**Thesis for doctoral degree (Ph.D.)**
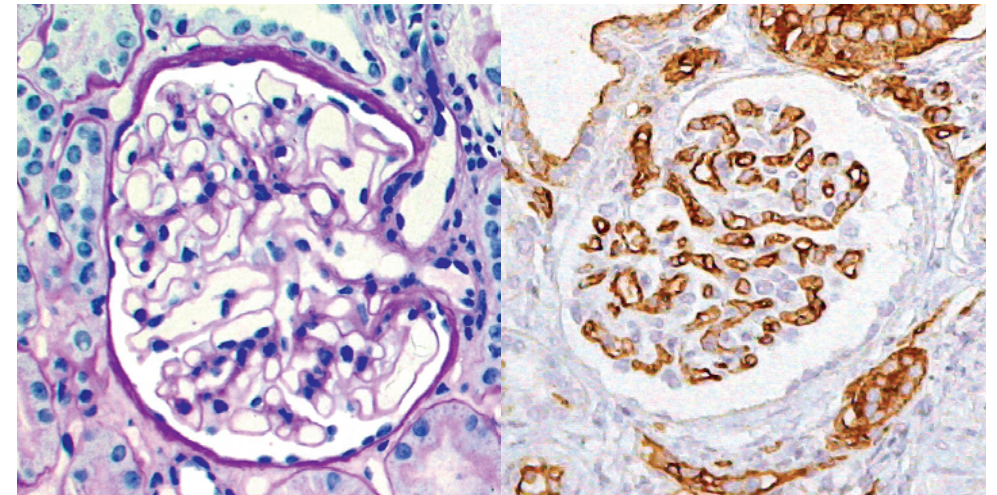**2008**

# AB0-INCOMPATIBLE KIDNEY TRANSPLANTATION USING ANTIGEN-SPECIFIC IMMUNOADSORPTION AND RITUXIMAB

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Stockholm 2008

Cover:

Right photo: Periodic acid Schiff-staining (PAS) of normal blood group A kidney tissue, kindly provided by Magnus Söderberg.

Left photo: Immunohistochemical staining of kidney tissue for the detection of A antigen, kindly provided by Anneli Hansson.

# LIST OF PUBLICATIONS

This thesis is based on the following papers, referred to by their Roman numerals:

Paper I. Tydén G, Kumlien G, Genberg H, Sandberg J, Lundgren T, Fehrman I. ***AB0- incompatible kidney transplantations without splenectomy, using antigen-specific immunoadsorption and rituximab.*** Am J Transplant. 2005 Jan;5(1):145-8.

Paper II. Genberg H, Kumlien G, Wennberg L, Berg U, Tydén G. ***AB0 -incompatible kidney transplantation using antigen-specific immunoadsorption and rituximab: a 3-year follow-up.*** Accepted for publication in Transplantation.

Paper III. Genberg H, Kumlien G, Wennberg L, Tydén G. ***No Rebound of A/B Antibodies after AB0-Incompatible Kidney Transplantation using Antigen-specific Immunoadsorption and Rituximab.*** Submitted in 2008

Paper IV. Genberg H, Hansson A, Wernerson A, Wennberg L, Tydén G. ***Pharmacodynamics of rituximab in kidney allotransplantation.*** Am J Transplant. 2006 Oct;6(10):2418-28.

# ABSTRACT

As the demand for kidney transplantation is constantly growing methods to expand the donor pool have become increasingly important. AB0-incompatibility has hitherto been regarded as an absolute contraindication to living donor donation. However, as AB0-incompatibility has accounted for the majority of living donor exclusions, efforts have been made to overcome this immunologic barrier. Successful desensitization protocols thus far, have combined plasmapheresis for antibody removal with splenectomy to reduce the antibody producing B-cell pool, in addition to quadruple immunosuppression. Although good graft function has been achieved, the high risks involved have been deterrent.

A protocol for AB0-incompatible kidney transplantation based on antigen-specific immunoadsorption and rituximab, in combination with standard maintenance immunosuppression (tacrolimus, mycophenolate mofetil and corticosteroids) was developed. We hypothesized that the anti-A/B antibodies could be effectively eliminated and good graft function achieved, without the complications of coagulopathy and transfusion reactions associated with plasmapheresis. Furthermore, we hypothesized that the substitution of splenectomy with a single dose of the B-lymphocyte depleting antibody, rituximab, would abolish the surgical risk and reduce the risk of infectious complications related to splenectomy.

From Sept 2001 to Oct 2007 a total of 39 patients underwent conditioning for AB0-incompatible kidney transplantation according to the protocol. Median follow-up was 2 years. In 38 out of 39 patients the anti-A/B antibodies could be effectively removed and transplantation performed as planned. The antigen-specific immunoadsorption was well tolerated without any serious side effects. Overall patient survival was 97.4% and graft survival was 86.8%. Kidney function was evaluated in a short and long term perspective, the results being equivalent to those of AB0-compatible living donor kidney transplantation. The incidence of antibody-mediated rejection was 2.6% and there was no significant rebound of anti-A/B antibodies during the study period. However, AB0-incompatible kidney transplantation was associated with an additional cost of approximately €32,000 compared with standard AB0-compatible living donor kidney transplantation.

B-lymphocytes were effectively eliminated long-term in peripheral blood as well as within the kidney transplant. In the lymphoid compartment, the B-lymphocytes were reduced. Despite B-lymphocyte depletion, there was no increased risk of infection following AB0-incompatible kidney transplantation compared with AB0-compatible transplantation.

We conclude that AB0-incompatible kidney transplantation using a protocol based on antigen-specific immunoadsorption and rituximab, in combination with triple immunosuppressive therapy is safe and effective. AB0-incompatibility following this protocol does not have a negative impact on graft function. AB0-incompatible kidney transplantation using this protocol is equivalent to standard AB0-compatible living donor kidney transplantation.

# CONTENTS

# LIST OF ABBREVIATIONS

| | |
|---|---|
| Ab | Antibody |
| AB0c | AB0-compatible |
| AB0i | AB0-incompatible |
| ALG | Antilymphocyte globulin |
| ATG | Antithymocyte globulin |
| BSA | Body surface area |
| CAN | Chronic allograft nephropathy |
| CD | Cluster of differentiation |
| CDC | Complement-dependent cytotoxic |
| CMV | Cytomegalovirus |
| CNI | Calcineurin inhibitor |
| DD | Deceased donor |
| DSA | Donor-specific antibodies |
| EBV | Epstein-Barr virus |
| FACS | Fluorescence-activated cell sorter |
| GFR | Glomerular filtration rate |
| HLA | Human leukocyte antigen |
| IA | Immunoadsorption |
| IF/TA | Interstital fibrosis/Tubular atrophy |
| IL | Interleukin |
| IL-2 | Interleukin-2 |
| IR | Resistance index |
| LD | Living donor |
| mAb | Monoclonal antibody |
| MMF | Mycophenolate mofetil |
| OPTN | Organ Procurement and Transplantation Network |
| RBC | Red blood cell |
| TA | Therapeutic apheresis |
| UNOS | United Network for Organs Sharing |
| WBC | White blood cells |
| XM | Crossmatch |

# KIDNEY TRANSPLANTATION

**The field of kidney transplantation has evolved during the past century. After several failed attempts from the beginning of the century onwards, the first successful transplantation was performed in 1952. Since then, kidney transplantation has developed as a therapeutic alternative, and become the treatment of choice for most patients with end-stage renal disease.**

**AB0-incompatible (AB0i) kidney transplantation is the focus of this work. Therefore other aspects of kidney transplantation will only be covered briefly.**

## History

Between 1900 and 1950 several attempts to transplant kidneys in humans were undertaken. The knowledge of transplant immunology was limited and no immunosuppression was used. Consequently, all of these transplantations were unsuccessful. However, through animal studies, the knowledge in transplant immunology grew and in the 1940's Billingham, Brent and Medawar could establish some principles of transplant immunology. By studies on mice, it was demonstrated that organs could successfully be transplanted between genetically identical subjects [1]. In concordance with these findings, first long-term graft survival in kidney transplantation was obtained using the homozygotic twin brother of the recipient as a donor. The Nobel Prize laureate Joseph Murray performed this kidney transplantation in 1952[2]. Since then the field of transplantation and transplant immunology has grown significantly. A major advancement was the introduction of cyclosporine in the beginning of the 1980's.

## Immunosuppression

When the first kidney transplantations were performed no immunosuppression was available. It soon became clear that transplantation of organs between individuals that were not genetically identical would, almost without exception, be rejected. To overcome this problem, attempts with full-body irradiation were undertaken. Although full-body irradiation provided some immunosuppressive effect, graft survival was poor and the side effects severe. Not seldom did the kidney recipients die of infections. This strategy was therefore abandoned.

In 1959, 6-mercaptopurin was introduced as an immunosuppressant. The closely related substance azathioprine was discovered shortly thereafter and had fewer side effects. Azathioprine in combination with corticosteroids soon came to replace other immunosuppressive therapies and remained the mainstay maintenance immunosuppressive therapy for several decades. With this, generally well tolerated, treatment, long-term graft survival was achievable. However the rate

of early graft loss was considerable with 1-year graft survival of approximately 50%.

When the calcineurin-inhibitor (CNI), cyclosporine, was introduced in the beginning of the early 1980's, short-term results improved significantly. In kidney transplantation 1-year graft survival increased from circa 50% to around 80%. In liver transplantation the effect was even more pronounced with 1-year graft survival increasing from 18% to 68%. Until the mid-1990s cyclosporine and azathioprine became the cornerstones in maintenance immunosuppressive therapy.

## Current immunosuppressive therapy

Over the past decade maintenance immunosuppressive therapy has become more diversified. New agents, such as the CNI tacrolimus (Prograf®) and the anti-metabolite mycophenolate mofetil (CellCept®) have been developed. In kidney transplantation a combination of three immunosuppressive drugs has been the standard of care for a long time. Today the most widely used immunosuppressive protocols combine tacrolimus, mycophenolate mofetil and corticosteroids[3]. Several studies comparing cyclosporine and tacrolimus have been conducted, generally showing equal efficacy of these two drugs. In a meta-analysis performed by the Cochrane library in 2005, the conclusion was that the use of tacrolimus re-

sulted in better graft function and fewer rejections but, on the other hand, was associated with an increased risk of post-transplant diabetes mellitus, compared with cyclosporine[4].

A major drawback to using CNIs is their potential for nephrotoxicity. They cause both acute arteriolar vasoconstriction, resulting in a decreased glomerular filtration rate (GFR), and chronic vascular and tubulointerstitial damage[5]. However, the therapeutic alternatives are few [3]. The latest conceptually new drug for maintenance immunosuppression on the market is sirolimus (Rapamune®) which was registered in 2001. The efficacy and tolerability of sirolimus in kidney transplantation is still in question[6, 7]. For this reason sirolimus is only used routinely at a few centers today[3]. Consequently, there is no clinically realistic alternative to the two CNIs cyclosporine and tacrolimus currently available. Thus, much focus today is aimed at the development of new low-toxicity protocols[8].

## Therapeutic antibodies

As induction and anti-rejection therapy, polyclonal antibodies to human lymphocytes, from rabbit and horse, have been in use in transplantation for several decades. These include anti-lymphocyte globulin (ALG) targeting both B and T lymphocytes, and antithymocyte globulin (ATG), primarily targeting the T lymphocytes. Although very effective, these antibodies are

immunogenic and treatment with them is associated with severe infusion-related reactions[9]. The field has expanded rapidly over the past decade and with the emergence of modern recombinant techniques, less immunogenic chimeric or humanized antibodies have been developed. The monoclonal humanized antibodies now constitute the fastest growing class of drugs[10].

Three monoclonal antibodies (mAbs) have been approved for use in transplantation but off-label use of other mAbs, primarily approved for use in hematological malignancies is not uncommon. The approved mAbs include the anti-CD3 murine T cell- depleting antibody muromonab-CD3 (Orthoclone OKT3®) registered in 1989, and the interleukin-2 (IL-2) receptor antagonists basiliximab, (Simulect®) and daclizumab (Zenapax®), both registered in the late 1990's.

### Muromonab-CD3 (OKT-3)

Muromonab-CD3 is the only approved mAb for the treatment of acute rejection. The drug is very potent but side effects are common. Early side effects include an acute severe influenza-like syndrome. A late but serious side effect is the development of lymphoma[11]. Its use in kidney transplantation today is limited.

### IL-2 receptor antagonists

The IL-2 receptor antagonists basiliximab and daclizumab are approved for induction therapy. Treatment with these mAbs is generally very well tolerated and the side effects are few. However, although the use of these mAbs is widespread, the clinical benefit has been questioned. In a Cochrane analysis, a reduced incidence of acute rejection was observed, but without any significant effect on patient or graft survival[12].

### Other therapeutic antibodies

Newer depleting antibodies used in kidney transplantation include the anti-CD20 mAb rituximab (Mabthera®, Rituxan®) and the anti-CD52 mAb alemtuzumab (Campath-1H). Rituximab was registered in 1997 for the treatment of lymphoma and alemtuzumab in 1999 for the treatment of chronic lymphocytic leukemia. As both agents induce a rapid and sustained elimination of cells expressing the target molecule, they have gained much interest in the field of transplantation.

*Rituximab is discussed in a separate section on B-cell depletion.*

The target molecule of alemtuzumab is CD52, a cell surface marker expressed on most mononuclear cells, including both B and T lymphocytes. The immunosuppressive effect is profound, as the majority of mononuclear cells are depleted for a long-term period[13]. Alemtuzumab is primarily used as induction therapy. Two randomized trials have been conducted, indicating that alemtuzumab induction enables a reduction in maintenance immunosuppression[14, 15]. Although

the use of alemtuzumab in transplantation is rapidly increasing, its use so far has been limited[16]. Another promising mAb in kidney transplantation is belatacept (CTLA4-Ig), a co-stimulatory blocker, in a clinical trial showing equal or better results compared with cyclosporine[17].

## Results

Kidney transplantation now has become an established treatment for end-stage renal disease. To date > 250,000 kidney transplantations have been performed only in the USA. Since the first kidney transplantation in Sweden in 1964, at total of 11,339 kidney transplantations has been performed here, as of 2007.

The prognosis after kidney transplantation as well as the quality of life is better compared with any type of dialysis[18]. Many centers report a 1-year patient and graft survival rate of >95% after living donor kidney transplantation[19, 20]. In reports from large registries, such as the Organ Procurement and Transplantation Network (OPTN)/United Network for Organs Sharing (UNOS) registry, similar results are found[21].

However, many factors may influence outcome. These include donor and recipient age, ischemia time, HLA matching, time on dialysis, primary kidney disease, immunization status and choice of immunosuppression[21, 22]. One of the most important factors

influencing the results is the donor source: In the UNOS data from 2002, 1-year graft survival was 95% following LD transplantation and 89% after DD transplantation[23]. The 5-year graft survival for LD kidney transplantation was 80% compared with 67% for DD kidney transplantation.

The 1- and 5-year patient survival, following LD kidney transplantation, was 98% and 90% in the same data base, and for DD transplantation 94% and 82%. (Data are based on transplantations performed in USA between 1997 and 2002). Recipients of living donor kidneys have a better graft and patient survival compared with deceased donor kidney recipients. The best patient and graft survival is obtained in LD kidney transplantation with HLA-identical siblings[21, 22].

In transplantations performed at the Karolinska University Hospital the difference in patient and graft survival rate depending on donor source is even more prominent. In an analysis of all kidney transplantations performed between 1990 and 2002, the graft survival at 10 years after LD kidney transplantation (n=363) was 70%, and patient survival 85%, compared with 40% and 55% following DD kidney transplantation (n=626) (*Figure 1*). Thus, graft survival was 57% higher and patient survival 65% higher in patients undergoing LD transplantation compared with those undergoing DD kidney transplantation (*unpublished data*). The discrepancy between Swe-

## Kidney transplantations in Stocholm 1990–2002
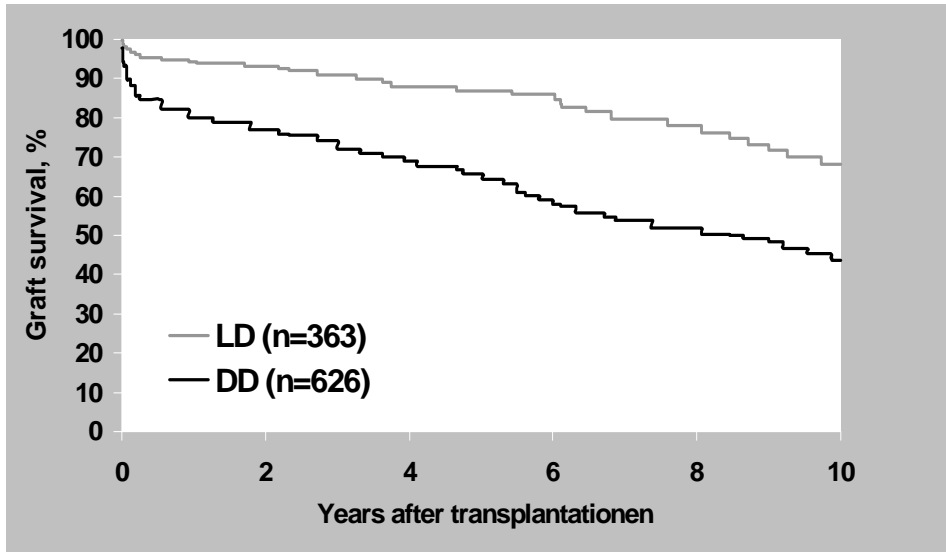


*Figure 1. Graft survival by donor source.*

dish and American data possibly reflects a different donor selection.

Despite a constant increase in kidney transplantations performed, owing to organ shortage, the number of patients waiting for a transplant is growing worldwide. New ways to expand the donor pool are therefore being explored. These include the use of donors after cardiac death (DCD), expanded criteria donors (ECD), paired exchange lists for living donors as well as the development of special immunosuppressive protocols to overcome immunological barriers, conventionally regarded as contraindications to living donor donation. Such protocols as usually referred to as desensitization protocols. *These are treated in a separate section*[24-26].

## Long-term results

A major challenge at present is to find ways to improve graft survival long-term. Long-term patient and graft survival did indeed increase after the introduction of cyclosporine by approximately ~60%. However, recent advances have been very modest. From 1988 to 1995 graft survival half-life increased by approximately 2 years[27]. In more recent analyses the rate of improvement is even less[28]. The major cause of graft loss is ascribed to chronic allograft injury, eventually affecting approximately 40% of all kidney transplants. The pathogenesis is multifactorial and includes CNI nephrotoxicity, hypertension and immune-mediated damage. The term chronic allograft nephropathy (CAN) has been used to describe such pathologic changes[29].

13

However the concept of CAN, as it was defined by the Banff classification of renal allograft pathology, has been criticized for being imprecise. A revision of the Banff criteria has therefore been undertaken. In the revised Banff classification, CAN is replaced with the term chronic allograft injury[30, 31]. The most typical chronic changes are now referred to as interstitial fibrosis and tubular atrophy (IF/TA). In the Banff 2005 criteria, the pathogenesis of the chronic changes, i.e. CNI nephrotoxicity, hypertension and chronic antibody-mediated rejection is emphasized. Information needed for correct therapy to be instituted will thereby not be omitted. Nonetheless, treatment of chronic allograft injury has so far often been ineffective.

An improvement in the long-term results would certainly be an effective approach to overcome the problems of organ shortage. The number of patients re-listed for kidney transplantation is at present accelerating.

## Immunologic barriers

As the demand for kidney transplantation is continuously growing, methods to expand the donor pool are becoming increasingly important. Living donor kidney transplantation does not only offer superior patient and graft survival compared with DD transplantation[32, 33], living donors is also a means to expand the total donor pool. However, immunologic barriers limits the potential use of living donors[24].

Preformed antibodies in the recipient, directed against various antigens expressed in the donor kidney, is the most common contraindication to LD donation[34]. Transplantation in the presence of such antibodies can evoke a hyperacute rejection. Commonly present preformed donor-specific antibodies (DSA) include AB0 antibodies and human leukocyte antigen (HLA) antibodies. Sometimes antibodies against endothelial cell structures of unknown specificity are found. These are referred to as non-HLA vascular endothelial cell (VEC) antibodies[35]. AB0 antibodies are natural antibodies, i.e. they are formed without prior antigen stimulation, whereas HLA antibodies and VEC antibodies may develop after exposure to foreign tissue. Such sensitizing events include previous blood transfusions, pregnancies and transplantations. Consequently, many of the patients listed for retransplantation have HLA antibodies, thus making them HLA-sensitized.

The immunological barriers posed by AB0-incompatibility and HLA-sensitization, account for an estimated 30-50% of all LD refusals. Therefore, effective desensitization, eliminating the donor-specific antibodies, could yield a substantial increase in the number of transplantations performed. For this reason, desensitization protocols are being developed.

## Desensitization protocols

The definition of desensitization is *"the reduction or abolition of reactions to a specific antigen or allergen"*. Herein, desensitization protocols refer to immunosuppressive protocols to allow for transplantation in the presence of donor-specific antibodies.

Current desensitization protocols are all based on the same principle of removing existing antibodies and preventing rebound of antibodies in the recipient after transplantation.

There are a number of apheresis techniques available for the removal of antibodies today. Generally several apheresis sessions are needed for efficient antibody removal, both prior to and after the transplantation. To prevent rebound, apheresis is combined with immunosuppressive therapy. The donor-specific antibody levels are monitored and transplantation performed when the antibodies are sufficiently reduced. This process normally takes 1-2 weeks[24]. For this reason desensitization is almost exclusively limited to LD transplantation. However, effective desensitization protocols are an important way to increase the number of LD kidney transplantations that can be performed.

## Summary

Over the past 50 years, kidney transplantation has evolved from a small-scale experimental treatment, to become the best therapeutic alternative for most patients with end-stage renal disease. However, graft survival is still limited and, in general, not long enough to maintain adequate renal function throughout the rest of the patient's life. As there is an ever-increasing demand for kidney transplantation, means to overcome the shortage of organs are gaining importance. Methods to expand the donor pool are being developed, including protocols to overcome immunological barriers, such as the one posed by the AB0 system. Successful AB0-incompatible kidney transplantation could lead to an increase in the number of living donor kidney transplantations performed by 20 to 30%.

Following is a summary of the field of AB0i kidney transplantation, including an analysis of protocols for AB0-incompatible kidney transplantation currently in practice, an overview of the evolution of AB0i transplantation as well as a brief discussion about new approaches.

# BLOOD GROUP SYSTEMS

To date 29 blood group systems have been recognized, together they comprise >300 antigens[36, 37]. Only a few of these are of importance in solid organ transplantation, i.e. blood group systems expressed on structures within the graft, such as the graft epithelium. The most important blood group

system is the AB0 system. Antibodies against the antigens of the AB0 system are naturally occurring. They are produced without any preceding sensitization event. In contrast, antibodies to antigens of the other blood group systems are normally only produced as a response to antigenic exposure, most commonly by blood transfusion or pregnancy. Apart from antigens of the AB0 system, antigens belonging to other blood group systems also expressed in the kidney, include antigens of the Lewis system, the Lutheran system, the Duffy system and the P system[38]. In kidney transplantation, the clinical relevance of antibodies against systems other than the AB0 system is not fully understood. To date there is evidence to support that Lewis antibodies may trigger antibody-mediated allograft rejection[39, 40]. The binding of Duffy antibodies to the Duffy antigen on RBCs is known to evoke a strong immunologic response. However, the importance of Duffy antibodies in kidney transplantation remains unknown. Potentially such antibodies could have clinical implications in kidney transplantation[41, 42]. Antibodies against the Lutheran and P system antigens, are normally not active at 37°C and are therefore of lesser importance[37].

Methods to determine blood groups are both serological as well as genomic. The density of the blood group antigen on the cell surface is sometimes but not always associated with the genotype of an individual. Sometimes

homozygous individuals express more antigen than heterozygous individuals, usually referred to as *"dosage"*. However, in the AB0 system, the Lewis system and the Duffy system dosage does not seem to occur[38, 43]. Thus, an individual with the genotype AA will express the same amount of A antigen on the cell surface as an individual of genotype A0. The antigen specificity of antibodies of the AB0, Lewis, and P blood group systems is most commonly an immunodominant sugar. The immunodominant sugar refers to the monosaccharide that determines the antigen specificity of the antibody formed[44].

*Blood group systems other than the AB0 system will not be discussed further.*

# The AB0 system

In 1900 Landsteiner discovered the first and most important blood group system, the AB0 system. According to the law he formulated, individuals of blood group A have antibodies against blood group B, while individuals of blood group B have antibodies against blood group A. AB individuals do not produce any AB0 antibodies and blood group 0 subjects have antibodies against both A and B (*Figure 2*).

As the A and B antigens are expressed on many cell types in the body, i.e. kidney, liver, intestine etc, and not only on erythrocytes, the same principles of AB0 matching generally applies in

**The AB0-system**

40%
**O** ag
anti A Ab
anti B Ab

45%
**A** ag
(A$_1$ ~36%, A$_2$ ~9%)

anti B Ab

10%
**B** ag
anti A ab

**A** ag**B** ag
no Ab

5%

The likelihood that two
unrelated individuals are:
- identical is 37.5%
- compatible is 26.75%
- incompatible is 35.75%
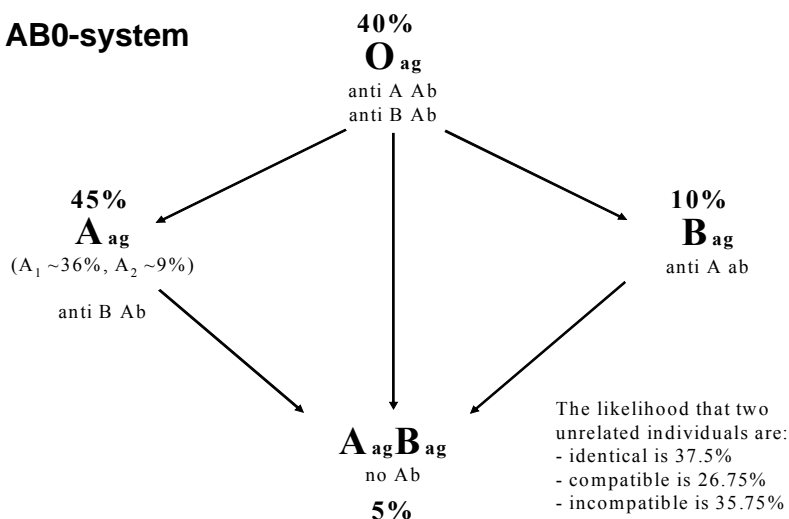
*Figure 2. The principles of AB0 blood group matching and the distribution of blood groups within the Caucasian population.*

*Blood group A antigens on core sacharride chain types 1-4*

A type 1: GalNAc$\alpha$1, 3(Fuc$\alpha$1, 2) Gal$\beta$1, 3GlcNAc$\beta$1-R

A type 2: GalNAc$\alpha$1, 3(Fuc$\alpha$1, 2) Gal$\beta$1, 4GlcNAc$\beta$1-R

A type 3: GalNAc$\alpha$1, 3(Fuc$\alpha$1, 2) Gal$\beta$1, 3GalNAc$\alpha$1-R

A type 4: GalNAc$\alpha$1, 3(Fuc$\alpha$1, 2) Gal$\beta$1, 3GalNAc$\beta$1-R

*Blood group B antigens on core sacharride chain types 1-4*

B type 1: Gal$\alpha$1, 3(Fuc$\alpha$1, 2) Gal$\beta$1, 3GlcNAc$\beta$1-R

B type 2: Gal$\alpha$1, 3(Fuc$\alpha$1, 2) Gal$\beta$1, 4GlcNAc$\beta$1-R

B type 3: Gal$\alpha$1, 3(Fuc$\alpha$1, 2) Gal$\beta$1, 3GalNAc$\alpha$1-R

B type 4: Gal$\alpha$1, 3(Fuc$\alpha$1, 2) Gal$\beta$1, 3GalNAc$\beta$1-R

transplantation, as in the transfusion of red blood cells (*Figure 2, 4 a and b*). The distributions of blood groups within the Caucasian population are displayed in *Figure 2*.

## Antigens of the AB0 system

The A and B blood groups are inherited in a co-dominant manner, while type 0 is recessive. The genes encoding the A and B genotype are found on chromosome 9p. The gene products are the enzymes glycosyltransferase (A gene) and galactosyltransferase (B gene). These enzymes are needed for the terminal monosaccharide to be added to the precursor oligosaccharide[45]. Individuals expressing both enzymes belong to the AB blood group. In blood group 0, the enzymes needed to add the terminal monosaccharide to the precursor oligosaccharide is lacking. However, in individuals of blood group 0, the H gene is expressed and encodes the enzyme fucose transferase. The final product is the precursor chain of antigen A and B (*Figure 3*) . Consequently, as the H antigen is a precursor of the A and B antigens, individuals of blood group A and B also express the H antigen and do therefore not produce antibodies against the H antigen. (*Figure 2*).

A very small number of individuals are of the Bombay or Reunion phenotype, characterized by the lack of the H antigen. These individuals produce H antibodies, whereas individuals of all other AB0 blood groups do not.

The blood group antigens are composed of a core saccharide, either at-

## Synthesis of AB0 antigens
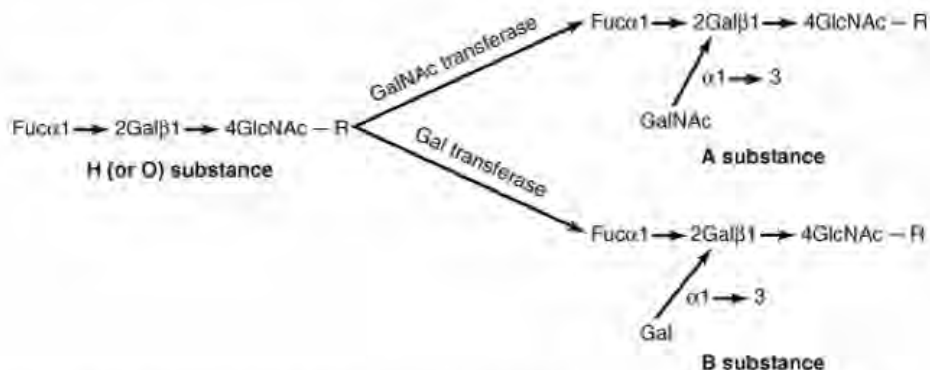
*Figure 3.*

*Figure 4 a.*

*In this immuno h i s t o - c h e m i c a l s t a i n i n g , antigen $A_1$ is visulized in brown. Photo k i n d l y provided by A n n e l i Hansson.*
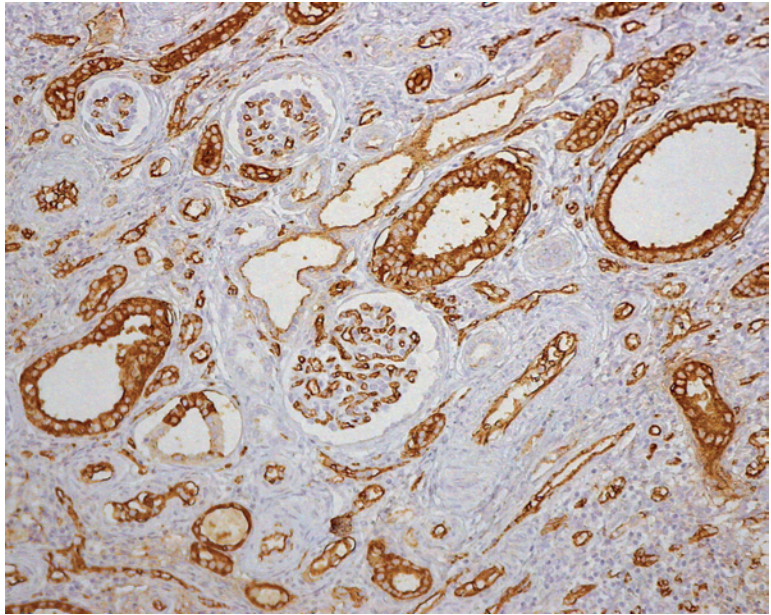
**Expression of $A_1$ and B antigen in kidney  tissue.**
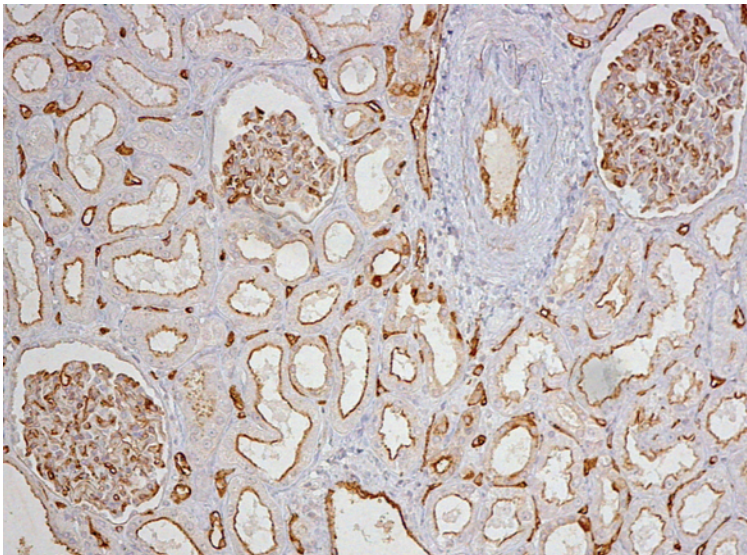


*Figure 4b.*

*In this immuno- histochemical s t a i n i n g , antigen B is visulized in brown. Photo k i n d l y provided by A n n e l i Hansson.*

tached to a lipid or to a protein, and terminal oligosaccharides. In blood group A and B the terminal oligosaccharide is a trisaccharide, whereas in blood group 0, the terminal oligosaccharide is a disaccharide.

There are 4 core chains known to day[45]:

Type 1 chain:
Galß1-3GlcNAcß1-R
Type 2 chain:
Galß1-4GlcNAcß1-R
Type 3 chain:
Galß1-3GalNAca1-R
Type 4 chain:
Galß1-3GalNAcß1-R

Thus, depending on the core chain and the terminal trisaccharide the AB0 antigens can be further subdivided [46]. *See page19.*

In blood group A, GalNAc is the immunodominant sugar, while Gal is the immunodominant sugar in blood group B[44].

The AB0 antigens are expressed on most cells in the body (*Figure 4 a and b*). However, the distribution of core saccharides varies in different organs. In the kidney as well as on the urothelial cells, core chain type 4 is the most common. On red blood cells however core chain type 2 is most frequently found[45].

## Blood group $A_1$ and $A_2$

Blood group A can be further subdivided into blood group $A_1$ and $A_2$. In the 1960's by studies on skin grafts, it was discovered that the $A_2$ phenotype was less immunogenic than the $A_1$, B or AB phenotype[47, 48]. The most apparent

## $A_2$ expression in kidney and liver.
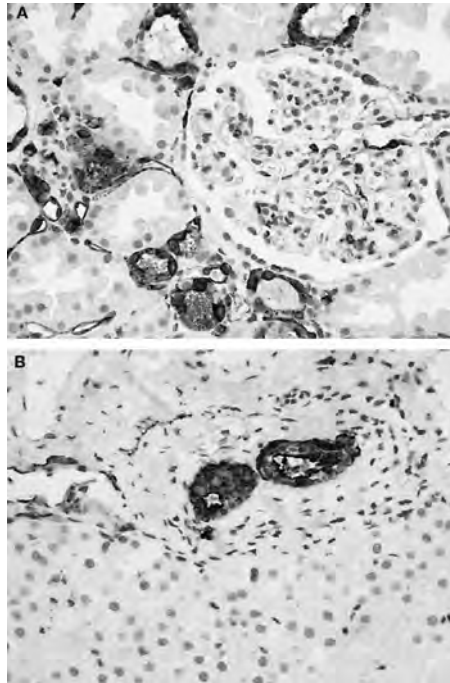


*Figure 5. Adapted from: L. Rydberg, U. Skogsberg, J. Mölne. **AB0 antigen expression in graft tissue: is titration against donor erythrocytes relevant?** Transplantation. 2007 Dec 27; 84 (12 S uppl): S 10–2. Top: $A_2$ expression in kidney. Bottom: $A_2$ expression in liver.*

difference between these subtypes is the density of antigen on the cell surface[49]. Consequently, in $A_2$ kidneys smaller amounts of A antigen are expressed compared with $A_1$ kidneys (*Figure 5*). The immunologic barrier posed by $A_2$ is therefore generally regar-

ded as weaker compared with $A_1$ or B.

However, there is also a qualitative difference in composition, as the $A_1$ subtype can make use of all four core saccharide chains whereas the $A_2$ subtype can only make use of core saccharide chain 1 and 2. Consequently, $A_2$-individuals may produce antibodies against the A-trisaccharide+core chain type 3 and 4. *The implications of such core chain-specific antibodies are discussed further below.*

## Secretor status

A distinction should be made about a person's *secretor* status. In so called secretors (genotype SeSe and Sese), A and B antigen is secreted from cells and soluble A/B antigen can be found in the circulation. As many microorganisms bind to A/B antigens on the cells surface, these soluble antigens may protect the cells against certain infections, by acting as blocking or neutralizing agents. *Secretor* phenotype is the most common in the population. In the *non-secretors*, (genotype sese), A/B antigens are not secreted. Thus, no soluble antigen is found in the circulation. It is known that such individuals are more susceptible to various infections, including infections caused by *Candida albicans*, *Streptococcus pneumoniae* and *Haemophilus influenzae*[38]. Moreover, the *secretor* genotype is related to the *Lewis* phenotype [50].

In AB0i transplantation it should be noted that the *secretor* phenotype is generally more immunogenic compared with the *non-secretor* phenotype, as the density of A/B antigen on the cell surface is higher in the *secretor* phenotype[51].

## Antibodies against the AB0 system

As mentioned, antibodies that can bind to the antigens of the AB0 system can occur naturally, i.e. without previous sensitization but they can also arise as a response to antigenic stimulation. Most commonly these antibodies are directed against the terminal trisaccharides of the A/B antigen. However, it has been shown that the specificity of some antibodies is a tetrasaccharide, i.e. the terminal trisaccharide + core chain[52, 53].

In further support of this observation, are studies from the 1970's, when it was noted that graft survival following AB0c kidney transplantation was superior in blood group 0 recipients compared with recipients of other blood groups[54]. As there was no matching for blood group $A_1$ and $A_2$, $A_1$ kidneys were transplanted into $A_2$ recipients. The existence of antibodies specific for $A_1$, i.e. A trisaccharide + core chain 3 or 4 in $A_2$ recipients receiving $A_1$ kidneys, has been proposed as one explanation for the reduced graft survival in non-0 kidney recipients.

The existence of core chain-specific antibodies can be of importance in AB0i kidney transplantation for seve-

ral reasons. Firstly, if erythrocytes are used as a surrogate of kidney tissue in the determination of anti-A/B antibodies, as is common practice today, the clinical relevance of the result may be equivocal[55]. Secondly, using antigen-specific immunoadsorption based on only the terminal A or B trisaccharide, to remove anti-A/B antibodies, such core chain specific antibodies will not be eliminated.

# AB0-INCOMPATIBLE KIDNEY TRANSPLANTATION

AB0-incompatible (AB0i) kidney transplantation has gained renewed interest over the past few years as new and more effective immunosuppressive therapies have evolved. The first AB0i kidney transplantation was performed in 1952 by Hume (USA)[56]. Partial graft function was obtained at first but on day 7 the kidney ceased to produce urine. The graft was eventually removed on day 17[56]. It became obvious that AB0-matching was very important for the transplantation to be successful. Nevertheless, a few (non-$A_2$) AB0i kidney transplantations were performed during the period of 1955 to 1987, without any special type of conditioning. Some of these were a result of AB0 mistyping, but a few were performed deliberately. Cook et al. analyzed the results of 25 DD AB0i kidney transplantations performed accidentally. Graft survival was only 4%

at one year. The authors concluded that the AB0 barrier remained intact after transplantation[57]. (The incidence of AB0 mistyping in this study was 0.5-1 per 1000 transplantations). Including these 25 transplantations, some 50 cases of AB0i kidney transplantation were performed during this time period, with an overall 1-year graft survival of approximately 10%[45, 56-60].

The first successful AB0i kidney transplantation was reported by Slapak et al. in 1981. It was a kidney recipient, who developed antibody-mediated rejection after accidental AB0 mismatch. He was effectively treated with plasmapheresis and the rejection resolved. Subsequently the same group performed another three AB0i kidney transplantations intentionally, with good outcome. They used both plasmapheresis and immunoadsorption for antibody removal. In 1982 in Belgium, Alexandre et al. instigated the first larger study on LD AB0i kidney transplantation[61, 62]. By 1989 a total of 39 LD AB0i kidney transplantations had been performed, with a 1-year graft survival of approximately 75%. According to Alexandre et al., splenectomy was necessary for the AB0i transplantation to be successful, as three patients in the very beginning, who where not splenectomized, lost their grafts. The protocol also included repeated plasmapheresis, the use of donor platelet transfusion and the administration of substance A (A trisaccharide) or substance B (B trisaccharide) depending

on the A/B incompatibility, antilympho-cyte globulin and triple maintenance immunosuppression (corticosteroids, azathioprine and cyclosporine). Based on their success, similar protocols were implemented at a few other centers worldwide, most notably in Japan.

In Japan the first AB0i kidney trans-plantations were performed in 1989. Protocols for AB0i kidney transplan-tation rapidly gained much attention, as the Japanese kidney transplant pro-grams heavily rely on living donors. Due to legislation, the number of decea-sed donors is very limited in Japan. Al-ready at an early stage the Japanese achieved results of the AB0i kidney transplantations similar to those of AB0c kidney transplantation, further supporting the AB0i programs[63, 64]. The AB0i programs in Japan have in fact been very successful and to date nearly 1000 AB0i kidney transplanta-tions have been performed.

Outside Japan, only a few AB0i kid-ney transplantations were performed between 1989 and 1999. Excluding transplantations with $A_2$ donors, some 10 cases were published during this pe-riod. Of these, all kidney recipients un-derwent splenectomy and the 1-year graft survival was 75%, a significant improvement compared with earlier results[45, 65].

# AB0 incompatible kidney transplantation using $A_2$ donors

In the 1960's it was demonstrated that organs from $A_2$ donors could be suc-cessfully transplanted into individuals of other blood groups. This finding together with a growing transplant waiting list for patients of blood gro-up 0, prompted the first clinical trial on $A_2$-incompatible transplantation. This clinical trial was conducted in Gothenburg, Sweden, between 1974 and 1988. A total of 23 DD kidney transplantations were performed, using only regular immunosuppression. Se-veral centers worldwide adopted this protocol. However, with growing ex-perience, it became clear that, a high anti-A/B antibody titre in the recipient, was strongly associated with an in-creased risk of early graft loss. One-year graft survival in the first 20 kid-ney transplantations performed in Gothenburg was 55%[66]. For this rea-son, at most centers, $A_2$-incompatible kidney transplantation was only per-formed with regular immunosuppres-sion if the anti-A/B antibody titres were low. Nelson et al. in Kansas, USA, having the largest series of $A_2$-incom-patible DD kidney transplantations, only accepted recipients with an anti-A/B antibody titre below or equal to 1:4[67].

In the beginning of the 2000's, AB0i kidney transplantation gained a rene-

wed interest both in Europe and the USA primarily owing to new therapeutic options that became available, such as rituximab and, in Europe, most notably the Glycosorb ABO® system *(as described below)*, but also as a corollary to the success in Japan.

# Risks in AB0-incompatible kidney transplantation

## Antibody-mediated acute rejection

Already in the 1950's, it was known that AB0-incompatibility would give rise to an antibody-mediated rejection hyperacute rejection, leading to graft loss within hours to days. For this reason AB0-incompatibility has been regarded as an absolute contraindication to transplantation for many decades. With the emergence of protocols to overcome AB0-incompatibility the incidence of hyperacute rejection was significantly reduced[62]. However, in many studies, a high incidence of acute antibody-mediated rejection has been observed following AB0i kidney transplantation[62, 63, 68-70]. With current desensitization protocols for AB0i kidney transplantation the incidence of antibody-mediated rejection has decreased[71].

## Chronic allograft injury

It has been demonstrated in several studies that the development of donor specific HLA antibodies after transplantation, is associated with a poor prognosis. However the risk of chronic allograft injury following AB0i transplantation is unclear. In an investigation by Gloor et al., the risk of chronic allograft injury was studied, comparing patients with either a positive crossmatch (XM) or AB0-incompatibility with a control group of consisting of conventional XM negative AB0c kidney recipients[72]. They observed an increased risk of chronic allograft injury in patients with a history of acute-antibody-mediated rejection. In *non-rejectors* the risk of chronic allograft injury was significantly lower, regardless of immunization status or AB0-incompatibility at transplantation. Thus, AB0-incompatibility *per se* did not increase the risk of chronic allograft injury. In a comparable study by Setuguchi et al. similar result was obtained[73].

## Infections

In order for the AB0i graft to be accepted, extensive immunosuppressive therapy is usually given. An increased risk of infection would therefore be expected.

In addition, the blood group antibodies may be protective of some diseases. Anti-B antibodies for example may reduce the risk of infections caused by *Salmonella*, *Shigella*, *Neisse-*

*ria gonorrhoeae* and *Escherichia coli*. Consequently depletion of these antibodies could further augment the risk of infection following AB0i transplantation[43]. There are a few publications on AB0i transplantation, evaluating the risk of infection[68, 74-78]. Only in one out five of these studies an increased risk of infection was actually observed, following AB0i kidney transplantation.

# Immunologic adaptation following AB0-incompatible kidney transplantation

Current results following LD AB0i kidney transplantation are similar to those of LD AB0c kidney transplantation[63, 72, 79] supporting that immunologic adaptation occurs after AB0i transplantation. Following AB0i transplantation several changes in gene expression have been observed, both within the graft itself and in the host's immune response. Exactly how the process of adaptation after AB0i kidney transplantation takes place is not fully known. Several mechanisms are probably of importance, including the up-regulation of immunoprotective genes within the transplant and a reduced anti-A/B antibody production after the AB0i kidney transplantation[80]. Such phenomena are usually referred to as *"accommodation"* and

*"tolerance"*. Although quite frequently used, there is no uniform definition of the terms accommodation and tolerance[72, 81, 82]. Herein the term *"adaptation"* will be used to describe all changes that occur following AB0i kidney transplantation, both in the recipient as well as within the transplant. *"Tolerance"* will refer to changes in the host's, i.e. the allograft recipient's immune defense and *"accommodation"* will refer to changes, other than down-regulation of A/B antigen, within the allograft, making it resistant to the recipient's immune defense. Other mechanisms that may be of relevance in the process of adaptation include a down-regulation of the A/B antigen expression, the development of donor/recipient endothelial cell chimerism, and the development of glycosyltransferase inhibitors.

## Tolerance

*"Actively acquired tolerance"* was first described in neonates, by Billingham et al in 1953[81]. In transplantation, tolerance is usually defined as *"a well-functioning graft lacking histological signs of rejection, in the absence of any immunosupressive drugs, in an immunocompetent host capable of accepting a second graft of the same donor origin while being able to reject a third-party graft."*[83]. Moreover *"Clinical operational tolerance"* refers to a state of stable and satisfying graft function without any immunosuppressive thera-

py[84]. Achieving tolerance in transplantation has been the ultimate goal ever since transplantations were first performed. In rodents, several successful protocols for the induction of tolerance exist today. In humans, tolerance after kidney transplantation is rare and in most published cases, induced by mere chance and not as a consequence of a specific immunosuppressive protocol. However, a few clinical studies on tolerance-promoting immunosuppressive protocols have been conducted, including two studies on alemtuzumab[85, 86], one study on CTLA4-Ig[17] and a trial on combined bone marrow and kidney transplantation in patients with myeloma[87]. In the latter study, clinical tolerance was possibly obtained in five out of six patients. In the other two studies the patients either rejected or the immunosuppression was not withdrawn. One difficulty is that there is no validated method to diagnose tolerance, available today[88]. In a study by Brouard et al. on the gene transcript in peripheral blood of 17 clinically stable kidney recipients, it was observed that gene clusters involved in immune activation and signal transduction were down-regulated whereas gene clusters involved in mitosis were up-regulated[89]. Furthermore, the study showed a reduction of proinflammatory cytokines by about 90% in the tolerant patients. In AB0i kidney transplantation, tolerance has been put forward as one of the mechanisms to explain long-term graft function. In several Japanese studies it has been reported that the majority of the AB0i kidney recipients stop producing anti-A/B antibody after transplantation, as a result of acquired tolerance to the organ[74, 90]. However, few patients in these studies have no detectable antibodies. In contrast, most patients continue to produce anti-A/B antibodies, although at low levels. Moreover, all of the patients are on immunosuppressive treatment. Thus, the evidence to support that tolerance has been achieved, is weak. Further studies will be needed before it is possible to conclude that tolerance is acquired after AB0i transplantation.

## Accommodation

Accommodation was first recognized in AB0i kidney transplantation in the 1980's[91]. One commonly used definition of accommodation is the *"survival of the graft with the absence of antigen-antibody reaction (i.e. acute antibody-mediated rejection) despite the presence of antigen on the endothelial cells within the graft and the presence of antibody in the recipients's blood"*, as formulated by Takahashi[68, 74].

In a study by Park et al., it was demonstrated that changes in the gene expression occurred in accommodating AB0i kidney grafts, including down-regulation of SMADs, protein tyrosine kinases and TNF-á and up-regulation of Muc1. These genes are involved in cell cycle regulation, cell growth,

apoptosis and immunoregulation. Their conclusion was that a pro-survival environment was created in accommodated AB0i kidneys[80].

## Down-regulation of A/B antigen

Maybe the most obvious way for the graft to adapt to the new environment, would be by down-regulation of the A/B antigen. However, such down-regulation has only rarely been demonstrated. For instance, Koestner et al. were able to show that an AB0i heart allograft actually converted from blood group B to O (*Figure 6*)[92]. In other studies, the antigen expression following AB0i transplantation has not changed after transplantation[68].

## Glucosyltransferase inhibitors

In a few cases of AB0i transplantation, an antibody against the A/B glycosyltransferase has been detected. This enzyme is needed for the A/B antigen to be produced. As 0-recipients do not express any glycosyltransferase, the A or B glycosyltransferase produced in the kidney transplant, can after AB0i transplantation potentially be recognized as foreign and evoke the production of anti-A/B glycosyltransferase antibodies in the kidney recipient. If these antibodies block the A/B glycosyltransferase, the kidney transplant can no longer produce the A/B antigen

## Conversion from blood group B to 0 in a cardiac AB0–incompatible allograft.
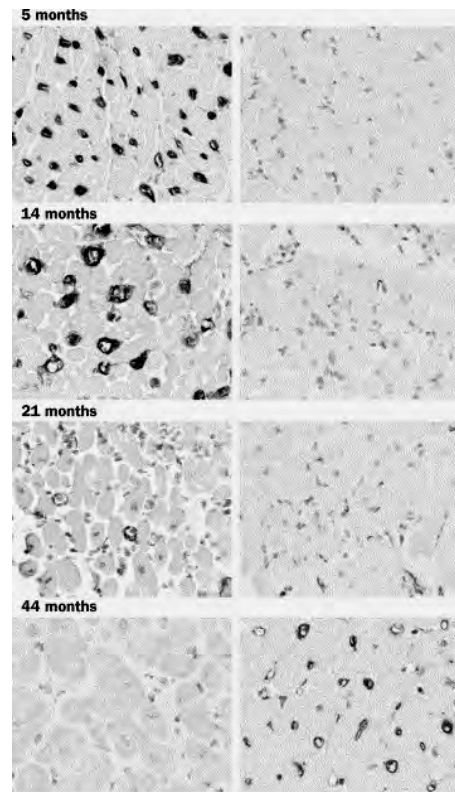


*Figure 6. Adapted from SC Koestner, A. Kappeler, T. Schaffner, T.P. Carrel, U.E. Nydegger, and P. Mohacsi, **Histo-blood group type change of the graft from B to O after ABO mismatched heart transplantation.** Lancet, 2004. 363(9420): p. 1523-5.*

and is thereby protected from anti-A/B antibodies[93-97]. The generation of glycosyltransferase antibodies following AB0i kidney transplantation, has not been studied at any larger scale. The importance of such antibodies for

long-term graft survival is therefore not known. However it has been demonstrated in several studies that AB0i organs continue to express A/B antigen also after transplantation[98, 99].

Possibly, a novel approach to AB0i transplantation, would be to synthesize an A/B glycosyltransferase inhibitor, or to produce therapeutic antibody against the glycosyltransferases.

## Donor/recipient endothelial cell chimerism

In the 1962 Medawar suggested that adaptation in transplantation would occur through the replacement of donor endothelial cells within the graft, with endothelial cells of the recipient[100]. Such phenomenon is often referred to as *chimerism*, signifying the mixture of cells of donor and recipient origin[101]. Observations of such *donor/recipient endothelial cell chimerism* has been reported but the clinical importance remains uncertain[102, 103]. In a recent study by Lagaaij et al., only in grafts with a history of severe rejection, a mixture of donor and recipient endothelial cells was found. In *non-rejecting* grafts, all endothelial cells were of donor origin[101]. In AB0i transplantation, *chimerism* could potentially protect the graft against immune-mediated injury, as the new endothelial cells would be of the recipient's blood group. However, such a hypothesis needs to be verified by further studies.

## AB0 desensitization

As mentioned earlier, all successful desensitization protocols to overcome AB0-incompatibility thus far are based on the same principles, to remove the preformed anti-A/B antibodies and to halt the production of new anti-A/B antibodies. These desensitization protocols have many features in common, which include:

1. Pre-transplant repeated therapeutic apheresis (immunoadsorption) for antibody removal
2. Reduction of the B-cell pool.
3. (Preemptive post-transplant immunoadsorption)
4. Maintenance immunosuppression

# Therapeutic apheresis

Therapeutic apheresis (TA) involves the removal of undesired blood components, such as toxins, lipids or antibodies. TA is a general term, used to describe several similar techniques. In AB0i transplantation, currently used TA techniques include plasma exchange/plasmapheresis, double-filtration plasmapheresis, protein A immunoadsorption and antigen-specific immunoadsorption. The major difference between these techniques is their level of selectivity.

## Unselective methods for anti-A/B antibody removal

### *Plasma exchange/plasmapheresis*

Plasma exchange or plasmapheresis is an unselective form of TA and the method that is still most commonly used. (The terms plasma exchange and plasmapheresis are used synonymously). Plasma exchange was first explored in dogs around 1930[104]. Some 20 years later plasma exchange was attempted in humans. In a report by Smolens et al. in the 1950's, 23 blood donors underwent plasmapheresis biweekly for a period of 40 weeks. The researchers demonstrated that repeated removal of 200 mL of plasma could be conducted safely. As a result they envisioned the use of human plasma donors as well as the production of antibodies for immunization purposes in humans[105, 106].

In the 1960's and 1970's plasma exchange as a means to treat disease caused by pathogenic blood components evolved. Plasma exchange was attempted in a number of conditions including hyperviscosity syndromes, cancer, hyperlipidemia, hepatic failure, acquired hemophilia, SLE, Goodpasture's syndrome etc[107].

Although plasma exchange has a proven benefit in the treatment of various conditions, the method has some limitations. As the pathogenic blood components are eliminated together with all other plasma components, many beneficial plasma proteins are also lost, including albumin, coagulation factors and immunoglobulins, limiting the clinical utility of the technique.

Moreover, in plasma exchange the administration of fluid substitution is necessary. This replacement fluid usually consists of albumin solutions or donor plasma.

Although associated with fewer side effects than donor plasma, albumin solutions do not replenish immunoglobulins or coagulation factors. On the other hand, donor plasma can elicit allergic reactions.

Nevertheless, in patients undergoing surgery, donor plasma is safer than albumin solution, as both coagulation factors and the platelet count are otherwise temporarily reduced by the plasma exchange[108-111].

As an alternative to plasma exchange, are other, more selective methods for therapeutic apheresis. These are generally both safer and more effective and therefore normally preferred [112].

On the other hand, plain plasma exchange does have some advantages over more selective apheresis techniques: Firstly, plasma exchange is readily available. As antibody removal is sometimes required urgently, in the treatment of antibody-mediated rejection, availability may be of importance. Secondly, in AB0i kidney recipients with donor specific antibodies other than

anti-A/B antibodies, plasma exchange allows for simultaneous removal of all antibodies, regardless of the antibody specificity. Thirdly, complement factors are also reduced by plasma exchange. As allograft injury caused by anti-A/B antibody is partly mediated via complement activation, a reduction of complement could be advantageous[99].

## Selective methods for anti-A/B antibody removal

To overcome the problems normally associated with plasma exchange, techniques for the selective removal of immunoglobulins have been developed. As no coagulation factors are removed, large plasma volumes can be processed, increasing the therapeutic efficacy compared with plasma exchange[113, 114].

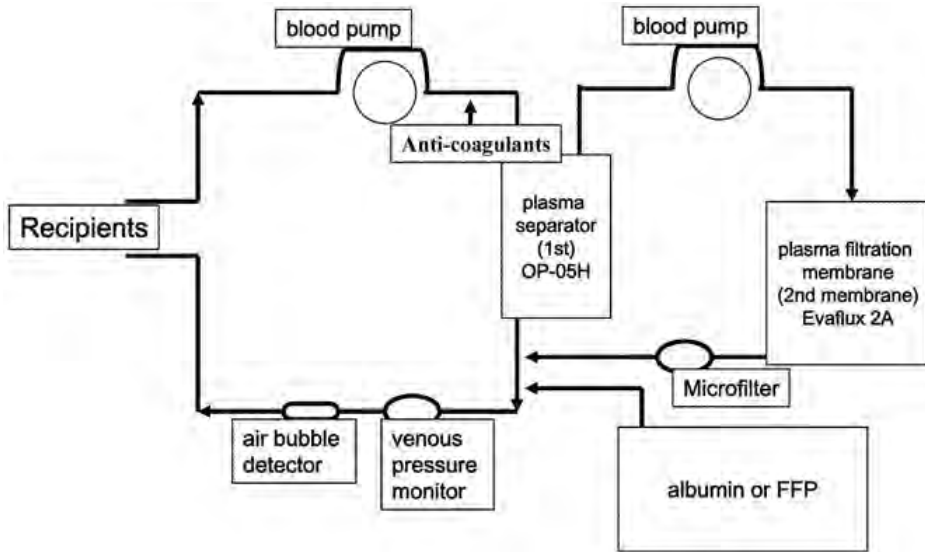## Double-filtration plasmapheresis



*Figure 7. Adapted from: K Tanabe.* **Double-filtration plasmapheresis.** *Transplantation. 2007 Dec 27;84(12 Suppl):S30-2.*

### *Double-filtration plasmapheresis*

Double-filtration plasmapheresis, a selective technique for the removal of immunoglobulins, was developed in

Japan[115, 116]. This technique allows for the removal of the plasma fraction containing the immunoglobulins. Two filtrations are performed, a primary filtration in a plasma separator, and a second filtration in a plasma fractionator. After separation of plasma from whole blood, the plasma is passed through the plasma fractionator where plasma components of a certain molecular weight are filtered out. The remaining plasma is subsequently returned to the patient, while the targeted plasma fraction is discarded. Removal of coagulation factors etc is hereby avoided and only small amounts of replacement fluid are needed[68] *(Figure 7).*

### *Protein A immunoadsorption*

Protein A immunoadsorption is an apheresis method selective for immunoglobulins. The system is based on a column containing protein A, derived from the cell wall of *Staphyloccocus aureus,* immobilized by the attachment to a matrix. Various matrices have been used, including colloidon charcoal, sepharose, glass or silica beads. During protein A immunoadsorption, the Fc part of the IgG molecule binds covalently to protein A. The IgG fraction of the plasma can thereby be eliminated. However, the binding affinity of different subclasses of IgG is variable, such that $IgG_1$, $IgG_2$ and $IgG_4$ bind with higher affinity compared with $IgG_3$. As a consequence these IgG subtypes are more efficiently removed.

Two systems for protein A immunoadsorption used to be commercially available, the Prosorba® system, based on protein A bound to a silica matrix (Fresenius Hemocare, Redmont, CA, USA) and the Immunosorba® system, based on protein A bound to a sepharose matrix (Fresenius Hemocare, St. Wendel, Saarland, Germany). The Immunosorba® system is a double-column system, two columns being installed in parallel, allowing for perfusion of one column, while the other is regenerated. Using the Immunosorba® system, IgG can very effectively be removed and the side effects are few. It has been demonstrated that one passage allows for removal of nearly 90% of IgG and ~55% of IgM and IgA, without any significant effect on the fibrinogen levels[113]. The Prosorba® system is a single-column system and treatment limited to 2000 mL of plasma per passage [117]. Although similar in the structure, a difference in therapeutic effect as well as in side effects has been observed, when comparing the two systems. As superior tolerability and efficacy was demonstrated for the Immunosorba® system, Fresensius Hemocare decided not to continue the production of the Prosorba® system *(personal communication)*[118].

## Antigen-specific immunoadsorption for anti-A/B antibody removal

In the 1970's the first reports on antigen-specific immunoadsorption in

## Antigen-specific immunoadsorption
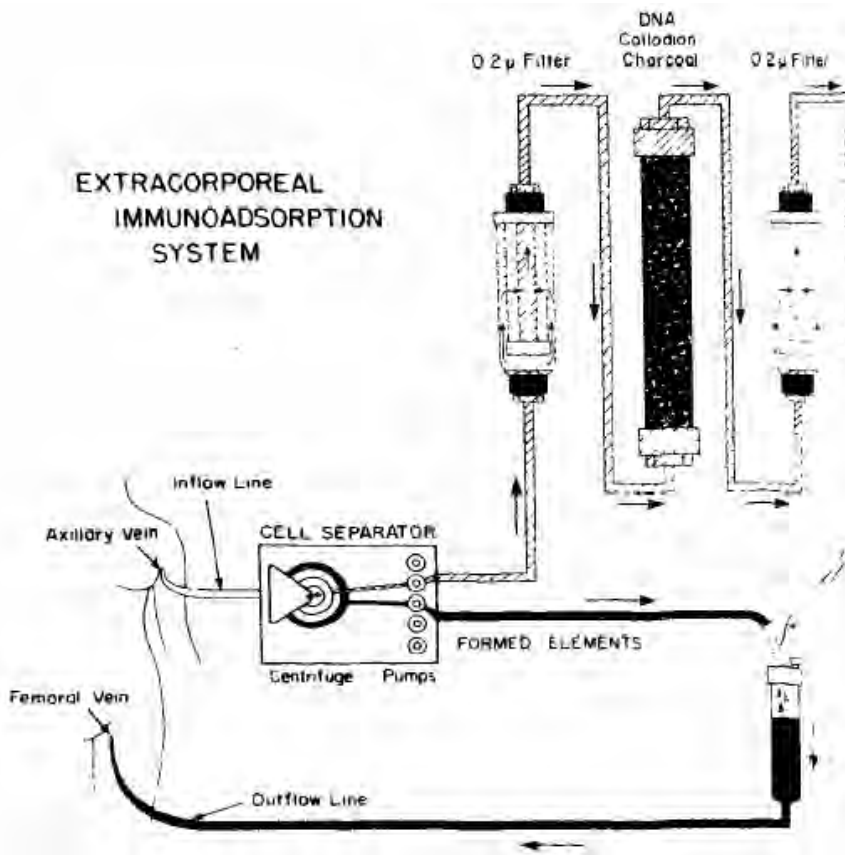


*Figure 8. Adapted from: DS Terman, G. Buffaloe, C. Mattioli, G. Cook, R. Tillquist, M. Sullivan, et al., **Extracorporeal immunoadsorption: initial experience in human systemic lupus erythematosus.** Lancet, 1979. **2**(8147): p. 824-7.*

dogs appeared[119, 120]. In 1979, Terman et al. reported on a patient with SLE, successfully treated with antigen-specific immunoadsorption of DNA antibodies. The extracorporeal immunadsorption system used, was composed of DNA immobilized on collodion-charcoal (*Figure 8*)[121]. A little later, Bensinger et al. performed the first clinical study on immunoadsorption for the removal of anti-A/B antibodies. In patients undergoing AB0i bone marrow transplantation, an immunosorbent composed of synthetic A or B trisaccharide, linked to a silica matrix, was used to eliminate the anti-A/B antibodies[122]. This product was later marketed as the Synsorb or Biosynsorb® system.

### Synsorb/Biosynsorb® system

The SynSorb/Biosynsorb® system was one of the first devices for antigen-specific removal of anti-A/B antibodies to be used clinically. The Synsorb® system refers to the original immunadsorbent, consisting of either A or B antigen bound to a silica matrix (Synsorb A and B), and the Biosynsorb® system refers to the same product, modified by a special polymeric membrane coating (Biosynsorb® A and B), (Chembiomed Ltd., Edmonton Research Park, Edmonton, Alberta, Canada)[123]. Using this system, transplantations were performed in a number of centers worldwide[53, 62, 68, 124]. However, supposedly due to side effects and limited efficacy, the columns were withdrawn from the market in the beginning of 1990's. Since then, they have not been commercially available. Reported side effects using the columns, included symptoms comparable to first-use syndrome in dialysis, such as sneezing, wheezing, short-

## The GlycosorbABO® system
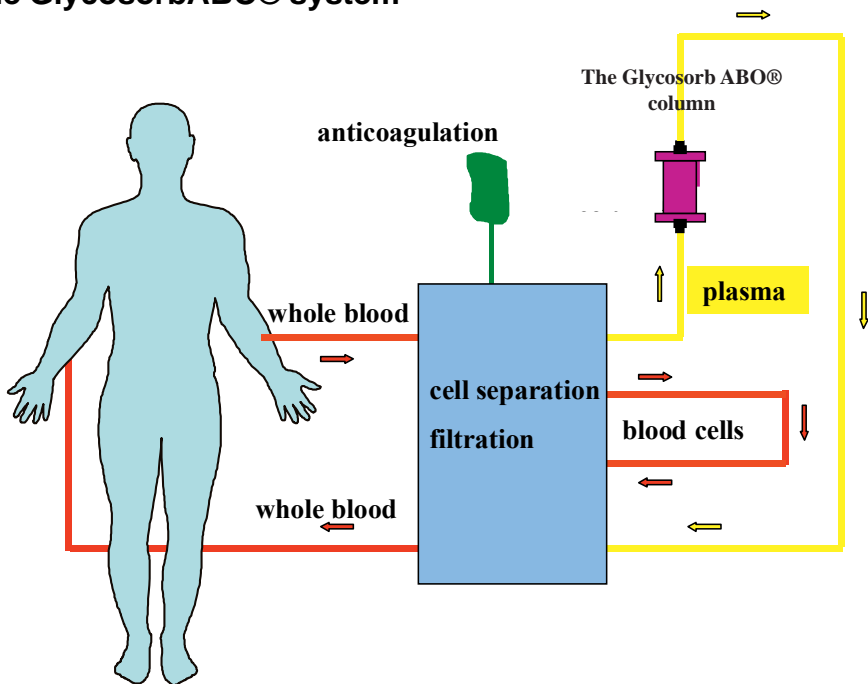


*Figure 9.*

ness of breath, back pain, chest pain, or sudden death[68]. The first-use syndrome is ascribed to a type of anaphylactic reaction to the column membrane. Moreover, in a study by Sakhrani et al., all patients developed trombocytopenia and 20 % of the patients experienced other serious side effects such as gastrointestinal bleeding and angioedema[125].

## BioSorbent

When the Synsorb/Biosynsorb® system went off market, there was no other system for antigen-specific removal of anti-A/B antibodies commercially available. As an alternative Rieben et al. developed a column, named BioSorbent, in which blood group antigens were bound to glass beads (BioSorbent A and B). In an *in vitro* study in 1995, good efficacy and biocompatibility was reported)[126]. However, this system has not been marketed yet.

## Glycosorb AB0 system

In 2001 a new system for antigen-specific immunadsorption for the removal of anti-A/B antibodies became available (Glycosorb ABO® system, Glycorex Transplantation AB, Lund, Sweden). Like its predecessors, this system is based on a column containing immobilized A or B trisaccharide (*Figure 9*). These terminal trisaccharides are bound covalently to a sepharose matrix, via a six-spacer hydrocarbon molecule [127].

The first clinical transplantation, using this system, was conducted at the Department of Transplantation Surgery, Karolinska University Hospital, Stockholm, Sweden in September 2001[128]. Since then 40 AB0i kidney transplantations have been performed here, using this system. The application of the Glycosorb AB0® system is further discussed in *Paper I, II* and *III.* In brief, this system has been very well tolerated and without any serious side effects.

## Novel approaches

A new device for antigen-specific removal of anti-A/B antibodies has been developed at the University of Pittsburgh, USA. This system is based on a hollow fiber membrane with antigens attached to the fiber lumen surface.

The system has two advantages over the Glycosorb ABO® system. One, it does not require plasma separation and two, it uses a tetrasaccharide antigen structure, allowing for the absorption of trisaccharide-specific antibodies, as well as, some core chain-specific antibodies. Thus far, the results of an *in vitro* testing of the device have been published with promising results[129].

Another system for antigen-specific removal of anti-A/B antibodies is under development, at the Swedish biotech company AbSorber, Stockholm, Sweden[46]. In this system, the terminal AB0 oligosaccharides are linked to the various blood group core chains on a mucin carrier-protein. The polyvalent expression of antigen on the carrier,

potentially increases the efficacy of the system. The company claims that their mucin-based immunoadsorber is, up to a 100 times, more effective than the Glycosorb AB0® system. The system is under development for anti-A/B antibody removal in AB0i transplantation, but also as diagnostic tool for blood typing and in the quantification of anti-A/B core chain-specific antibodies. The company envisions that the product will be commercially available within three years[130].

# B-cell depletion

B-cell depleting therapies have been included in the vast majority of AB0i protocols since the very first reports by Alexandre et al in the 1980's[62, 69, 70]. Until recently, B-cell depletion could only be achieved surgically, by removal of the spleen, or pharmacologically using chemotherapeutic agents. Often a combined approach was used. Today therapeutic alternatives also include the mAbs rituximab and alemtuzumab. In this section, theses therapeutic options are further discussed.

## Surgical B-cell depletion

### *Splenectomy*

In the 1970's, before the introduction of cyclosporine, splenectomy in the AB0c setting was used in transplantation as an adjunct to the immunosuppression. However, the benefit of this procedure was unclear[131].

In AB0i transplantation, after the first successful transplantations in the 1980's, the general belief was that splenectomy was necessary in order to avoid hyperacute rejection[62, 132]. In a study by Alexandre et al, three consecutive AB0i kidney transplantations, into non-splenectomized patients, failed, whereas 10 out of 11 transplants, performed in splenectomized patients, functioned. This observation is a commonly used argument in favor of splenectomy[68]. Another often cited study, when advocating the necessity of splenectomy, comes from Salamon et al. In their study from 1985, a patient of blood group A, who received a combined pancreas-spleen transplant, from a blood group 0 donor, developed severe hemolysis caused by anti-A antibodies, presumably produced by the spleen transplant[133]. A conclusion in this study was that the spleen served as an important locus for the anti-A/B antibody production.

By performing splenectomy, the B-cell population was partially reduced, including some of the antibody-producing plasma cells. Most protocols for AB0i transplantation have therefore included splenectomy at time of transplantation. At nearly all Japanese transplant centers, splenectomy has been part of the AB0i protocol[134]. According to the Japanese, splenectomy is beneficial, improving graft survival[68]. In the USA, splenectomy has also been included in most protocols for AB0i kidney transplantation until very recently[69].

Nevertheless, splenectomy carries

some obvious risks.

First of all, extra surgical risk is added to the transplantation. Secondly, asplenism is known to be associated with an increased risk of infection, especially with encapsulated bacteria[135-138]. However, in a study by Takahashi et al., they were not able to demonstrate any increased risk of infection when comparing splenectomized AB0i kidney recipients with non-splenectomized AB0c recipients, but in this study the number of patients was small (n=33)[68]. Regardless, the therapeutic efficacy is limited, as a considerable fraction of the B-cell/plasma cell population, resides in compartments other than the spleen, such as the bone marrow and lymph nodes.

## Pharmaceutical B-cell depletion

Although current strategies for the medical management of transplant patients are largely focused on the prevention and treatment of T cell mediated processes, there is evidence to support that B cells play a role in cellular rejection, both in AB0i transplantation and in conventional AB0c transplantation. Possible mechanisms for this include:

- Reduced antigen-presentation and subsequently a reduced activation of T helper cells[139].

-Induction of B cell tolerance [140, 141].

-Reduced antibody production[142]..

Therapies targeting the B-cell population could therefore be effective in the prevention of acute rejection, which traditionally is regarded as a T cell-mediated event[143-146]. Therapies for B cell suppression/depletion used today, include deoxyspergualin, cyclophophamide, alemtuzumab and rituximab. Of these agents, only rituximab is B cell-specific, as the others also effect other cell types, such as the T cells. Herein, rituximab is in focus, being part of the protocol we have developed for AB0i transplantation.

### *Rituximab*

Rituximab is a chimeric mouse/human antibody of the $IgG_1$ subtype directed at the transmembrane protein CD20[147]. The target molecule CD20, functions as a calcium-channel and is involved in cell-cycle regulation[148]. CD20 is expressed on all mature B cells but not on hematopoetic stem cells, the antibody-producing plasma cells or on any other cell type[149]. Rituximab evokes a long-term depletion of B cells[150].

### Mechanisms of action

There are two principle hypothesis to explain the function of rituximab. One, CD20 mAb alters $Ca^{2+}$ transportation, leading to a disruption of the cell cycle and apoptosis[151]. Two, CD20 mAb activates innate immune system[152].

There is strong evidence in support of the latter hypothesis. Firstly, the pe-

ritoneal cavity of mice is naturally devoid of cells belonging to the innate immune system. When rituximab is injected into the mouse peritoneal cavity, the B cells within the peritoneal cavity are coated with rituximab but not eliminated. This finding indicates that B-cell depletion is not mediated solely by the induction of apoptosis[153]. Secondly, in rituximab-treated macrophage-deficient mice, B-cell depletion is reduced. This finding suggests that B-cells depletion is mediated principally by monocytes/macrophages via Fc-gamma-receptors. This is often referred to as antibody-dependent cell-mediated cytotoxicity (ADCC) [10, 147].

However, several other mechanisms may play a role in the activation of the innate immune system, including: Complement-dependent cytotoxicity (CDCC). In *in vitro* studies rituximab binding has initiated the complement cascade, resulting in the formation of a membrane attack complex and cell destruction[154]. Antibody-dependent cellular phagocytosis (ADCP), possibly mediated via NK-cells, may also play a role[155].

## Indications

In 1997 rituximab was registered in the USA for the treatment of B-cell lymphoma. Since the beginning of the 2000's rituximab has gained much interest in renal transplantation. Although the approved indication for rituximab treatment is limited to B cell lymphoma and rheumatoid arthritis, ri-

tuximab has been explored as a therapeutic option for virtually all autoimmune diseases and for various indications in transplantation, including induction therapy[156, 157], and in the treatment of renal allograft rejection[158].

Rituximab is generally well tolerated and serious side effects are rare, which in part explains its popularity. There are several ongoing randomized controlled trials evaluating rituximab in kidney transplantation. They include trials on rituximab as induction therapy, in the treatment of rejection and in highly sensitized patients awaiting renal transplantation, as a means to reduce HLA antibodies.

Splenectomy used to be mainstay therapy for the reduction of the antibody-producing B cell/plasma cell population in AB0i transplantation but is now increasingly becoming replaced by rituximab[156].

## Pharmacokinetics and pharmacodynamics

It has been demonstrated in lymphoma patients that, the serum-concentration of rituximab correlates to the level of B-cell depletion and to the clinical effect[159]. Moreover, the clearance and volume of distribution is correlated to BSA but there is great inter-individual variability in the concentrations of rituximab obtained[160].

Several assays for the measurement of the rituximab concentration in blood have been developed, including enzy-

me-linked immunosorbent assays (ELI-SA) using polyclonal and monoclonal antibodies against the rituximab idiotype or the mouse Fv part, as well as flow cytometry assays, detecting the binding of rituximab to CD20[159, 161, 162]. However, there are no international standards or recommendations available today for rituximab measurement. In studies determining the concentration of rituximab, methods measuring both free and bound rituximab have been used, making it difficult to compare the results in different studies. Optimal concentration is not known for any patient group to day[163]. Furthermore, elimination $T_{1/2}$ of rituximab varies in different studies but is generally long (4-20 days). Rituximab has been the detectable in the circulation of non-Hodgkin's lymphoma patients for as long as 6 months[150, 159, 160, 164].

The optimal dose of rituximab in kidney transplantation is not known today. In a study on the effects of rituximab in uremic patients, the B cells were completely eliminated even in the group receiving the lowest dose of 50 mg/ $m^2$ BSA[164]. However, in lymphoma patients the most commonly used regimen consists of one dose of 375 mg/ $m^2$ BSA of rituximab per week over a four-week period[153]. In kidney transplantation, most commonly a single dose of rituximab 375 mg/ $m^2$ BSA is given[156, 157, 165]. We have demonstrated that such an approach is safe and effective. A single dose of rituximab 375mg/$m^2$ BSA evokes a long-term elimination of B cells in peripheral blood. We have also demonstrated that rituximab eliminates B cells within the graft (*paper IV*). This observation has been confirmed in another study[166]. Furthermore, we have used rituximab in the treatment of rejection in kidney transplantation as well as in liver transplantation. In the liver graft B cells are also depleted (*Figure 10a-d at the next page*). In the lymphoid compartment, single-dose of rituximab 375 mg/ $m^2$ BSA does not induce a complete B cell depletion but a reduction of the B cells has been observed in studies on lymph nodes and spleen *(Paper IV)*[167].

### Alemtuzumab

The effects of alemtuzumab are discussed in a separate section above. There are several publications showing a beneficial effect of alemtuzumab induction in kidney transplantation[14, 15]. There are also a number of ongoing clinical trials evaluating alemtuzumab in kidney transplantation. However, alemtuzumab has thus far not been evaluated in AB0i transplantation.

### Deoxyspergualin

Deoxyspergualin (gusperimus) is an antiproliferative agent primarily inhibiting antibody production as well as T-cell costimulation. The drug is a synthetic analogue of spergualin, produced by the bacterium *Bacillus laterosporus*. The first reports on the immunosupressive properties of deoxyspergualin were published in 1990. De-

## B cells in liver allograft biopsies, in a patient with multiple episodes of acute rejection, before and after rituximab treatment.
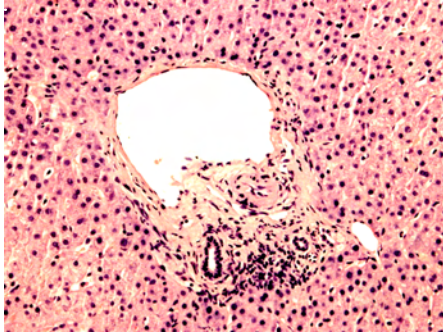


*Figure 10a. Hematoxylin and eosin staining of a liver biopsy during treatment with tacrolimus, MMF, corticosteroids and daclizumab.*
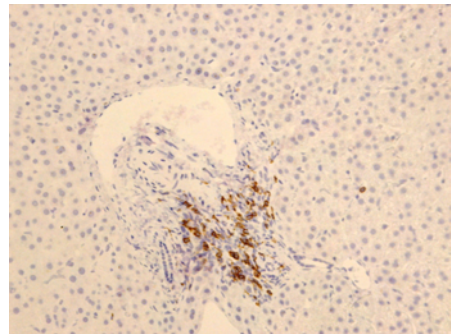


*Figure 10b. Immunohistochemistry for B cell detection of the same liver biopsy as in 10a, using CD20 as a B cell marker, before treatment with rituximab.*
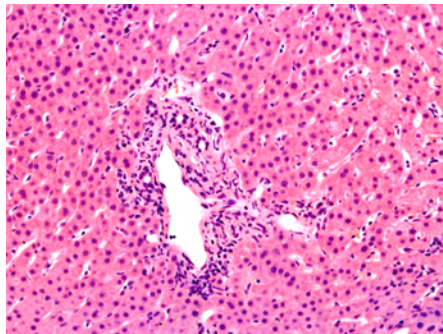


*Figure 10c.Hematoxylin and eosin staining of a liver biopsy after treatment with rituximab in combination tacrolimus, MMF, corticosteroids and daclisumab.*
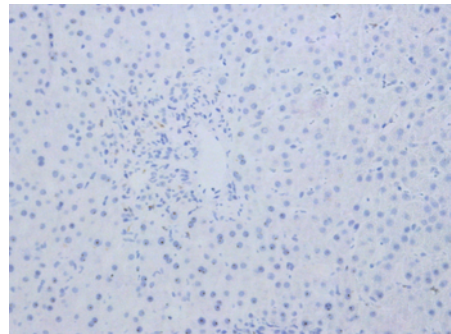


*Figure 10d.Immunohistochemistry for B cell detection of the same liver biopsy as in 10c, using CD20 as a B cell marker, after treatment with rituximab.*

oxyspergualin is administered intravenously and usually given in cycles of 10-14 days.

In Japan it is approved for treatment of steroid-resistant rejection in kidney transplantation. In a randomized clinical trial, deoxyspergualin showed equal efficacy to OKT-3 in the treatment of steroid-resistant rejection. A common side effect is transient leucopenia, but otherwise, known serious side effects are few[168]. In transplantation, deoxyspergualin is currently evaluated in two clinical trials in the USA, in islet-

cell transplantation and in kidney transplantation, in combination with alemtuzumab. However, in a pilot study on tolerance-induction in kidney transplantation, the researchers were not able to show any additive effect when comparing alemtuzumab + deoxyspergualin with alemtuzumab alone but the number of subjects in this study was small (n=10)[86]. In Europe deoxyspergualin holds orphan drug status for the treatment of Wegener's granulomatosis although it has been evaluated as a treatment for rejection[169-171].

## Cyclophosphamide

Cyclophosphamide is an alkylating chemotherapeutic agent related to nitrogen mustard. By inhibition of the lymphocyte cell cycle, it suppresses the clonal expansion of both B and T cells. Consequently the drug has a marked immunosuppressive effect. However side effects such as nausea, bone marrow depression, hemorrhagic cystitis and transient alopecia are common[172]. The drug is also cardiotoxic and requires careful monitoring[173]

Cyclophosphamide is primarily used in the treatment of leukemia and lymphoma and during conditioning for hematopoetic stem cell transplantation. In addition cyclophosphamide is established treatment for some glomerulonephritides[174]. In Japan it has been used as a B cell depleting agent in AB0i kidney transplantation as induction therapy, from –2 weeks before transplantation to 3 months after transplantation[68]. Cyclophosphamide has not been evaluated in any clinical trial in kidney transplantation.

Finally, in terms of B cell depletion/ B cell suppression in AB0i transplantation, a recent study has demonstrated that AB0i kidney transplantation can be successfully performed completely without B cell depletion, omitting splenectomy, anti-CD20 mAb and other B cell suppressing agents[175]. This observation warrants further studies, to evaluate the actual need of B cell depletion in AB0i transplantation.

# Other agents used in AB0-incompatible kidney transplantation

## Intravenous immunoglobulin

Intravenous immunoglobulin (IVIG) is an immunomodulating agent frequently used in desensitization[176-180]. The function is not fully understood but IVIG has a proven effect in the treatment of many autoimmune diseases[181]. It has been demonstrated that IVIG exerts its immunomodulatory effect through several mechanims, including anti-idiotype binding of alloantibodies, inhibition of cytokine and antibody production and inhibition of complement[177, 182-184]. Moreover, in a randomized trial, IVIG showed similar efficacy to OKT-3 in the treatment of steroid-resistant kidney allograft rejection[185]. However, treatment with IVIG is associated with some potentially serious side effects

including thrombosis, myocardial infarction, and anaphylaxic reactions[181]. In the protocol we developed for AB0i transplantation, IVIG (0.5g/kg) was initially given for six days but one of the first patients developed arterial thrombosis of the kidney during IVIG infusion. The protocol was thereafter changed to only include a single-dose of IVIG (0.5g/kg) the day before transplantation *(Paper II)*.

### *Maintenance immunosuppression*

Standard triple therapy maintenance immunosuppression is usually used in AB0i transplation, consisting of a combination of tacrolimus, mycophenolate mofetil and corticosteroids. *See Current immunosuppression, for more information.*

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# AIMS OF THE STUDY

1. To evaluate a protocol for AB0-incompatible kidney transplantation based on antigen-specific immunoadsorption and rituximab.

2. To evaluate kidney function in a short and long term perspective following AB0-incompatible kidney transplantation based on antigen-specific immunoadsorption and rituximab.

3. To study the effects on the anti-A/B antibody production follwoing AB0-incompatible kidney transplantation based on antigen-specific immunoadsorption and rituximab.

4. To study the effects of rituximab in AB0i kidney transplantation.

# PATIENTS

In *Paper I, II and III* patients undergoing AB0i kidney transplantation at the Department of Transplantation surgery, Karolinska University Hospital, Huddinge were enrolled. The patients were transplanted between Sept 2001 and Oct 2007. In *paper IV*, all kidney recipients treated with single-dose rituximab were included in the study. They were all transplanted at the Department of Transplantation surgery, Karolinska University Hospital, Huddinge. The indications for rituximab therapy varied within this group of patients. The majority were AB0i kidney recipients but some patients underwent kidney transplantation against a positive flow cytometric cross-match or some received rituximab as anti-rejection therapy. In *Paper II and IV*, a control group consisting of patients undergoing AB0c kidney transplantation was used.

# METHODS AND MATERIALS

## *Immunosuppressive protocol*

Removal of anti-A/B antibody was achieved by repeated antigen-specific immunoadsorption (GlycosorbABO®; Glycorex Transplantation AB, Lund, Sweden) preoperatively on day -6, -5, -2 and -1. In addition, a single dose of rituximab 375 mg/m$^2$ (BSA) was given on day -30 and oral immunosuppression (tacrolimus, mycophenolate mofetil and prednisolone) instituted on day -10. On day -1, intravenous immunoglobulin (Gammagard S/D®; Baxter, Lessine, Belgium) 0.5 g/kg was administered. Postoperatively, immunoadsorption was performed on days 2, 5 and 8. Additional immunoadsorptions were performed preoperatively if A/B antibody titres exceeded 1:4 after the last preoperative session and postoperatively if there was a rise in A/B antibody titres with concomitant impairment in kidney function.

Since the introduction of the protocol in 2001 some changes have been made: initially the protocol did not call for any postoperative immunoadsorptions but as the A/B antibody titres in the first two patients rose in the early postoperative period, three pre-emptive immunoadsorptions after transplantation were added. Moreover, low-molecular heparin and colloids (dextran 70 or hydroxyethyl starch) were given to reduce the risk of thrombosis of the graft. After bleeding complications in four out of the first seven cases this strategy was abandoned and thrombosis prophylaxis was only given to patients with increased risk of thrombosis or multiple arterial anastomoses. In addition, six doses of intravenous immunoglobulin (0.5 g/kg) were originally given, one dose on day -1 and five doses postoperatively. However, as mentioned, one of the first

patients developed thrombosis of the renal artery, resulting in graft loss. As administration of intravenous immunoglobulin is known to be associated with an increased risk of thromboembolic events, the dosage was reduced to single-dose administration on day -1, as described above. In addition, the tacrolimus target trough level, originally 20 ng/mL, was later on reduced to 15 ng/mL and the mycophenolate mofetil starting dose was reduced from 3 g per day to 2 g per day for the adult patients, the primary reason being not well tolerated side effects. Overall the amount of immunosuppression has been reduced over time.

All patients received *Pneumocystis jiroveci* prophylaxis (sulfametoxazol/trimethoprim) for 6 months. All cytomegalovirus (CMV)-incompatible patients received CMV prophylaxis: the AB0c group was given valaciclovir or valganciclovir for 3 months. The AB0i group received valganciclovir for 3 months followed by valaciclovir for another 9 months. All CMV-compatible AB0i patients were given valaciclovir for 12 months as herpes simplex prophylaxis.

## Anti-A/B antibody measurements

In the quantification of anti-A/B antibodies, a hemagglutination technique of titration was used. The titration was initially done using a tube technique and from February 2005 by a gel tech-

nique. The newer gel technique yielded an anti-A/B IgG titre half a titre-step higher than the previous method. Approximately 2/3 of the samples were analyzed by gel technique.

## Flow cytometry of peripheral blood

Flow cytometry was conducted on samples of venous blood in EDTA. To obtain a leukocyte suspension, ammonium chloride lysing solution was added to samples of venous blood to eliminate erythrocytes and serum immunoglobulins. The leukocyte cell suspension was mixed with the antibody reagents and incubated for 15 min. After the addition of buffered sodium chloride (pH 7.0) (PBS) the samples were centrifuged. The cell suspension was diluted in PBS to achieve a cell rate of 1000–1200 cells/second. The flow cytometry was performed in a FACSCalibur flowcytometer™ (BD Biosciences, San Jose, CA, USA) using CELLQuest software (BD Biosciences) for statistical analyses.

## Flow cytometry of lymph nodes

Lymph nodes were extirpated from the inguinal area during kidney transplantation or other surgical procedures. The lymph nodes were cut in a standardized manner to allow for histologic and flow cytometric analyses from adjacent areas of the tissue. Pieces of tissue of

approximately 8 mm$^3$, were inserted into polyethylene chambers, known as Medicons, (BD Biosciences) together with 1 mL of PBS. The Medicons were placed in a Medimachine™ (BD Biosciences) and the tissue disaggregated for 15–20 s in order to obtain a cell suspension. Ammonium chloride lysing buffer was added to the cell suspension and flow cytometry was performed using the technique describe above for peripheral blood. CD19 and CD20, expressed on all normal mature B cells, were used as specific B-cell markers.

## Histopathology

Kidney tissue samples were fixed in 4% buffered formalin and embedded in paraffin and 1–2 μm thick sections were cut on a rotation microtome. Routine stainings, including hematoxylin & eosin, periodic acid Schiff (PAS) and Ladewig were done and the tissue samples assessed by a transplant pathologist. Diagnosis of acute cellular and antibody-mediated rejection was based on the Banff criteria[30, 31, 186, 187].

## Immunohistochemistry of kidneys and lymph nodes

Immunohistochemical stainings were performed on formalin fixed, paraffin embedded 1–2 μm thick sections of kidney tissue and inguinal lymph nodes,

using a TechMate™500 Plus machine (Dako, Glostrup, Denmark). CD20 and CD79á were used as B-cell markers. Monoclonal anti-CD20 clone L26, anti-CD79á clone JCB117 and polyclonal anti-CD3 (Dako) were used together with polyclonal anti-CD5 (Novocastra Laboratories Ltd, Newcastle upon Tyne, UK). In order to assess the prevalence of B cells, the sections were evaluated in a blinded fashion and the lymphocytes semi-quantitatively graded on a scale ranging from 0 to 5 as outlined below. For each antibody, one section was randomly chosen and evaluated.

Grading used for the semi-quantitative analyses in the immunohistochemical studies: (The numbers refer to the percentage of cells staining positive in relation to the entire population of lymphocytes within the tissue.)

    0 = 0% positive cells
    1 = 1–10% positive cells
    2 = >10–25% positive cells
    3 = >25–50% positive cells
    4 = >50–75% positive cells
    5 = >75% positive cells

## ETHICS

The regional ethics board approved the studies.

# RESULTS

## Paper I.

Tydén G, Kumlien G, Genberg H, Sandberg J, Lundgren T, Fehrman I. *AB0-incompatible kidney transplantations without splenectomy, using antigen-specific immunoadsorption and rituximab*. Am J Transplant. 2005 Jan;5(1):145-8.

### Aims:

To evaluate a protocol for AB0i living donor kidney transplantation based on antigen-specific immunoadsorption and rituximab.

### Results

The first eleven kidney recipients undergoing AB0i kidney transplantation according to the protocol were included in the study. In this pilot study, technical feasibility of the protocol was demonstrated. Furthermore, the protocol was well tolerated and it allowed for effective anti-A/B antibody removal. Patient and graft survival short-term results was 100% and kidney function good in the majority of kidney recipients. We concluded that blood group-incompatible renal transplantation without splenectomy, could be performed with excellent results, using antigen-specific immunoadsorption in combination with a single dose of rituximab and a single dose of IVIG incombination with standard immunosuppression.

## Paper II.

Genberg H, Kumlien G, Wennberg L, Berg U, Tydén G. *AB0 -incompatible kidney transplantation using antigen-specific immunoadsorption and rituximab: a 3-year follow-up.* Accepted for publication in Transplantation.

### Aims:

To evelute the protocol for AB0i kidney transplantation based on rituximab and antigen-specific immunoadsorption long-term.

### Results

The first 20 kidney recipients undergoing AB0i kidney transplantation according to the protocol were compared with a group of AB0c living donor kidney recipients maintained on the same basic immunosuppression (tacrolimus, mycophenolate mofetil and corticosteroids) and transplanted during the same period. Mean follow-up in the study was 3 years. This study showed that, the results following AB0i kidney transplantation were similar to those of AB0c LD kidney transplantation, not only short-term but also in a longer perspective. We concluded that AB0i transplantation following this protocol did not have a negative impact on graft function in the long term.

## Paper III.

Genberg H, Kumlien G, Wennberg L, Tydén G. *No Rebound of A/B Antibo-*

*dies after AB0-Incompatible Kidney Transplantation using Antigen-specific Immunoadsorption and Rituximab.* Submitted in 2008.

## Aims:

To study the effects of the protocol for AB0i kidney transplantation using antigen-specific immunoadsorption and rituximab, on the anti-A/B antibody titres.

## Results

In previous studies a rebound of anti-A/B antibodies after AB0i kidney transplantation has been observed and associated with a poor prognosis. Moreover, high baseline anti-A/B antibody titres (before any immunosuppressive therapy) have been correlated with an increased risk of antibody-mediated rejection and graft loss. In this study all AB0i kidney recipients transplanted at our center, were included (n=39). We studied the effects of rituximab and the antigen-specific immunoadsorption, during conditioning and the early postoperative period. In addition the anti-A/B antibody levels long-term were evaluated. We concluded that, AB0i kidney transplantation using antigen-specific immunoadsorption in combination with rituximab, effectively depletes anti-A/B antibodies. There is no significant rebound of anti-A/B antibodies although splenectomy was not performed. Our data show that a persistent low-grade anti-A/B antibody production following AB0i kidney transplantation is common but does not have any negative impact on graft function.

## Paper IV.

Genberg H, Hansson A, Wernerson A, Wennberg L, Tydén G. *Pharmacodynamics of rituximab in kidney allotransplantation. Am J Transplant. 2006 Oct;6(10):2418-28.*

## Aims:

To study the effect of single-dose rituximab on the B-cell population in kidney allograft recipients.

## Results

Kidney recipients receiving either rituximab induction or rituximab as anti-rejection therapy were studied (n=49). B-cells were measured repeatedly after treatment in peripheral blood using flow cytometry. The effect of rituximab on the B-cell population within graft as well as within the lymphoid compartment was also studied. The study clearly showed that rituximab effectively eliminates B-cells in peripheral blood and that the effect is long-term. Rituximab also reduces the B cell population in lymph nodes and completely depletes B cells within the transplant.

## DISCUSSION

The constantly growing demand for renal replacement therapy has lead to

a renewed interest in AB0i kidney transplantation. In the Caucasian population the probability that two unrelated individuals are AB0-incompatible is around 35%[188]. Consequently AB0-incompatibility is the most common contraindication to living donor transplantation. Successful AB0i kidney transplantation is therefore a means to substantially increase the number of living donor transplantations performed.

AB0i kidney transplantation was first attempted at any larger scale in 1970's, using $A_2$ donors for DD kidney recipients of blood group 0 and B. Only regular immunosuppression was used. As experience grew, it became clear that low initial anti-A/B antibody titres, were a prerequisite for the transplantation to be successful. In the largest series from Kansas, only 0 and B recipients with an antibody titre below or equal to 1:4 were accepted, significantly limiting the number of potential candidates. In our own material, only 1/24 patients undergoing conditioning for anti-A antibody removal had such a low antibody titre at baseline. Primarily owing to poor results, most centers attempting AB0-incompatible kidney transplantation using $A_2$ donors without conditioning, had abandoned this strategy by the end of 1980's[189]. To overcome major AB0-incompatibility (non-$A_2$-incompatibilities) in kidney transplantation, Alexandre et al. developed the first successful protocol in the 1980's. The protocol was based on plasmapheresis for antibody removal, in combination with splenectomy to reduce the antibody-producing B cell pool, in addition to an extensive immunosuppressive regimen. Since then, most successful desensitization protocols for AB0i kidney transplantation have been based on these principles. Although good graft function has been achieved using such protocols, the high risks involved have been deterrent. Consequently AB0i kidney transplantation has only been performed at the minority of transplant centers worldwide. A few centers attempted AB0i transplantation in the 1980's with varying success. In many of these centres, the AB0i programmes were abandoned by the end of the 1980's. Between 1989 and 1999, AB0i kidney transplantation was only conducted regularly at Japanese institutions.

In 2001 a protocol for AB0i kidney transplantation based on antigen-specific immunoadsorption and rituximab in combination with standard immunosuppressive therapy (tacrolimus, mycophenolate mofetil and corticosteroids), was introduced at our centre.

We hypothesized that the anti-A/B antibodies could be effectively eliminated and good graft function achieved, without the complications of coagulopathy and transfusion reactions associated with plasmapheresis. Furthermore, we hypothesized that the substitution of splenectomy with a single dose of the B cell depleting agent, rituximab, would abolish the surgical risk and re-

duce the risk of infectious complications related to splenectomy. The Glycosorb ABO® system was a new invention, not evaluated in the clinical setting when the first transplantation was performed at our institution.

However, an *in vitro* analysis showed promising results and as antigen-specific immunoadsorption is specific for anti-A/B antibodies[127, 190], we assumed that complications associated with plasma exchange such as coagulopathy and transfusion reactions could be avoided[108, 109, 191].

In a pilot study, this protocol was evaluated, showing that both tolerability and efficacy were good. The protocol has in fact been very well tolerated without any serious side effects. To date, 40 kidney transplantations using this protocol have been performed at our centre. In addition, in a collaborative study with the transplant centers in Uppsala, Sweden and Freiburg, Germany, we demonstrated that the protocol could be implemented elsewhere, without difficulty[71].

After the early reports on successful AB0i kidney transplantation using the Glycosorb ABO® system *(Paper I)* our protocol for AB0i kidney transplantation has been adopted by > 20 transplant centers worldwide and some 200 AB0i transplantations performed[192]. Today AB0-incompatible kidney transplantation is also performed at a few centres in the USA and Australia [156]. Their protocols are primarily based on plasmapheresis with or

without rituximab.

Today transplant centers worldwide report long-term results in AB0i LD transplantation, equivalent to those of AB0c LD transplantation. We have the same experience. Recent data support that AB0i LD kidney transplantation is a superior alternative to DD AB0c transplantation and possibly not inferior to LD AB0c transplantation.

However, desensitization is associated with an increased cost[76, 193]. The AB0i transplantations using our protocol cost an additional €32,000 compared with standard AB0c LD transplantation.

Furthermore, at the time of the introduction of the protocol for AB0i kidney transplantation, the experience of rituximab induction in kidney transplantation was very limited. Its safety had been confirmed in studies on lymphoma patients. Rituximab was also known to rapidly eradicate the B-cell population in peripheral blood. According to studies on lymphoma patients the B-cells would repopulate within 3-12 months after treatment.

One observation, using the protocol for AB0i transplantation, was that the incidence of acute rejection, both cellular and antibody-mediated rejection, was very low. Only one of the first 20 AB0i kidney recipients experienced acute rejection. As several centers reported a considerably higher incidence of acute rejection in the AB0i kidney recipients compared with the AB0c LD kidney recipients, especially in antibo-

dy-mediated rejection, this finding was unexpected.

To further evaluate the effect of rituximab as induction therapy in kidney transplantation, a clinical multicenter trial was undertaken, named the MANTRA-study. In this study the efficacy and safety of rituximab induction was studied in AB0c kidney transplantation. The study was designed as a double-blind placebo controlled trial, the inclusion criteria being first or second kidney transplantation in adult patients with a flow-PRA < 50%. Primary outcome measures in the study were the composite endpoint of biopsy-proven rejection, graft loss or death during the first 6 months following transplantation. Secondary outcome measures were renal function at 6 months, the incidence of infections, the incidence of rituximab-related adverse events and the incidence of malignancies. In total 140 patients were enrolled in the study. Preliminary analysis of the results shows that rituximab induction is safe. It also confirms that rituximab in kidney transplantation effectively eliminates B-cells in peripheral blood for at least six months. Being the first placebo-controlled trial on rituximab some important information can most certainly be gained from this study.

# FUTURE PROJECTS

## A study of adaptation in AB0-incompatible kidney transplantation

So far we have not observed any negative impact of the AB0-incomaptibility using the protocol we developed for AB0i kidney transplantation. As the results both short-term and in a long-term perspective, are similar to those of AB0c LD kidney transplantation, we believe that adaptation occurs following AB0i transplantation.

We aim to investigate the process of adaptation follwing AB0i kidney transplantation. As a first step, an assessment of histopathologic changes related to chronic allograft injury will be performed in AB0i kidney biopsies, as the absence of such histopathologic changes, will support the hypothesis that adaptation takes place.

Moreover, during the process of adaptation, several mechanisms may come into play, including a down-regulation of the antigen expression, an up-regulation of immunoprotective genes and, the development of endothelial cell chimerism within the graft. Changes in the AB0i kidney recipient, leading to adaptation, may include the development of anti-glycosyltransferase antibodies as well as the halted production of graft-specific anti-A/B antibodies, *see below*. In order to compre-

hensively investigate the process of adaptation, following AB0i kidney transplantation, all of these hypothetical mechanisms will be studied.

The regional ethics board has approved this study.

## An analysis of anti-A/B antibody specificity

Although the epitope of most A/B antibodies, is the terminal trisaccharide of the A/B antigens, it has been shown that the epitope of a subset of anti-A/B antibodies, is the terminal trisaccharide + core chain. Thus far, we have not analyzed the specificity of the anti-A/B antibodies in the patients undergoing AB0i kidney transplantation at our center. However, we have observed that the majority of AB0i kidney recipients continue to produce antibody also after the transplantation. As the kidney function following AB0i kidney transplantation according to our protocol, is equivalent to that of AB0-comptible LD kidney transplantation, the presence of these anti-A/B antibodies in the circulation does not seem to affect the allograft. One possibility would be that these antibodies bind to subtypes of A/B antigen expressed on the erythrocytes used for the titration, but not expressed in the kidney transplant, i.e. core chain specific antibodies. Furthermore, in eight patients thus far, the anti-A/B antibodies have not been removed as efficiently as expected using the Glycosorb ABO® system. This system is composed of the terminal A or B trisaccharide bound to a sepharose matrix. Hence, any core chain specific anti-A/B antibodies will not be adsorbed by this system. This observation further supports the existence of such core saccharide chain-specific antibodies.

The regional ethics board has approved this study.

## A 3-year follow-up of the MANTRA-study

In kidney recipients receiving single-dose rituximab induction, the effects of this therapy will be evaluated at 3 years after transplantation. Eligible for this study are patients initially included in the MANTRA-study. The aim is to evaluate patient and graft survival, kidney function, the incidence of late acute rejection and the incidence of malignancy, after rituximab induction in kidney transplantation, long term.

One hypothesis is that rituximab in combination with tacrolimus, mycophenolate mofetil and corticosteroids will inhibit the production of donor-specific antibodies and thereby improve long-term graft function. Another hypothesis is that rituximab induction reduces the risk of lymphoma and does not increase the risk of other malignancies.

# Implementation of a protocol for cross-match positive kidney transplantation

Similar to AB0-incompatibility, a positive cross-match is generally an absolute contraindication to transplantation. To overcome this problem we have developed a desensitization protocol, very similar to that used for AB0i transplantation. Instead of antigen-specific immunoadsorption for anti-A/B antibody removal, the removal of HLA antibodies is done either by plasmapheresis or by protein A immunoadsorption using the Immunosorba® system. At present this protocol is evaluated in kidney transplantation against a positive fluorescence-activated cell sorter (FACS) cross-match (XM) in patients with a negative complement-dependent cytotoxicity (CDC) XM[194]. A positive FACS XM in combination with a negative CDC XM, indicate the presence of HLA-antibodies at low levels. The results so far are promising, nine out of 11 patients converting to a negative FACS XM during conditioning for transplantation. As a future step, we want to extend the protocol to patients with a positive CDC XM. At present, CDC XM-positive kidney transplantation is primarily performed at two centres in the US[195, 196] and to a limited extent elsewhere. The HLA barrier is seemingly more difficult to cross than the AB0 barrier, and few reports show results following CDC XM-positive transplantation equal to those of standard XM-negative transplantation.

However, as the numbers of patients requiring retransplantation are increasing, effective desensitization protocols are certainly needed.

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