIGEN IN KIDNEY

Normal adult kidney tissue expresses ABH antigens in endothelial cells and in epithelial cells of tubules and collecting ducts. Epithelial and mesangial cells of the glomerulus do not. The expression of ABH is influenced by the secretor status and shows a regional variation with a distal gradient of increasing expression from the glomerulus to the transitional epithelium of the renal pelvis and bladder. ABH antigens are expressed by the distal convoluted tubules and collecting ducts. In the distal tubules, the presence of A antigen is independent of the secretor status. The ABH determinants are carried by at least type 2 and type 4 chains, and are expressed at apical membranes and in the cytoplasm of tubular epithelial cells. Type 4 chains are mainly found in renal tissue from A_1 secretors and in reduced amounts in A_2 individuals and A non-secretors (*9*).

3 ABO INCOMPATIBLE KIDNEY TRANSPLANTATION

3.1 EARLY EXPERIENCE

One of the limitations for kidney transplantation has been the ABO blood group system. Major ABO incompatibility, where the recipient has preformed circulating antibodies with specificity against antigens expressed in the transplanted organ, causes hyper acute rejection of the transplanted organ.
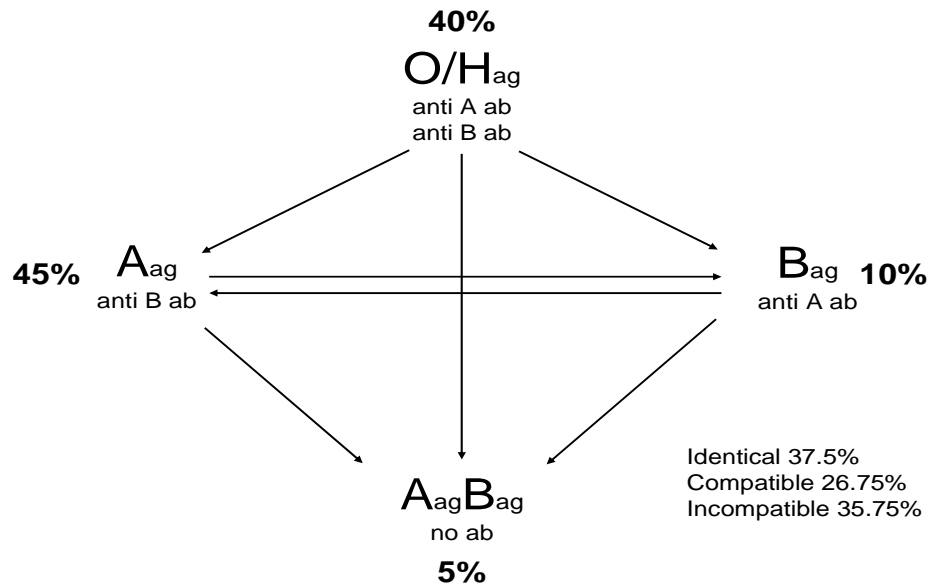

40%

O/H_{ag}
anti A ab
anti B ab

45% A_{ag}
anti B ab

B_{ag} **10%**
anti A ab

$A_{ag}B_{ag}$
no ab

5%

Identical 37.5%
Compatible 26.75%
Incompatible 35.75%

*Figure 1. The distribution of blood groups within the Caucasian population and
the principles of ABO blood group matching.*

If ABO-incompatible kidney transplantations can be performed, the pool of living kidney donors can theoretically be increased with 30-40%. However, until recently ABO-incompatible kidney transplantations could only be performed after reinforced immunosuppressive protocols in combination with splenectomy to prevent rebound of ABO-antibodies.

A report of 25 accidental ABOi kidney transplantations was published in 1987 (*24*), only 4% 1-year survival was seen. The first successful ABOi kidney transplantation was reported by Slapak et al in 1981 (*25*). Also this was an accidental ABOi kidney transplantation and the patient developed antibody-mediated rejection, but in this case the rejection could be resolved after treatment with plasma exchange and immunoadsorption. In the 1980´s Alexandre et al in Belgium performed the first larger study on ABOi kidney transplantation (*26, 27*). In a group of 39 LD ABOi kidney transplantations, 1-year graft survival was 75%. Three early patients who were not splenectomized lost their grafts and therefore it was concluded that splenectomy was necessary for successful ABOi kidney transplantation. The protocol also included repeated plasma exchange plus donor platelet transfusions and administration of A or B trisaccharide to further deplete anti-A/B. Immunosuppression was based on corticosteroids, azathioprine and cyclosporine plus antilymphocyte globulin.

Based on the Belgian success, similar protocols were implemented in a few other transplantation centers worldwide. Between the late 1980´s and the late 1990´s ABOi kidney transplantations has been performed predominantly in Japan. Deceased donors are not used in Japan due to legislation and the predominant use of living donors allows time for pre-transplant antibody depletion. The Japanese ABOi kidney transplant programs have been very successful and almost 1000 transplants have been performed (*28, 29*). During this period, outside Japan few ABOi kidney transplantations were performed, with the exception of transplanting kidneys from donors with blood type A2 into recipients with low anti-A antibody titers (*11, 30, 31*).

3.2 A_2 DONORS

The first clinical trial using A_2 kidney donors for transplantation into patients with blood group O was performed in Gothenburg, Sweden between 1974 and 1988. Using only standard immunosuppression and no antibody depletion 23 DD kidney transplantations were performed. One-year graft survival was 55% for the first 20 patients (*31*). The protocol was implemented in several other centers, but with increased experience it became clear that high titers of anti-A/B antibodies in the recipient was correlated with high risk of early graft loss and A_2-compatible kidney transplantation was only performed with standard immunosuppression if anti-A/B titers were low. The largest series of A_2-incompatible DD kidney transplantations were performed in USA, and in this series only recipients with anti-A/B titers no higher than 1:4 were accepted (*32*).

3.3   RENEWED INTEREST

Following the success in Japan, there has been a renewed interest in ABOi kidney transplantation since the early 2000´s, both in USA and Europe. The use of rituximab as a new therapeutic option in transplantation has been a major focus of the new protocols. The new Glycosorb ABO® column for antigen-specific depletion of ABO-antibodies was first used in a clinical setting at Karolinska University Hospital Huddinge, starting in September 2001, and has since gained widespread use, especially in Europe.

Patients are selected to the ABOi waiting list based on, among other clinical parameters, the titers of anti-A/B antibody. The evaluation of and the number of antigen-specific immunoadsorptions performed and finally if the transplant itself can be performed is dependent on the recipients anti-A/B titers. Thus, the determination of anti-A/B antibody levels is of crucial importance in these protocols.

4   QUANTIFICATION OF ABO BLOOD GROUP ANTIBODIES

4.1   HEMAGGLUTINATION

4.1.1   Background

The IAT hemagglutination technique is being used to identify alloantibodies against blood group antigens. Some alloantibodies against RBC antigens such as Kidd, can be present in very low titers in the circulation after primary immunization but rapidly increase after secondary immunization and cause sometimes severe delayed hemolytic transfusion reactions. Thus it is necessary for the blood banks to be able to identify some low titer alloantibodies. Low ionic strength saline solution (LISS), albumin and polyethyleneglycol (PEG) are agents that enhance the antigen-antibody reaction. When the IAT technique is used for titration of anti-A/B, local variations of the technique has an impact on the titer results.

Originally hemagglutination was performed using glass tubes and ocular rating of agglutinates that sometimes dissolves during the procedure. This technique is still in use in blood banks around the world more than a century after Karl Landsteiner´s ground-breaking experiment (*4*) that led to the first understanding of the ABO blood group system. In the 1990´s the gel microcolumn hemagglutination technique was developed (*33*) and it has several advantages compared with tube hemagglutination technique: the agglutinates are more stable and easier to rate, the technique is quicker and smaller volumes of sample and reagent is needed. The lower cost has been a main reason the tube technique is still much in use. However, hemagglutination is at best a semi-quantitative technique and without standardization it can not be used for inter-center comparison.

4.1.2   Evaluation

The compilation of data from 60 consecutive kidney transplantations performed in three centers with the same protocol revealed marked differences regarding preoperative titers of anti-A/B antibodies (*34*). The three centers all used tube hemagglutination techniques that differed regarding practically every detail. In February 2006 Stockholm switched to gel microcolumn hemagglutination technique. Comparing results from the same samples revealed method-related differences regarding titer levels. When the three centers repeated the comparison using the same gel microcolumn technique the variability was markedly decreased as described in paper III. When plasma dilutions and RBC suspensions were sent out and analysed with the same method the results were even more reproducible *(unpublished data, Figure 2)*. The remaining differences are probably due to systematic differences regarding the rating of agglutinates. This could probably be addressed by using existing automated systems for rating of agglutinates.
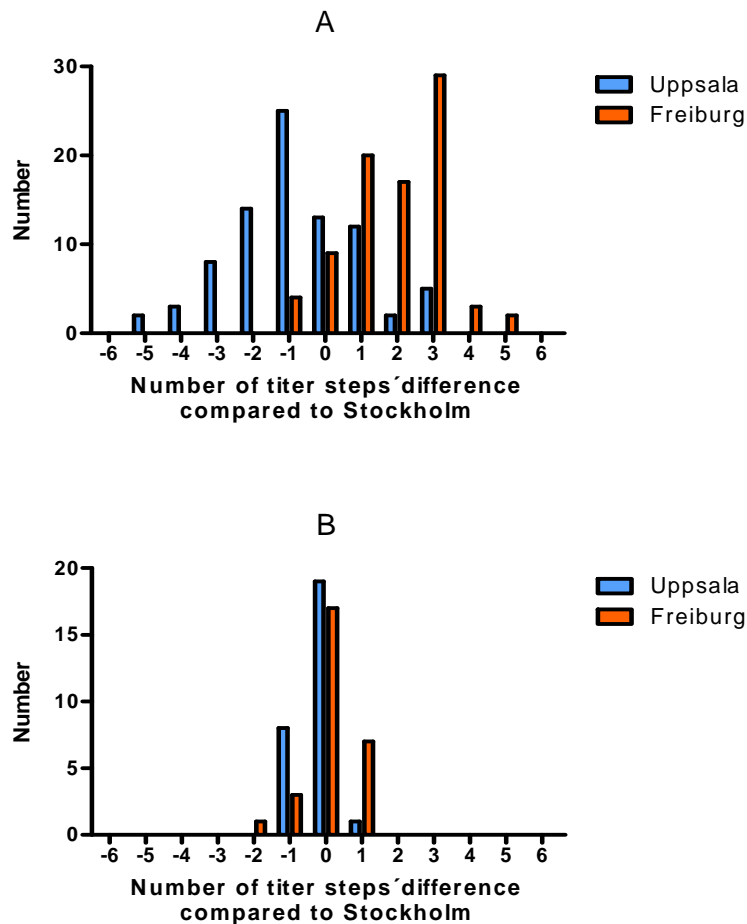
Figure 2A: Comparing the same samples titrated with different hemagglutinantion methods.
Figure 2B: Comparing the same samples titrated with the same gel hemagglutination method,
with correction for dilution errors.

4.2   OTHER TECHNIQUES

4.2.1   ELISA-technique

An indirect ELISA system for quantification of human anti-A/B IgG and IgM was
introduced in 1989 (*35*). Blood group antigens A or B were used to sensitize polystyrol
micro titer plates and bound antibodies were detected with polyclonal or monoclonal
anti-Ig antibodies. Correlation between anti-A IgG and titer was excellent (p<0.05).

4.2.2   Flow Cytometry

In 2005 a flow cytometric method for isotype-specific detection of anti-A/B IgM, IgG
and IgG subclasses was introduced (*36*).

4.2.3 Surface Plasmon Resonance – SPR

Anti-A/B of IgG type can be rapidly quantified by surface plasmon resonance (*37*). The method also evaluates antibody avidity but works less well for detection of IgM antibodies.

4.2.4 KODE-technology

Glycolipids have a natural ability to insert into red cell membranes. Based on this the serological properties of RBC´s can be modified using synthetic antigen of different concentrations to manufacture standardized test RBC´s (*38*). This system is not yet commercially available.

5 REMOVAL OF ANTI-A/B ANTIBODIES

5.1 UNSELECTIVE METHODS

5.1.1 Introduction

Apheresis may be described as a process by which blood being removed from a subject is continuously separated into component parts, usually to allow a desired component to be retained while the reminder is returned to the subject. The term apheresis includes many types of procedures. Apheresis is used both for donation of blood products, donation of hematopoietic stem cells for transplantation and for many types of therapeutic apheresis treatments. Separation can be performed using filter or centrifugation technique. In general, more adverse effects are correlated with filter technique than with centrifugation technique. With centrifugation technique, many adverse effects are correlated to substitution fluids (*39*).

Modern apheresis machines are being increasingly automated for increased safety and standardization. Earlier centrifuge apheresis machines used discontinuous separation of whole blood with large extracorporeal volumes (ECV) during treatment. Continuous blood flow, smaller ECV, more controlled administration of anticoagulants and increased use of prophylactic calcium infusion when citrate is used as anticoagulant, has led to increased patient safety compared with earlier practices (*40*).

5.1.2 Plasma exchange - TPE

In therapeutic plasma exchange (TPE) treatment, patient plasma is removed from the circulation using centrifugation or filtration technique. The patient plasma is subsequently discarded and substituted with other fluids, such as albumin, other colloids or donor plasma *(Figure 3)*.
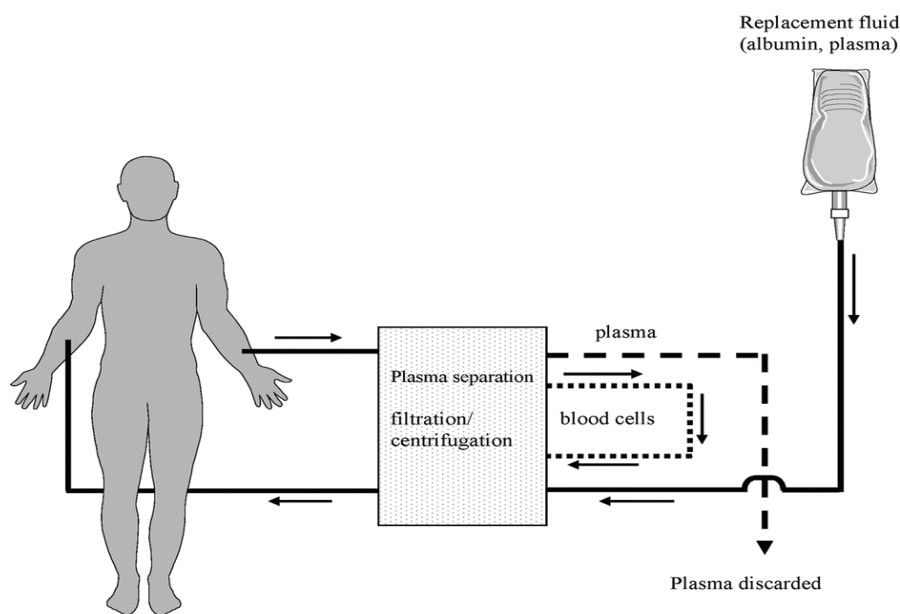
Plasma exchange

Figure 3: Principles of Therapeutic Plasma Exchange

TPE is still the predominant form of therapeutic apheresis (*41*). Theoretically, as a rule of thumb, 65% of the targeted plasma protein can be depleted after the exchange of 1.0 to 1.5 plasma volumes *(Figure 4)*.
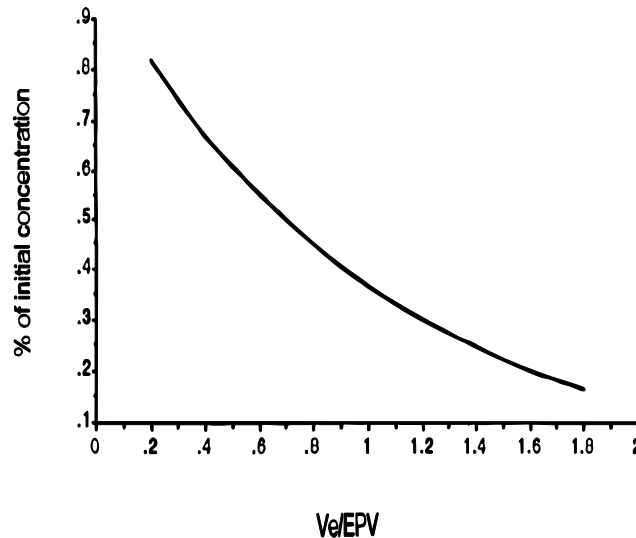

Figure 4: Concentration reduction vs No. of plasma volumes processed
Adapted from: Kaplan AA, A Practical Guide to Therapeutic Plasma Exchange, Blackwell Science, 1998

In clinical practice however, usually only about 45-50% is removed after 1.0 plasma volume (personal observation), probably due to redistribution of plasma proteins during the procedure.

Coagulopathy can develop as a result of removal of large volumes of plasma (*42, 43*), and it may be necessary to substitute with plasma, especially if surgical procedures are planned shortly after TPE. However, plasma substitution is the cause of the majority of adverse effects related to TPE (*39*), especially if plasma from female donors is used and the plasma is stored for more than a week before use (*44*). In spite of this, TPE still plays an important role in ABO-incompatible kidney transplantation (*45*), especially in countries where more selective methods are not available.

5.2 SELECTIVE METHODS

5.2.1 Introduction

Selective apheresis methods often uses filters and the matrix used in apheresis filters is of importance for their biocompatibility. Silica is known to cause various adverse reactions and all filters containing silica as a matrix have been withdrawn from the market. Sepharosis is known to be inert and is used in several currently commercially available apheresis filters.
Porous polyacrylic beads coated with negatively charged polyacrylic acid is the basis for selective whole blood LDL-apheresis filters that bind the positively charged LDL-cholesterol molecule (*46*) *(Figure 5)*.
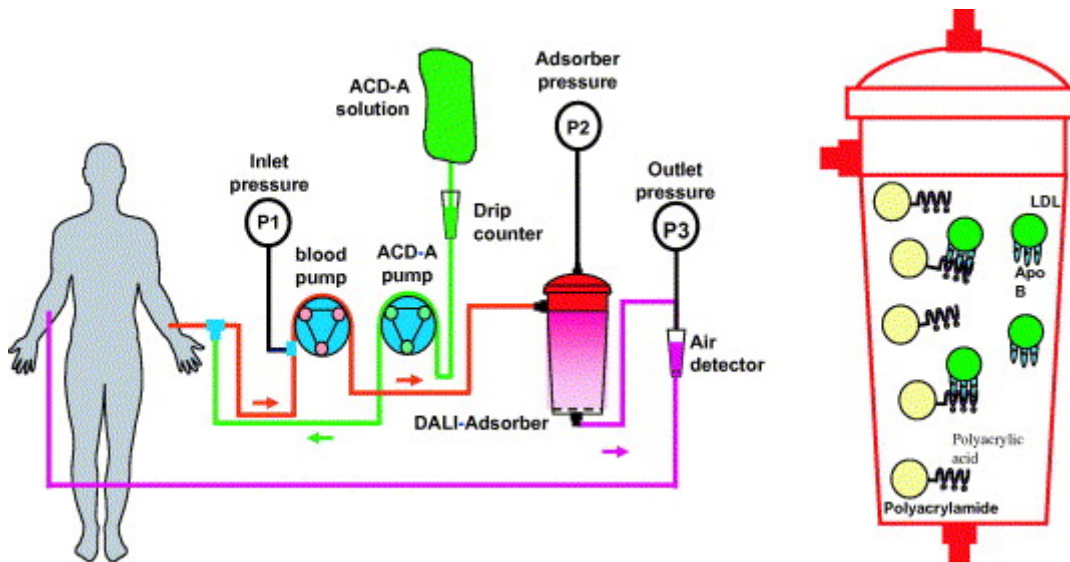
Figure 5: Principal overview of the DALI-system for depletion of LDL from whole blood using porous polyacrylic beads coated with polyacrylic acid. Adapted from Stegmayr B, A survey of blood purification techniques. Transfus Apher Sci 2005;(32):209-20.

The negative charge, of both whole blood LDL-apheresis filters and dextrane sulphate based apheresis filters for separating LDL from plasma, is correlated with adverse effects thought to be caused by bradykinin activation. If combined with ACE-inhibitors, these adverse effects can be severe (*47*).

5.2.2 TPE plus incubation with incompatible RBC´s

A technically simple but laborious system for depletion of anti-A/B antibodies uses plasma exchange plus incubation of patient plasma with graft incompatible erythrocytes before retransfusion of antibody depleted plasma (*48*). This system has the advantage of a reduced cost and the disadvantage of exposure of the patient to an increased number of blood donors.

5.2.3 Double filtration plasmapheresis

This two-step selective technique for removal of immunoglobulins was developed in Japan (*49, 50*). Plasma is separated by filtration and subsequently processed in a second plasma filter where plasma components of a certain molecular weight is separated. The remaining plasma is returned to the patient while the targeted plasma fraction is discarded. With this technique, only a small volume of patient plasma is discarded. Depletion of coagulation factors is avoided and only a small amount of substitution fluid is needed (*51*). *(Figure 6)*
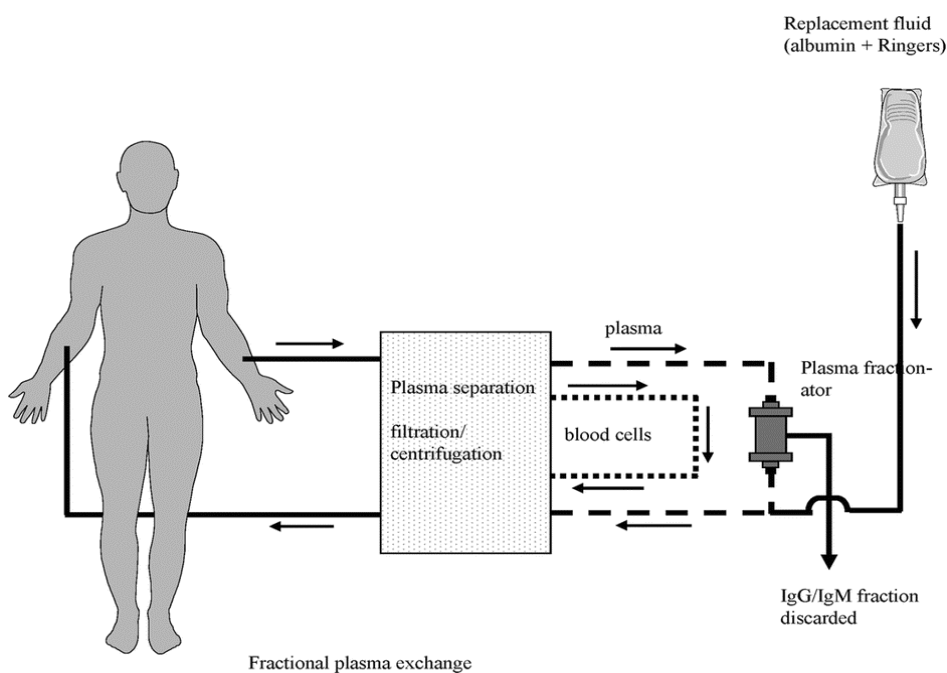
Figure 6: Principles of Double Filtration Plasmaferesis

5.2.4 Protein A immunoadsorption

Protein A immunoadsorption is another method for selective removal of immunoglobulins. It is based on protein A, derived from the cell wall of *Staphylococcus aureus,* immobilized by the attachment to a matrix, usually sepharose as in the Immunosorba® system (Fresenius Hemocare, Germany). During immunoadsorption, the Fc part of the IgG molecule binds covalently to protein A. Because the column is saturated during treatment, the system consists of two columns that are repeatedly being regenerated and used alternately. Immunoglobulins are removed effectively with depletion of about 87% of total IgG during one session (*52*) and the side effects are few (*52*). However, IgM and IgG of the IgG3 subclass are removed with less efficacy. *(Figure 7)*
A similar column, Prosorba® (Fresenius Hemocare, USA), based on protein A bound to a silica matrix has been removed from the market due to adverse effects (*53, 54*)

5.3 ANTIGEN-SPECIFIC IMMUNOADSORPTION

5.3.1 Introduction

If an identified substance needs to be removed from the circulation of a patient in order to remove a pathogenic substance or to remove graft-specific antibodies before transplantation it is an advantage to use specific methods if possible. If no plasma is removed, beneficial substances such as immune antibodies, memory cells, plasma proteins and coagulation factors are not depleted. Also, if no substitution fluids are needed, the risk for side effects decreases.
The first reports on antigen-specific immunadsorption were published in the 1970´s and described experiments performed on dogs (*55, 56*). The system used DNA immobilized in collodion charcoal and a few years later a patient with SLE was successfully treated (*57*).

5.3.2 Synsorb®/Biosynsorb®

In 1981, the first clinical study on immunoadsorption for removal of anti-A/B antibodies was published (*58*). The system used A or B trisaccharide linked to a silica matrix and was firstly used in patients undergoing ABO incompatible bone marrow transplantation. This filter was later marketed under the name Synsorb®. A modified product with a polymeric coating was later marketed under the name Biosynsorb® (*59*). This filter was used in several transplantation centers (*17, 27, 51*) However, both products were withdrawn from the market in the early 1990´s due to adverse effects. Reported side effects are similar to first-use syndrome in dialysis with shortness of breath, chest pain, back pain, and even sudden death (*51*).

5.3.3 BioSorbent

Rieben et al developed a column with synthetic A or B trisaccharides covalently coupled to macroporous glass beads via polyacrylamide. An *in vitro* study published in 1995 (*60*), demonstrated good efficacy and biocompatibility with human plasma but this system has not been marketed yet.

5.3.4 Glycosorb ABO® column

A new system for antigen-specific removal of anti-A/B antibodies became available in 2001. The Glycosorb ABO® column is based on synthetic terminal A or B trisaccharide, covalently linked to a sepharose matrix via a six-spacer hydrocarbon molecule. The first clinical transplantation using this system was performed at Karolinska University Hospital Huddinge in September 2001 (*61*) and since then 45 patients have received ABOi kidney transplantations at our center. The system has been very well tolerated and without any serious side effects (*34, 62-64*). *Figure 7:*
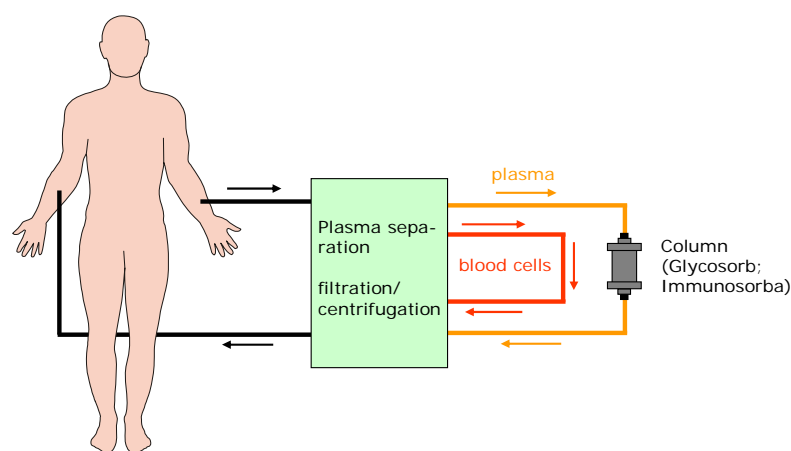
Immunoadsorption

5.4 EXPERIMENTAL MODELS

5.4.1 Whole blood filter for depletion of anti-A/B

A hollow fiber apheresis filter with antigen attached to the fiber membrane surface allows depletion of antibodies from whole blood without prior plasma separation. Synthetic terminal A-trisaccharide is attached to polyacrylic acid. An experimental filter with a total luminal area of 24 cm^2 was found to deplete up to 70% and 35% of monoclonal anti-A IgM (Immucor) antibody from the reservoirs of 7mL of buffer with initial concentration 1μg/mL and 5μg/mL, respectively (65). Clinical use will require a larger filter. The depletion of anti-A/B from whole blood is an advantage but the use of polyacrylic acid may be a disadvantage. The negative charge of polyacrylic acid can activate the bradykinin system and cause adverse effects in clinical use, especially if combined with ACE-inhibitors.

5.4.2 Absorber ABO column

The Swedish biotech company AbSorber is developing another antigen-specific apheresis filter. In this system the terminal ABO oligosaccharides are linked to the various blood group core chains on a mucin carrier-protein which allows depletion of chore chain specific antibodies as well as trisaccharide specific antibodies. The choice of antigen potentially increases the efficacy of the system (66).

5.4.3 In vivo depletion of anti-A

In an experimental mice model (67), blood group antibodies are eliminated in a two step approach. Similar to human blood group O or B individuals, mice have naturally occurring antibodies against blood group A carbohydrates in their sera. Mice B cells with receptors for A carbohydrates and belonging to the CD5$^+$CD11b$^+$ B-1a subset have phenotypic properties similar to those of human B cells. ABO blood group antibodies are produced by B-1 cells (CD5$^+$CD11b$^+$ B-lymphocytes). Cyclosporin (Cya) treatment blocks the differentiation from B-0 to B-1 cells. B-lymphocyte differentiation to antibody producing cells is prevented with Cya treatment. Existing antibodies are eliminated using infusion of synthetic blood group antigen linked to BSA plus anti-BSA antibodies which leads to elimination of antigen-antibody complexes in the spleen.

Figure 8: From Irei, T et al. Blood 2007;(110):4567-4575

15
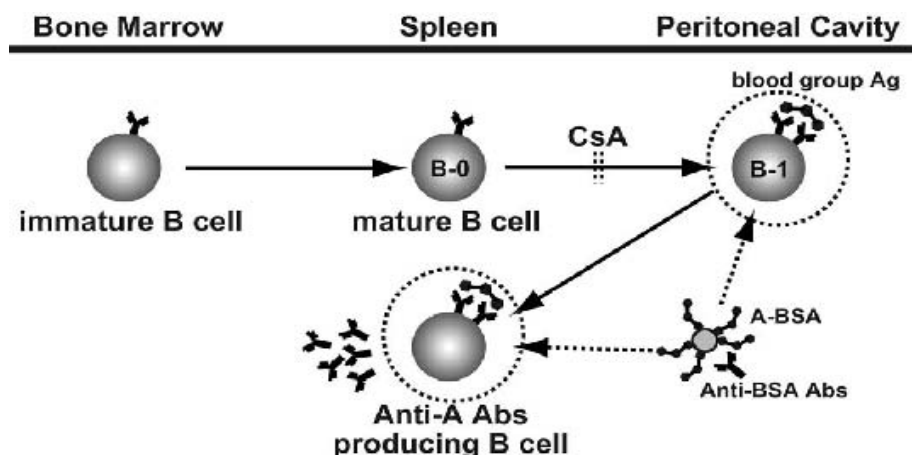
6 PHOTOPHERESIS

6.1 BACKGROUND

Photopheresis or extracorporeal photopheresis (ECP) was introduced by Edelson and coworkers in 1987 for the treatment of Sézary syndrome and other cutaneous T-cell lymphomas (CTCL) (*68*). ECP is an apheresis-based immunomodulatory therapy where autologous mononuclear cells are treated with a light sensitive agent that is subsequently activated with ultraviolet irradiation type A (UVA). After PUVA-treatment, cells are retransfused to the patient. The mechanism of action for the immunomodulatory effect remains largely unclear.

6.2 TECHNIQUES

Traditionally, 8-methoxypsoralen is used in ECP in spite of psoralens being a large group of light sensitive substances with different characteristics, some of which are currently being used for the inactivation of pathogens in blood products. The ECP-treatment can be performed by using a specialized apheresis machine (THERAKOS UVAR®XTS™) or by using any apheresis machine for separation of mononuclear cells combined with subsequent PUVA-treatment using a separate UVA-irradiation device (*69, 70*). The UVA-irradiation device Bio-Génic, previously marketed by Vilber-Lourmat, France, is now CE-certified and marketed by Macopharma, France, under the name MACOGENIC™ (*71*). The latter system allows more flexibility for the apheresis department as a Cobe Spectra Apheresis machine can be used to perform many different types of apheresis procedures, including ECP.

6.3 INDICATIONS

ECP is approved by the U.S. Food and Drug Administration (FDA) for the treatment CTCL. A multitude of T cell mediated diseases have been treated with ECP but most commonly rejection after organ transplantation (*72*) and GVHD after hematopoietic stem cell transplantation (*73-76*). There are a few case reports on the use of ECP in kidney transplantation (*77-79*). ECP has been used both to treat and to prevent rejection after cardiac transplantation (*80, 81*). A large number of ECP treatments have been performed since the introduction in the early 1980´s but there are no reports of serious side effects.

6.4 MECHANISM OF ACTION

Psoralens transport passively over the cellular membrane and into the nucleus where they intercalate in the DNA helix. After activation with UVA, the molecule binds covalently to the DNA strands and this triggers a chain of reactions. It has been shown that lymphocytes and monocytes react differently to PUVA treatment. Lymphocytes go into apoptosis (*82*) and monocytes differentiate into antigen-presenting cells (*83*). The apoptotic cells are thought to induce immunomodulation after retransfusion. Induction of regulatory T cells during ECP treatment has been reported and is suggested as a key mechanism of action (*84, 85*).

7 AIMS OF THE STUDY

7.1 PAPER I

To introduce a new protocol for ABO incompatible kidney transplantation without splenectomy, based on antigen-specific immunoadsorption and rituximab.

7.2 PAPER II

To evaluate the safety and efficacy of the new Glycosorb ABO$^{®}$ column for antigen-specific depletion of ABO blood group antibodies.

7.3 PAPER III

To investigate if inter-center differences regarding preoperative levels of ABO blood group antibodies were method-related, and if so to investigate if gel microcolumn hemagglutination technique can be standardized to allow inter-center comparisons.

7.4 PAPER IV

To evaluate mid to long term results of the protocol for ABO incompatible kidney transplantation introduced in Paper I.

7.5 PAPER V

To evaluate the results of post transplant photopheresis treatment of acute cellular rejection, that is refractory to conventional therapy.

7.6 PAPER VI

To investigate if photopheresis treatment as anti-rejection prophylaxis after kidney transplantation a) is safe b) improves the clinical outcome and c) induces immunological tolerance.

8 RESULTS AND DISCUSSION

8.1 PAPER I

8.1.1 Background

In this paper, a new protocol for ABO incompatible kidney transplantation is introduced. Historically, ABO incompatible kidney transplantations had only been undertaken after reinforced immunosuppressive protocols including quadruple drug immunosuppression plus B-cell specific drugs and splenectomy to prevent rebound of ABO antibodies. These reinforced protocols had limited use due to increased morbidity and even mortality. Also, ABO antibodies were depleted preoperatively using unspecific methods, most often plasma exchange (TPE). During a TPE session, several liters of patient plasma are discarded and substituted with colloid solutions and/or donor plasma.

8.1.2 Material and Methods

In the new protocol, conventional triple drug immunosuppression was used plus a single infusion of rituximab, anti-CD20 monoclonal antibody, instead of splenectomy. Moreover, a new antigen-specific apheresis technique, the Glycosorb ABO® column, was used for removal of anti-A/B from the patient´s circulation before transplantation. Four patients were reported and the donor/recipient blood group combinations were A_2/O, B/O, B/A and A_1/O.

8.1.3 Results

The clinical results were comparable with ABO compatible kidney transplantation and no serious side effects of the new protocol were noted. The patient group was small but the clinical results were so encouraging that data was published.

8.2 PAPER II

8.2.1 Background

Data from the 15 first patients receiving ABO incompatible kidney transplantations with the new protocol that was introduced in Paper I, were evaluated regarding safety and efficacy of the new antigen-specific apheresis filter.

8.2.2 Material and Methods

8.2.2.1 Glycosorb ABO® column

The new Glycosorb ABO® column contains synthetic terminal A or B trisaccharides covalently linked to a Sepharose® particle matrix with a patented six-hydrocarbon spacer molecule. The columns are steam sterilized, have luer-lock fittings and are European Council certified as a medical device since November 2001. In vitro testing of human plasma revealed no unfavorable effects. Since September 2001, we have had the opportunity to be the first center to evaluate this new apheresis filter in a clinical setting.

8.2.2.2 Regeneration

The column is registered for single use only but circumstances in the beginning of the project, with a severe shortage of columns for a patient already in treatment, forced us to develop a routine for regeneration. The columns can easily be regenerated using a similar method as for regeneration of protein A columns. The bound antibody is eluted with a citrate solution and the columns are stored at +4C filled with buffer and preservative. We have reused columns up to 10 times with preserved function. No infectious complications correlated to reuse of columns have been noted.

8.2.2.3 Patients

In this group of 15 patients, the donor/recipient ABO blood groups were A_1/O (n=5), A_2/O (n=2), B/O (n=4), B/A (n=2) and A_1B/B (n=2).

8.2.2.4 Apheresis

The Cobe Spectra apheresis system is not designed for column treatment but the TPE apheresis program was modified for use with the column.

8.2.2.5 Transfusion

Usually blood transfusion is not needed in kidney transplantation but sometimes bleeding complications occur and thus is it important to prevent transfusion of graft incompatible plasma into these patients. Therefore special transfusion regulations were introduced and managed through the blood bank database ProSang.

8.2.2.6 Antibody depletion

Serum samples were collected from the patients before and after each IA. Plasma samples were collected immediately after the column at the end of each IA to investigate the performance. Anti-A/B titers were analyzed at the serology lab of the Department of Transfusion medicine using a tube hemagglutination technique. Immunoglobulins were analyzed at the Department of Clinical immunology. Analyses of coagulation factors were performed at the Department of Clinical Chemistry.

8.2.2.7 Titration of anti-A/B antibody

Titration was performed using a tube hemagglutination technique. Direct agglutination in saline (NaCl) was interpreted as IgM titer and indirect agglutination with monoclonal IgG was interpreted as IgG titer. Kidney donor erythrocytes were used as test erythrocytes. With each sample, a previously titrated patient sample was reanalyzed as internal control.

8.2.3 Results

8.2.3.1 Anti-A/B titers

During each preoperative IA, both IgM and IgG anti-A/B titers were lowered by a median of two dilutions (range one to four dilutions). Titers were lowered to 1:4 before kidney transplantation was performed. In three of the four adult patients receiving A_1 kidneys, more than four IA´s were needed to achieve this.

8.2.3.2 Immunoglobulin levels

Levels of IgM, IgA, IgG as well as IgG subclasses IgG1, IgG2 and IgG3 were significantly reduced already after the first IA. Levels of specific antibody against protein (tetanus) antigen was not affected (n=12), whereas levels of specific antibody against carbohydrate (pneumococcal) antigen were lowered in two of 12 patients.

8.2.3.3 Coagulation factors

Fibrinogen was significantly lowered but only to slightly subnormal levels. Activated partial thromboplastin time, antithrombin and fibrin D-dimer were analyzed but results were highly variable and inconclusive.

8.2.3.4 Clinical outcome

Clinical outcome for the 15 patients was excellent and even better than compared with ABOc kidney transplant recipients. No rejections were seen, neither cellular nor humoral. No serious side effects related to the new protocol was noted.

8.3   PAPER III

8.3.1 Background

The data from Paper I and a following publication from our center in 2005 (*62*) gained interest in other transplant centers and soon the new protocol was implemented mainly in European centers. Data from 60 consecutive ABOi kidney transplantations performed in Stockholm, Uppsala and Freiburg, Germany, revealed significant differences regarding preoperative anti-A/B titers (*34*). We wanted to investigate if these differences were method related, and if so we wanted to investigate if hemagglutination could be standardized to allow inter-center comparisons of clinical data.

8.3.2 Material and Methods

As a first step, the same samples from 21 healthy blood donors were analyzed regarding anti-A/B titers in the three centers, using current local methods. In Stockholm gel microcolumn hemagglutination technique was used and in the other two other centers different tube techniques were used. As a second step the same number of samples were analyzed in the three centers using the same gel hemagglutination method and the same test RBC´s.

8.3.3 Results

Results revealed method related differences that correlated with the different anti-A/B levels in the patient groups (*34*). When the same method and the same test RBC′s were used, variability of titer results was significantly reduced.

8.4 PAPER IV

8.4.1 Background

The protocol for ABOi kidney transplantation that was introduced at our center in 2001 showed excellent short-term clinical results. In this study we wanted to assess mid-term to long-term results.

8.4.2 Material and Methods

Twenty ABOi kidney recipients (15 adult, five <16 years) with more than 12 months follow-up was included in the study and were compared with a group of ABOc kidney recipients (30 adult, 18<16 years). The study was designed as a comparative retrospective, cross-sectional study. Baseline and clinical data were collected and statistically evaluated. To assess transplant function, s-creatinine, change in s-creatinine from 3 months posttransplant (Δs-creatinine), GFR (glomerular filtration rate) as well as proteinuria at 1, 2 and 3 years posttransplant was compared.

8.4.3 Results

Mean follow-up was three years. There was no significant differences regarding patient survival, graft survival, incidence of acute rejections or infectious complications. The conclusion is that ABOi kidney transplantation with this protocol is equivalent with ABOc transplantation, not only short-term but also long-term.

8.5 PAPER V

8.5.1 Background

In spite of modern immunosuppressive protocols, acute rejections still occur after kidney transplantation. For acute cellular rejection, standard first line therapy is high dose corticosteroid injection for three consecutive days. As second line therapy, anti T cell antibody is given. Most commonly, polyclonal anti-thymocyte globulin (ATG) or monoclonal OKT-3, directed against CD3 epitopes in the T cell receptor complex, is used. At present there is no pharmacological third line therapy.

8.5.2 Material and Methods

We treated seven consecutive kidney transplant recipients, with acute cellular rejections that did not respond to conventional first and second line rejection therapy, with extracorporeal photopheresis (ECP). Kidney biopsies confirmed ongoing acute cellular rejections after antibody therapy was given. Autologous mononuclear cells were collected with Cobe Spectra apheresis system and cells were PUVA-treated separately

before retransfusion into the patients. A median of 10 ECP (range 6-26) treatments were given. No protocol biopsies were performed after ECP treatment.

8.5.3 Results

Clinical evaluation after six and 12 months revealed that none of the patients had lost the graft function. Serum creatinine levels indicated preserved kidney function in five patients and improved kidney function in two. Since patients with acute rejections that do not respond to anti T cell antibody treatment usually lose the graft function, we found the results in this small group of patients very encouraging and aimed to further explore the use of ECP to induce tolerance in kidney transplant patients.

8.6 PAPER VI

8.6.1 Background

Based on the encouraging results in the pilot study, we aimed to investigate if ECP can be used as prophylaxis for acute rejection after kidney transplantation.

8.6.2 Material and Methods

The study was initially planned as a randomized study but because not all patients had a functioning apheresis access, this was changed into a case control study. Patients receiving first time ABOc kidney transplants were included and immunosuppression was given with prednisolone, azathioprine and tacrolimus. Ten patients were given additional treatment with eight ECP sessions on postoperative days five to 27. Another 10 patients did not receive ECP treatment and constitute the control group. Flow cytometric analyses of mononuclear cells were performed regularly during ECP treatment to investigate innate and adaptive immunity.

8.6.3 Results

Event-free survival time was not significantly different in the two groups. No ECP related side effects were noted. A significant increase of $CD4^+CD25^{hi}FoxP3^+$ regulatory T cells was induced during ECP treatment. We found that ECP treatment was safe in this group of patients but that our patients groups were too small to reveal any difference in clinical outcome. The fact that an adaptive immune response with a tolerogenic shift is induced during ECP treatment is promising.

8.7 DISCUSSION

Many patients with end-stage renal disease will never receive their ideal treatment, kidney transplantation, due to the severe shortage of organs for transplantation.
In this thesis, the shortage of kidneys for transplantation is addressed in two different ways.

 The pool of living donors can be expanded if ABOi kidney transplantations can be done safely. In the first part of the thesis, Paper I-IV, a new protocol for ABOi kidney transplantation with conventional immunosuppression and without splenectomy is introduced and evaluated. The protocol has proved to be safe and with excellent clinical outcome, equal to ABOc kidney transplantation both short and long term. A new and specific apheresis technique for depletion of anti-A/B, the Glycosorb ABO® column, has been implemented and evaluated. It was shown that anti-A/B antibodies were safely and readily depleted with the new technique. Through collaborative analyses is has been shown that the semi-quantitative gel hemagglutination technique for determination of anti-A/B can be standardized to allow inter-center comparison of clinical results.

The short-term graft survival after kidney transplantation is excellent and has improved much over the last decades whereas long-term graft survival has not.

In the second part of the thesis, Paper V-VI, the long-term graft survival has been addressed by investigating if extracorporeal photopheresis (ECP), an immunomodulatory apheresis technique, can be used to induce tolerance after kidney transplantation. The good clinical result in the pilot study and the fact that a significant increase of tolerogenic $CD4^+CD25^{hi}FoxP3^+$ regulatory T cells is induced during ECP treatment is promising.

9 FUTURE PROJECTS

9.1   ADAPTIVE IMMUNE RESPONSE AFTER PHOTOPHERESIS TREATMENT

Paper VI reveals that $CD4^+CD25^{hi}FoxP3^+$ regulatory T cells increase significantly during ECP treatment. Thus, an adaptive immune response with a tolerogenic shift is induced. We aim to further explore the adaptive immune response after ECP treatment. The study plan includes flow cytometry, cytokine release and molecular biology techniques. In the first study we plan to use mononuclear cells, that would otherwise be discarded (buffy coat), from healthy blood donors and thus approval from the local ethics committee will probably not be needed.

9.2   PHOTOPHERESIS FOR THE TREATMENT OF GVHD

Evaluation of some 40 patients with acute or chronic GVHD who were treated with photopheresis at Karolinska University Hospital Huddinge after not responding to conventional GVHD treatment. The study has been approved by the local ethics committee.

9.3   FLOW CYTOMETRIC ANALYSIS OF ABO BLOOD GROUP ANTIBODIES

Flow cytometric analysis of frozen plasma samples from patients who have received ABO incompatible kidney grafts at our center, to investigate IgG subclass distribution and occurrence of antibodies with core chain specificity.

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