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EDITORIAL

Impact of donor-specific antibodies on the outcomes of kidney graft: Pathophysiology, clinical, therapy

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Abstract

Allo-antibodies, particularly when donor specific, are one of the most important factors that cause both early and late graft dysfunction. The authors review the current state of the art concerning this important issue in renal transplantation. Many antibodies have been recognized as mediators of renal injury. In particular donor-specific-Human Leukocyte Antigens antibodies appear to play a major role. New techniques, such as solid phase techniques and Luminex, have revealed these antibodies from patient sera. Other new techniques have uncovered alloantibodies and signs of complement activation in renal biopsy specimens. It has been acknowledged that the old concept of chronic renal injury caused by calcineurine inhibitors toxicity should be replaced in many cases by alloantibodies acting against the graft. In addition, the number of patients on waiting lists with preformed anti-human leukocyte antigens (HLA) antibodies is increasing, primarily from patients with a history of renal transplant failure already been sensitized. We should distinguish early and late acute antibody-mediated rejection from chronic antibody-mediated rejection. The latter often manifets late during the course of the post-transplant period and may be difficult to recognize if specific techniques are not applied. Different therapeutic strategies are used to control antibody-induced damage. These strategies may be applied prior to transplantation or, in the case of acute antibody-mediated rejection, after transplantation. Many new drugs are appearing at the horizon; however, these drugs are far from the clinic because they are in phase Ⅰ-Ⅱ of clinical trials. Thus the pipeline for the near future appears almost empty.

Key words: Donor-specific antibodies; Solid-phase techniques; Complement activation; Renal transplantation; Antibody-mediated rejection; Desensitization; New drugs for B-cells

Core tip: Clear evidence exists that shows that donor-specific-HLA antibodies (DSAs) are the primary players in the acute and chronic deterioration of graft. The emergence of sensitive techniques that detect DSAs, together with advances in the assessment of graft pathology, has enabled an improved understanding of antibody-mediated graft injury. Acute and chronic antibody-mediated rejection conditions have changed the nomenclature during recent Banff conferences and have enabled the dismissal of older terminologies, such as chronic allograft nephropathy. Therapies aimed at B cells and plasma cells and that control complement activation will be extremely important for improving long-term outcomes in kidney transplantations.

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INTRODUCTION

Despite improvements in renal transplantation outcomes,

kidney allograft loss remains substantial and is associated with increased morbidity, mortality and costs[1,2]. Clearly, the identification of the critical pathologic pathways underlying allograft loss and the development of therapeutic interventions that improve the duration and quality of allograft function are among the most important targets for transplant medicine. One of the most important advances of the past decade has been the realization that the insufficient control of the humoral arm of a recipient's immune system by current immunosuppressive regimens[3] is the factor primarily responsible for allograft dysfunction and loss[4-6].

ALLOGRAFT ANTIBODY EVOLUTION IN TRANSPLANTATION

The induction of allograft injury alloantibodies induced has now superseded the historical dogma that allograft losses were caused by the toxicity of calcineurin inhibitors (CNIs) and by chronic allograft nephropathy (CAN). Indeed, nephrotoxicity and CAN as causes of late graft failure are being challenged by the findings of the Long-Term Deterioration of Kidney Allograft Function (DeKAF)[6-8] and other studies[9,10].

In addition, recent therapeutic strategies that have permitted the human leukocyte antigens (HLA) to be crossed have created a new population at risk of antibody-mediated rejection (ABMR), which has enabled these patients to be studied over an extended time period.

The emergence of sensitive techniques that detect donor-specific anti-HLA antibodies (DSAs) and other HLA and non-HLA antibodies together with advances in the assessment of graft pathology have expanded the spectrum of ABMR.

The different technologies used by researchers and the significance of alloantibodies found by these technologies led recently to a consensus conference that elaborated upon consensus guidelines for testing and clinical management issues associated with HLA and non-HLA antibodies in transplant recipients[11].

As a consequence of this increase in knowledge, the term CAN was deleted in the Banff'05 meeting report[12]. In the Banff'07 and Banff'09 conferences[13,14], the concept of ABMR was further evaluated, and ABMR was definitively included in the Banff classifications.

The Banff'11 meeting report[15] and the recent Banff'13 conference (unpublished data) further elaborated upon new concepts in ABMR, which included the significance of C4d-negative and C1q-positive ABMR.

DETECTION OF ANTIBODIES AND THEIR SIGNIFICANCE

Preformed antibodies targeted against HLAs or antibodies formed *de novo* after transplantation predispose to either acute or chronic ABMR. These antibodies can be detected using several techniques.

A complement-dependent lymphocytotoxicity (CDC) cross-match is typically performed to detect cytotoxic DSAs. The main disadvantages of the CDC assay are that it is subjective and cumbersome and will only detect complement-fixing antibodies[16]. Indeed, ABMR has occurred in patients with a negative cross-match. This observation may indicate that the CDC lacks the sensitivity required to detect some clinically significant antibodies; moreover, acute ABMR can occur in recipients with immunological memory and undetectable levels of circulating HLA antibodies at the time of transplant[17]. Cross-match (XM) and antibody detection techniques have improved with time and show increased sensitivity and specificity[18,19].

Flow cytometry (FC) is another cell-based technique that was introduced more than 20 years ago to improve sensitivity. This test also lacks specificity, and with the introduction of solid-phase assays (SPA), the use of FC has been superseded. The introduction of SPA detection, while providing greater sensitivity than CDC assays, has resulted in a new paradigm with respect to the interpretation of DSAs. Although SPA using the Luminex instrument has permitted the detection of antibodies not detectable by CDC, the clinical significance of these antibodies is not fully understood. In addition, SPA testing raises technical issues that require resolution and careful consideration when interpreting antibody results. SPA, such as flow cytometry using antigen-coated micro particles, enzyme-linked immunosorbent assays (ELISAs) and Luminex, are now used to determine the specificity of anti-HLA antibodies and to better interpret positive CDC-XM results.

ELISA has an advantage over the CDC because is more sensitive and detects antibodies that not fix complement. However, non-specific binding to other immunoglobulins may occur in patients with autoimmune disorders. When detecting antibodies using flow panel-reactive antibody (PRA) beads, micro-particles are coated with purified HLA molecules[19]. The fluorescence is then measured using flow cytometry and the level of fluorescence is indicative of the level of antibody binding.

Luminex technology also uses pools of HLA class Ⅰ or Ⅱ antigen-coated micro-particles. These beads are colored with a combination of two dyes. Serum reactivity is assessed based on the fluorescent signal of each HLA-coated micro particle[19]. The Luminex platform enables the determination of DSAs specificity by using single HLA-coated beads and provides a relative indication of the antibody strength and level in the circulation by returning results to the user in the form of mean fluorescence intensity (MFI)[20]. However, MFI is not standardized across labs, and there is some arbitrariness in determining the MFI thresholds. Molecules with equivalent soluble fluorochrome (MESF) and maximum fluorescence values, obtained using the Luminex machine, enable more standardized measures of antibody strength[21,22].

According to the consensus publication by the National Conference to Assess Antibody-Mediated Rejection in Solid Organ Transplantation[23], a current positive CDC or anti-human immunoglobulin-CDC (CDC-AHG)

Table 1 Technological advantages and limitations of luminex human leukocyte antigens single antigen bead

Technological advantages	Technological limitations
Qualitative: enables precise identification of all antibody specificities in complex sera (DSA)	Some positive results can be caused by antibodies to denatured HLA
Comprehensive: distinguishes antibodies to all common alleles for HLA-A, HLA-B, HLA-C, HLA-DRB1, HLA-DRB3/4/5, HLA-DQA1, HLA-DQB1, HLA-DPA1, HLA-DPB1	Occasional high background binding requiring repeat testing and absorption protocols
Semiquantitative: enables determination of antibody levels (high, intermediate, and low)	Variable HLA protein density on beads. Blocking factors may cause false-negative or misleading low assessment of antibody levels (prozone?); IgM and C1 can block IgG binding
Sensitive: enables detection of weak antibody testing	
Rapid: enables real-time antibody monitoring for DSA. Pre-transplantation and post-transplantation antibody monitoring (assist diagnosis of ABMR). Virtual XM	Lot-to-lot variation requiring validation. Vendor-specific variation
Enables detection of non-HLA-specific antibodies (*e.g.*, MICA)	
Detection and differentiation between immunoglobulin class and isotype (*e.g.*, complement fixing and non-complement fixing C4d and C1q	Reagents not standardized

ABMR: Antibody mediated rejection; DSA: Donor specific HLA antibodies; HLA: Human leukocyte antigens; MICA: Major histocompatibility complex class I-related chain A; SAB: Single-antigen beads; XM: Cross-match.

cytotoxicity cross-match (CXM) predisposes to a high risk of ABMR or early graft loss. A current positive CDC or CDC-AHG CXM is a contraindication for transplantation unless DSAs can be reduced using desensitization protocols. A positive flow CXM or a remote (historic) positive CDC or CDC-AHG CXM poses an intermediate risk for early acute rejection and may require augmented immunosuppression.

The wide use of the Luminex technique with its increased specificity and sensitivity did uncover a new paradigm. Using the Luminex technique DSAs have been found in patients who show a negative classic CDC. Several studies have shown that these results represent a risk factor, but not a formal contraindication for transplantation[24-26].

These facts lead to workshops and Consensus Guidelines to further understand these technologies[11,27].

The recent consensus guidelines highlighted the technological advantages and limitations of Luminex as shown in Table 1.

In addition, the consensus guidelines[11] considered the following modifications to SPA for detecting new antibodies and assessing their functionality.

C4d assay

The C4d assay[28,29] shows superior specificity compared with the CDC. The C4d assay requires complement activation to occur and is influenced by complement regulatory factors. Clinical data obtained using various modifications of the C4d assay have shown that the presence of C4d+ antibodies correlates with graft survival in the kidney and hearts[28,29].

C1q assay

The C1q assay was designed to distinguish complement-fixing from non-complement-fixing antibodies and does not require complement activation other than the binding of C1q to the antibody[30]. It detects antibodies capable of binding complement and initiating the classical pathway.

The results of this technique still remain under debate. Although some authors[31] have reported no correlation with the clinical course in kidney transplant patients, others[32] have reported that both C1q and C4d Luminex assays show increased sensitivity and specificity and that they can be useful for both pre-transplant risk assessment and post-transplant monitoring.

Detection of antibodies targeted to non-HLA antigens

The endothelial cell is the principal target used to detect non-HLA antibodies involved in ABMR. Historically, different assay systems have been used to identify and characterize AECA including CDC[33], flow cytometry[34] and immunofluorescence[35].

The primary limitation is that the endothelial cells used for the detection and characterization of AECA have been derived from third-party donors, and that the cells used show different protein expressions and distinct phenotypes[36]. Surrogates of endothelial cells, such as MICA may be useful. However, MICA is not expressed constitutively on the endothelium; rather, its expression is induced under conditions of cellular stress.

Lymphocyte XM tests fail to detect AECA. The cross-match ONE assay is a Food and Drug Administration (FDA)-approved endothelial ECXM technique that uses endothelial cell precursor cells found in the peripheral blood at a frequency of 1%-2%[37]. An advantage of this test is that it detects DSAs and can be used to test for antibodies targeted to T lymphocytes, B lymphocytes and endothelial cells in the same assay[38].

Proteomics approaches using protein extracts from different sources, including cell lysates and protein microarrays are being used for antibody screening and identification of specificities[39,40].

A variety of non-HLA targets have been identified including MICA, vimentin, angiotensin Ⅱ type 1 receptor, tubulin, myosin and collagen Ⅴ. In general, single antigen bead (SAB) testing permits reassessment of the im-

munologic risk for kidney transplantation. Traditionally, high panel-reactive antibody, re-transplant and deceased donor grafts have been associated with increased risk. However, the risk factors for ABMR are DSAs, reduced HLA matching and evaluation of DSAs using different techniques[41].

PATHOPHYSIOLOGY

An increasing body of evidence suggests that patients with high titers of anti-HLA antibodies (particularly if they are donor-specific) that develop either pre-transplant or post-transplant, show a worse outcome. At any given time, approximately 25% of transplant recipients show antibodies against HLA antigens when evaluated using the newest, highly sensitive and specific techniques for DSAs monitoring[42,43]. Moreover, antibodies against non-HLA have also been implicated in ABMR[44]. Antibodies may mediate endothelial injury *via* complement-dependent or independent mechanisms by transducing signals that are pro-inflammatory and proliferative[45].

Preformed or *de novo* DSAs clearly cause acute and chronic ABMR; however the role and scope of non-HLA antibodies in mediating graft injury and loss remains less certain[46].

One hypothesis is that alloantigen sensitization occurs based on non-HLA polymorphic differences between the donor and the recipient [*e.g.*, major-histocompatibility-complex (MHC) class I -related chains A and B (MICA and MICB, respectively)]. Unfortunately progress in this area has been limited by a lack of validated clinical assays for non-HLA alloantibodies, the confounding presence of HLA-DSAs and, in the case of MICA antibodies, a lack of proof of specificity[47].

A second hypothesis is that auto antigen sensitization occurs due to exposure of cryptic epitopes after tissue injury or inflammation (including vimentin, K-α I tubulin, collagen V and agrin).

Although anti-HLA antibodies are responsible for the majority of antibody-mediated injuries, they do not underlie all ABMRs. In addition, as discussed above, the major histocompatibility antigens and a large number of minor antigens have been recognized as possible antibody targets[48-50].

Endothelial cells are targets for immune-mediated assaults *via* anti-endothelial cell antibodies (AECAs). The *de novo* development of circulating anti-endothelial cell antibodies, rather than pre-existing antibodies, is associated with post-transplant allograft rejection[51].

Apoptotic endothelial cells (ECs) release a bioactive C-terminal fragment of perlecan called laminin G-like 3 (LG3)[52]. LG3 behaves as a neo-antigen and induces the production of anti-LG-3 antibodies. Recently, these anti LG-3 antibodies have been documented to be novel accelerators of immune-mediated vascular injury and to obliterate remodeling[53].

Vimentin[54], collagen V[55] and K-α 1 tubulin[56] are involved in the ABMR of organ other than kidney as neo-antigens. The apoptosis of ECs and subsequent exposure of neo-antigens may induce an autoimmune response.

An autoantibody specific for angiotensin Ⅱ receptor type 1 has been associated with the development of hypertensive vasculopathy and acute renal allograft dysfunction[57]. Antibodies directed towards MHC class I polypeptide-related sequences A (MICA) and B (MICB), and not classical HLA molecules, have been implicated in transplant rejection in recipients who were otherwise well-matched for HLA due to the contribution of MICA antigens towards the activation of cellular and humoral immune responses[58].

The HLA complex encodes molecules crucial for the initiation and proliferation of the immune response. It is highly polymorphic and polygenic and its proteins are co-dominantly expressed. The *HLA* genes that are involved in the immune response belong to classes I and Ⅱ, which are structurally and functionally different. Recently, DSAs have been reported to activate endothelial cells, thereby increasing their potential to recruit and bind recipient leukocytes and increasing the potential for allograft inflammation[59,60].

Approximately 30% of patients on waiting list show detectable levels of HLA antibodies[61]. After transplantation, 25% of non-sensitized patients develop *de novo* HLA-DSAs.

In both groups of patients, the presence of these antibodies increases the risk of subsequent ABMR[9]. The development of a histological test to identify antibody-mediated complement activation on transplant biopsies (C4d staining) has provided a method for flagging potentially deleterious interactions between antibodies and the graft endothelium. In addition, molecular techniques, such as gene expression profiling, have enabled the identification of subclinical endothelial cell damage that can be present even in the absence of complement activation or detectable DSA[62]. Recent studies have documented the role of B cells and antibodies in transplantation. A study by Lynch *et al*[63] described a technique that may enable a more global assessment of B-cell reactivity to the allograft. Their results suggest that humoral responses to the allograft may be more common than previously appreciated. Antibodies reactive to donor human leukocyte antigen molecules, minor histocompatibility antigens, endothelial cells, red blood cells or auto antigens may trigger or contribute to rejection at both early and late time points after transplantation[64]. Often, the immune system shows an integrated response that results in allograft rejection involving parallel or simultaneous T cell mediated rejection (TCMR) and ABMR[65]. Antibody-mediated injury to the allograft is initiated by DSAs binding to HLA antigens or to other targets on the allograft endothelium. If DSAs are complement-activating, the classic complement pathway is rapidly activated *via* IgG binding and C1q activation[66]. This process typically results in the rapid loss of the allograft. Alternatively, DSAs can bind endothelial cell targets and stimulate cell proliferation or induce antibody-dependent cell-mediated cyto-toxicity with interferon γ release[45]. These processes appear to be more important for the development of the chronic

antibody-mediated injury that is more dependent on natural killer (NK) cells than the complement[67]. Antibodies may also bind HLA and other targets and incompletely activate the complement system without causing apparent injury. This process is referred to as accommodation[68].

ABMR is a continuous process, and its oscillation is characterized by fluctuations in DSAs, C4d deposition and dynamic and multidirectional glomerulitis and/or capillaritis scores[69]. The time to diagnosis of ABMR is highly dependent on the population studied. Early-onset ABMR (typically occurring within the first months after transplantation) is observed predominantly in patients with preformed DSAs, whereas late acute ABMR occurs primarily in patients who develop *de novo* DSAs after transplantation. Indeed, the pathologic and clinical manifestations may vary, including hyper-acute humoral rejection, acute humoral rejection, indolent or subclinical humoral rejection, "C4d"-negative humoral rejection and late acute humoral rejection.

ACUTE ABMR

Hyper-acute ABMR

The pathology of hyper-acute rejection overlaps completely with acute ABMR. It arises within minutes or a few hours after transplantation in pre-sensitized patients who have circulating HLA, AB0, or other alloantibodies to the donor endothelial surface antigens[70]. The outcome is always poor.

Acute ABMR

The diagnosis of acute ABMR relies upon the criteria shown: (1) morphologic evidence of acute tissue injury: acute tubular injury, neutrophils and/or mononuclear cells in PTC and/or glomeruli and/or capillary thrombosis, fibrinoid necrosis/intramural or trans-mural inflammation in arteries; (2) immuno-pathologic evidence for antibody action: C4d and/or (rarely) immunoglobulin in PTC; Ig and complement in arterial fibrinoid necrosis; and (3) serologic evidence of circulating antibodies to donor HLA or other anti-donor endothelial antigen. The endothelial injury has been recently reviewed completely by Drachenberg and colleagues[71]. Although acute ABMR generally occurs within the first year after transplantation in pre-sensitized patients[72], it may also develop years after transplantation and is often triggered by a decrease in immunosuppression (iatrogenic, non-compliance or malabsorption)[5,73-75].

Several patients with acute ABMR show a negative cross-match, which may be due to low level DSAs that are undetectable[76] or to *de novo* DSAs[77].

Recently, an increased risk of acute ABMR has been associated to elevated pre-transplantation soluble B-cell activating factor (BAFF)[78], whose neutralization may be an interesting therapeutic strategy.

Recently, Orandi *et al*[79] examined the long-term effect of early acute ABMR on kidney allograft and patient survival in 201 adult kidney transplant recipients who developed acute ABMR within the first year after transplantation. Each recipient was matched with 5 control patients. The majority of recipients were sensitized. Allograft survival rates at 1, 5 and 10 years in the group that developed acute ABMR were significantly lower than in the control group.

In another study[60] of a cohort of 355 adult kidney transplant recipients, all with a negative CDC-XM, C1q-fixing DSAs did not predict acute ABMR or allograft loss; however, the presence of class Ⅰ DSAs (versus class Ⅱ donor specific antigens) predicted acute ABMR and allograft loss.

Indolent or subclinical acute ABMR

Chronic rejection is often preceded by the occurrence of an acute ABMR due to the fact that modern therapeutic strategies fail to deplete antibody secreting plasma cells from the spleen and bone marrow of patients[80].

In addition, kidney transplant recipients who develop *de novo* DSAs are now recognized to often show pathologic features of indolent and slowly progressive microvascular abnormalities, which are referred referred to occasionally as subclinical acute ABMR[16,77,81]. The appearance of *de novo* DSAs likely results from inadequate immunosuppression and represents a dynamic process that begins early after transplantation and continues at varying levels thereafter.

C4d negative acute ABMR

Initial evidence for C4d-negative acute ABMR emerged in 2009 based on the work of the teams in Paris[69] and Edmonton[62]. The latter study demonstrated high endothelial-specific gene expression in kidney transplant biopsy samples with DSAs but without C4d. In this study, C4d-negative acute ABMR was characterized by the high intragraft endothelial gene expression of allo-antibodies, by histology typical of chronic or acute ABMR and by poor outcomes. Several hypotheses have been generated to explain the lack of complement deposition despite the evidence of micro-vascular inflammation and persistence of DSAs in the circulation. The low sensitivity of C4d[13,82] could be due to technical issues including the type of fixative used and the different methods used to detect C4d (immunofluorescence versus immunochemistry). Moreover, as documented by the Edmonton study, some DSAs, although showing poor complement-fixing ability, may nonetheless activate endothelial cells[62]. Another possibility is that the various prophylactic strategies used to prevent ABMR may decrease the burden of complement activation within capillaries[80].

Given the concerns over the lack of sensitivity of C4d for kidney transplantations, a working group was established at the 2011 Banff conference to refine the criteria used for diagnosis of ABMR in the kidney[15]. Although the 2013 Banff Conference, held in Fortaleza (Brazil) in August 2013, has ended, to the best of our knowledge, this work remains in progress.

Late acute ABMR

If the majority of early-acute ABMR depends upon pre-

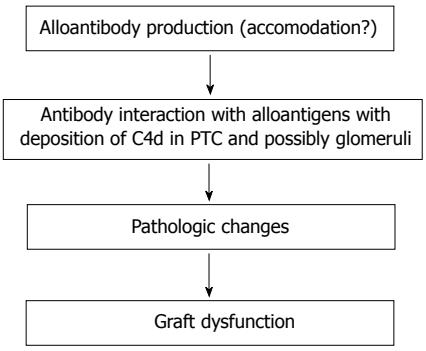

Figure 1 Stages of chronic antibody-mediated chronic rejection. PTC: Peritubular capillaries.

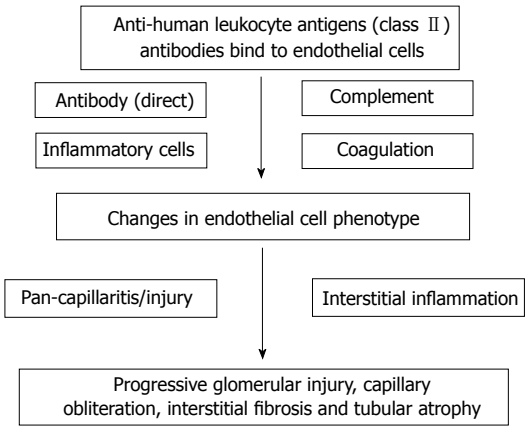

Figure 2 Proposed pathogenetic mechanisms for transplant glomerulopathy.

formed DSAs and primarily occurs in sensitized patients, late-acute ABMR often depends upon *de novo* DSAs.

De novo DSAs appear in 25% of non-sensitized patients[77]. *De novo* DSAs are often linked to late-acute ABMR and are characterized as occurring in patients who are young, with frequent non-adherence or suboptimal immunosuppression[74]. The observation that many cases of *de novo* DSAs are associated with prior therapy non-adherence or with a history of a clinical acute cellular rejection episode, suggests that immunosuppression is a potent inhibitor of the activation of mature, naïve B cells[83]. However, the observation that some cases of *de novo* DSAs formation appear in compliant patients suggests that either T cells capable of helping naïve B cells emerge despite immunosuppression or that some allo-reactive B cells may differentiate into antibody-secreting cells in the absence of T cell assistance. The antibody-producing cells may also originate from an existing population of memory B cells that do not require T-cell mediated activation[84].

CHRONIC ABMR

The clinical significance of chronic ABMR has been increasingly documented in recent years with some data suggesting that it may represent the leading cause of late allograft loss[4]. In contrast to acute ABMR, chronic ABMR is a long-term process that develops in sequential steps over a period of months to years[85]. Chronic ABMR has been proposed to arise over a series of stages or states[86]. The first common event is the production of alloantibodies followed by antibody interaction with alloantigens, resulting in the deposition of C4d in peritubular capillaries (PTC) and possibly glomeruli, followed by pathologic changes and graft dysfunction (Figure 1). Diagnostic features of chronic ABMR may include the presence of DSAs, transplant glomerulopathy (TG), peritubular capillary basement multilayering and the presence of C4d[27].

TG and PTC basement multilayering represent the histological hallmark of chronic ABMR. Transplant glomerulopathy is a morphological pattern of chronic kidney injury that lacks detectable immune-complex deposits and is associated with poor kidney transplant outcomes. It is primarily an endothelial pathology that affects kidney microcirculation endothelium, which is observed as a duplication (double contours) and/or multilamination of capillary basement membranes together with the substantial replacement of endothelial fenestrations with a continuous endothelial lining[87]. DSAs, particularly HLA antigen class Ⅱ antibodies may cause insidious graft injury and therefore constitute a central causative factor of transplant glomerulopathy (Figure 2). Although the international Banff consensus criteria classify transplant glomerulopathy as chronic ABMR if the pattern is accompanied by detectable DSAs and diffuse or focal linear C4d positivity in peritubular capillaries[4-6], Mauiyyedi *et al*[88] detected the deposition of C4d in peritubular capillaries in 61% of biopsies from patients showing chronic rejection with transplant glomerulopathy. In addition, a study by Regele *et al*[89] reported the presence of C4d in peritubular capillaries in 34% of patients with transplant glomerulopathy and this staining presaged the later development of transplant glomerulopathy.

Pathologic patterns of chronic ABMR are observed in renal biopsies performed either for clinical indications or for protocol at a much later date after kidney transplantation[5-83]. In addition to reduced immunosuppression and non-adherence, early acute rejection appears to play a relevant role during late chronic ABMR. Indeed, several years ago, Cosio *et al*[90] documented that in 1-year surveillance biopsies, the degree of inflammation at 1-year post-transplant predicts the loss of graft function and graft failure independently of function and other variables (Figure 3).

Recently El Ters *et al*[91] reported that early acute rejection, even in the absence of pre-transplant DSAs, increases the risk of alloimmune allograft loss late after transplantation and that the phenotype of this late loss is chronic ABMR. The hypothesis of this study was that the formation of new DSAs, particularly class Ⅱ DSAs, may be a consequence of early acute rejection[92]. El Ters *et al*[91] noted that the presence of inflammation in 1-year protocol biopsies correlated with early acute rejection, presensitization, re-transplantation and HLA mismatch. He also observed that chronic ABMR was responsible for 43% of allograft loss.

Table 2 Anti-antibodies main drugs to date in use and mechanism of action

Steps	Cells or mechanisms involved	Drugs	Mechanism of action
Exposure to antigen	B Cells	Rituximab	Binds CD20 on B cells and mediates cell lysis
		ivig	Multiple B cell apoptosis, decrease in B-cell proliferation
Secretion of alloantibodies	Plasma cells; antibodies	Bortezomib	Decrease donor-specific alloantibody production
			Mechanical removal of alloantibodies
		Plasma- exchange	Multiple B cell apoptosis, decrease in B-cell proliferation
		ivig	
Binding of antibodies to the graft	Complement activation	Eculizumab	Blocks cleavage of terminal complement C5 and halts the process of complement-mediated cell destruction

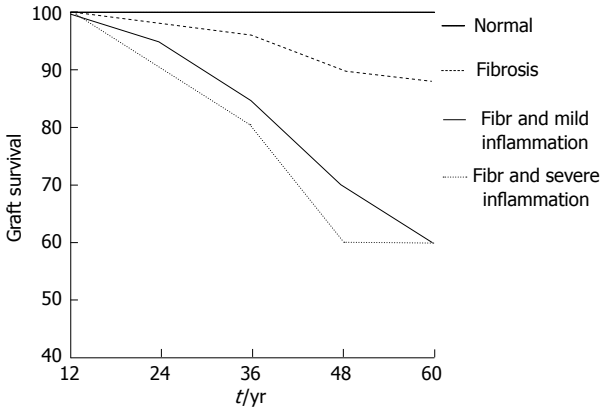

Figure 3 Five year post-transplant graft survival according 1-year post-transplant surveillance biopsy.

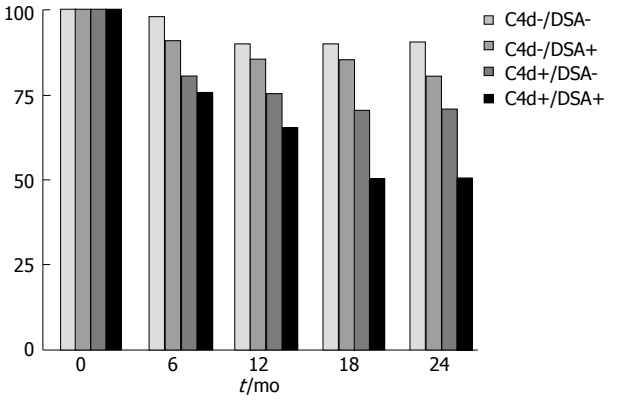

Figure 4 Deterioration of kidney allograft function study. Graft survival at 2-year according presence of donor-specific-HLA antibodies (DSAs) and/or C4d. HLA: Human leukocyte antigens.

In surveillance biopsies performed at 3 years after transplantation, Willicombe[93] reported that, despite excellent serum creatinine values, only one-third of biopsies were normal and that lesions appeared to correlate with the risks of immunological injury.

The 5-year follow-up data of the patient cohort from the DeKAF study[94,95] documented the role of antibodies in late graft dysfunction. Indeed, these studies showed a great number of patients with inflammation accompanying fibrosis or scarring, and their graft survival correlated with the presence of DSAs and/or C4d (Figure 4).

The therapeutic approach to these conditions is one of the major challenges to date in the treatment of transplanted patients.

Finally, recent studies[96,97] examining BAFF, a B-cell stimulating molecule, showed that the appearance of soluble BAFF levels early after transplantation correlated with the *de novo* development of DSAs and, ultimately, with the progression to chronic active ABMR in pediatric and adult first kidney transplant recipients who were highly desensitized prior to transplantation.

Hill *et al*[98] described a new insight into the pathogenesis of chronic ABMR. DSAs-positive patients showed a striking acceleration of arteriosclerosis. Pathologic examination revealed that the inner intima is hypercellular with actively proliferating myofibroblasts that lay down collagen that often overlies older, condensed collagen of pre-transplantation donor origin.

THERAPY

The primary drugs or systems used are shown in Table 2 and are divided based on their action on the different maturation steps of B cells. The primary therapeutic strategies used are the following: (1) removal of antibodies; (2) inhibition of antibody production; (3) complement inhibitors; (4) intravenous immunoglobulins; and (5) splenectomy.

Removal of antibodies by plasmapheresis or immunoabsorption

Plasmapheresis (PP) and immunoabsorption (IA) techniques have been used to remove alloantibodies. PP is not specific for immunoglobulins (Ig) removal and requires replacement with fresh frozen plasma and albumin. IA shows high affinity for binding Igs and has the advantage of specificity over PP.

However, due to the tendency of DSAs to rebound and return to baseline levels, several repeated treatments are required[99] or an additional inhibitor of antibody production is required.

Inhibition of antibody production

Rituximab (anti-CD20): Rituximab is a chimeric murine/human monoclonal antibody that binds CD20 on pre B and mature B lymphocytes[100,101]. Recently rituximab has been documented to also prevent an anamnestic

response in patients with cryptic sensitization to HLA[102].

BAFF blockade: BAFF, also known as B lymphocyte stimulator (Blys), is a member of the tumor necrosis factor cytokine family and is expressed primarily on T cells and dendritic cells for B-cell co-stimulation. BAFF binds to the receptor B-cell maturation antigen (BCMA), to the transmembrane activator (TACI) and to BAFF-receptor (BAFF-R) for B cell survival, proliferation and maturation[103].

BAFF blockade is a possible future therapy for renal transplantation. These drugs are highly promising because they selectively target B cells. Nevertheless no clinical trial is active in the field of transplantation although, these drugs have either been approved or are being examined for other diseases in large studies.

The best BAFF blockade drug is belimumab, which is a fully human recombinant IgG monoclonal antibody targeted against BAFF[104].

Bortezomib: Bortezomib is a proteasome inhibitor that is primarily used to treat acute ABMR or to decrease *de novo* DSA levels post-transplantation[105,106]. In further pilot studies, the authors used bortezomib in desensitization protocols with encouraging results[107,108].

Complement inhibitors

Eculizumab: Eculizumab is a humanized monoclonal antibody targeted against complement protein C5 that binds the C5 protein with high affinity and inhibits its cleavage to C5a and C5b, thereby preventing the generation of the terminal complement complex C5b-9. Eculizumab is used for the treatment of paroxysmal nocturnal hemoglobinuria and for atypical hemolytic uremic syndrome. Stegall *et al*[109] documented a decrease in post-transplant acute ABMR in sensitized renal transplant recipients, indicating its usefulness for desensitization protocols. Case reports have documented the effective rescue treatment of severe complement activation and reversal of acute ABMR by eculizumab in AB0-incompatible kidney-pancreas transplants and re-transplanted kidney recipients[110,111].

Intravenous immunoglobulins

Intravenous immunoglobulins show pleiotropic effects: They neutralize circulating anti-HLA antibodies *via* anti-idiotypic antibodies, inhibit complement activation by binding C3b and C4b and neutralizing C3a and C5a[112]. They also inhibit the expression of CD19 on activated B cells and induce the apoptosis of B cells[113]. Intravenous immunoglobulins (IVIGs) also show inhibitory effects on cellular immune responses with no specific inhibitory effects on the immune system by binding to Fcγ receptors on macrophages, neutrophils, platelets, mast cells and NK cells.

IVIGs are used to decrease PRA levels in highly sensitized patients, in desensitization protocols of AB0-incompatible and XM-positive patients and in the treatment of ABMR.

Splenectomy: Splenectomy has been used in desensitization protocols and in the treatment of refractory acute ABMR[114,115]. Splenectomy removes a major source of lymphocytes, but the effect on the immune system is permanent and places the patients at risk for the development of sepsis.

As discussed in the pathophysiology chapter, we should distinguish the following: (1) acute ABMR; and (2) chronic ABMR.

Acute ABMR

Early acute ABMR often occurs in patients with DSAs prior to transplantation with a CDC-XM-positive with the donor. Even after successful desensitization strategies and successful kidney transplantations acute ABMR occurs in up to 40% of recipients. A later occurrence of acute ABMR is typically noted in patients with *de novo* DSAs and often after the reduction of immunosuppression or non-adherence[116,117].

We should now distinguish between the prevention and the treatment of acute ABMR.

Prevention of acute ABMR: Patients waiting for a transplant may be highly immunized and many show detectable DSAs in their serum. Sensitized patients who are DSAs-negative with negative XM-CDC may be transplanted safely. They will likely require more immunosuppressive therapy and an induction therapy[118-120].

The different desensitization protocols apply primarily to DSA-positive patients who are XM-CDC positive. The majority of the current protocols are modified version of the high-dose IVIG initiated at the Cedars-Sinai Medical Center or of the PP with low-dose IVIG initiated at John Hopkins Hospital[121].

Jordan initially provided[122] high dose IVIGs (2 g/kg) to cross-match-positive recipients, and the patients received a kidney transplant when their CDC T cell XM became negative. Due to the high rate of acute ABMR, Jordan[123] decided to use alemtuzumab induction treatment and added rituximab to the protocol to decrease the acute rejection rate.

More recently, Vo *et al*[124]at the Cedars-Sinai reported on the 24-mo outcomes of the aforementioned desensitization protocol and showed a 2-years graft survival of 84% in 76 hyper immune XM-positive recipients.

The other approach to desensitization comprises the use of PP and low-dose anti cytomegalovirus IVIG (CMV Ig). This approach was first adopted in 1998 at John Hopkins Hospital in XM-incompatible living donor kidney transplant candidates[125]. Patients received PP and CMV Ig at 100 mg/kg after each PP, combined with tacrolimus and mycophenolate mofetil. In a recent study, Montgomery *et al*[126] successfully desensitized 211 DSA-positive recipients of living donor kidneys with PP and low-dose IVIG.

A differing approach is the use of peri-transplant

immunoabsorption rather than plasmapheresis. In 68 patients with deceased donors, Bartel and colleagues used peri-transplant IA followed by post-transplant IA and obtained excellent transplant outcomes[127].

Overall, over the last 13 years, almost 1000 patients with DSAs underwent kidney transplants and used varying desensitization protocols. The patient and graft survival rates are 95% and 86%, respectively, at the 2-year median follow-up. The primary issue is the high rates of acute rejection and of ABMR in particular (28%)[128]. New drugs are being developed to reduce this high rate of ABMR.

Stegall *et al*[109] added eculizumab during the pre-post-transplant period in DSAs-positive patients and obtained 7.7% post-transplant acute ABMR compared with 41.2% in the control group. However, at 2-years after transplantation the incidence of chronic ABMR was similar between the two groups. Chronic ABMR remains a major issue when transplanting hyper-immune patients.

A different option is to use the proteasome inhibitor bortezomib. In pilot studies, bortezomib has been used in desensitization protocols with encouraging results[107,108]. It is being used in a current ongoing a prospective iterative trial of proteasome inhibitor-based desensitization[129]. The trial has been approved by the International Review Board (IRB) and is being conducted under the auspices of FDA. Preliminary data suggest that bortezomib-based desensitization regimens comprising only two cycles (8 doses) may consistently reduce immunodominant HLA antibody levels and that multiple treatments with bortezomib (two-cycle regimen) may enable highly sensitized patients to undergo transplantation without IVIGs.

Treatment of acute ABMR: Acute ABMR in kidney recipients responds poorly to corticosteroids and antithymocyte agents alone, which are the standard treatment for acute cellular rejection.

International guidelines do not define an evidence-based treatment for acute ABMR. The kidney disease improving global outcomes guidelines (KDIGO) recommend the use of one or more of the following: corticosteroids, PP, IVIG, anti-CD20 antibodies or lymphocyte-depleting antibodies[130].

Two studies have individually reviewed the current approach to the treatment of acute ABMR[46] and the randomized controlled trials treating acute ABMR[131].

Although the literature suggests that plasmapheresis with or without low-dose IVIG or high-dose IVIG alone shows evidence of efficacy against acute ABMR and that they may be considered for the standard of care (SOC), these treatment regimens have not been standardized or optimized.

Approaches vary based on the amount of replacement volume, type of replacement fluids, number of PP sessions and the dose, timing and formulation of IVIGs used.

Other agents, such as rituximab, bortezomib and eculizumab have been used occasionally in conjunction with the above-mentioned therapies.

Of these treatments, rituximab has been used most frequently, and two studies in particular have evaluated rituximab as part of a combination treatment approach[132,133]. The latter study included 54 patients and compared a historical group treated with plasma exchange and IVIGs with a later group receiving a single dose of 500 mg/m^2 rituximab in addition. The use of rituximab was associated with a 90% 2-year graft survival compared with 60% in the control group. Nevertheless, the benefit of adding rituximab remains in question when examining all published patient series.

Several case reports and series have been published on the use of bortezomib in the treatment for acute AMBR.

The largest series of 20 patients treated with bortezomib was reported by Flechner[134]. Using this treatment regimen, a graft survival rate of 85% at 10 mo post-transplant was achieved. The mean decrease in the dominant DSA in MFI values was 50%. However, the side effects of the treatment were considerable. One of the most recent studies compared 10 bortezomib-treated patients with a historical group of 9 rituximab-treated patients and achieved a graft survival of 60% with bortezomib compared with only 11% with rituximab at 18 mo[135].

Taken together, these preliminary results on bortezomib in acute ABMR are promising; however carefully performed, controlled studies are required to prove its benefits.

In the setting of kidney transplantation, there is emerging but limited evidence that eculizumab is efficient in treating acute ABMR[136]. Thus far, only a few reports exist in the literature on the use of eculizumab in refractory acute ABMR[110,111]. Stegall *et al*[109] reported the largest study of eculizumab in renal transplantation in a desensitization strategy. In this study, eculizumab appeared to show no impact on DSAs production after transplantation. In addition, the incidence of chronic ABMR appeared unchanged either by the prevention of early ABMR or by the prolonged complement blockade.

Splenectomy: One last option to salvage a graft with acute therapy-resistant ABMR is rescue splenectomy and its use has been reported by at least three groups[137-139]. The majority of patients underwent this surgery prior to the advent of eculizumab, and in the future, splenectomy may be avoided by using eculizumab instead. Splenectomy is recommended only in resistant cases of acute ABMR where bortezomib or eculizumab have already failed.

In summary, the first step for managing acute ABMR includes steroid pulses and/or antibody removal with PP or IA and IVIGs. The second step in patients with persistent allograft dysfunction includes the use of bortezomib and/or rituximab. The third step in resistant acute ABMR includes eculizumab and rescue splenectomy.

Chronic ABMR: In contrast to acute ABMR, chronic ABMR is a long-term process that develops in sequential steps over months to years[84].

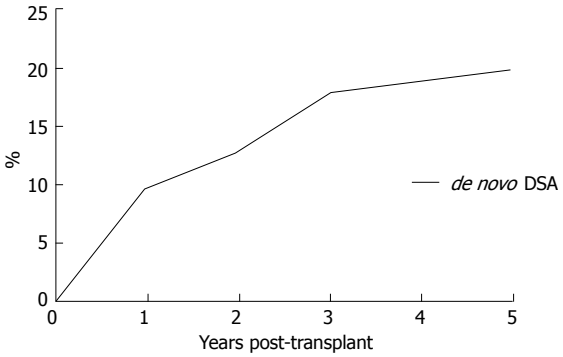

Figure 5 Actual 5-year post-transplantation cumulative incidence of de novo donor-specific-human leukocyte antigens antibodies (DSA). DSA: Donor-specific-human leukocyte antigens antibodies.

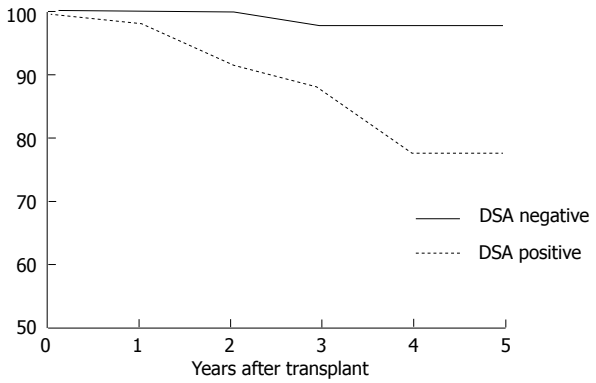

Figure 6 Probability of graft loss within 3 years after *de novo* donor-specific-human leukocyte antigens antibodies appearance. DSA:Donor-specific-human leukocyte antigens antibodies.

In theory, every option available to treat acute ABMR may also be applied to chronic ABMR. However, there are no controlled trials in the literature regarding the treatment of chronic ABMR. The only treatment option with some reported benefit is a combination of rituximab and IVIGs[140].

With respect to established chronic ABMR, there have only been three case series treated with this combination therapy[141-143]. DSAs decreased only in some patients, and the therapy showed limited effects in cases with massive proteinuria, more severe peritubular capillaritis and previous acute rejection.

Very few patients have received bortezomib as a rescue treatment for chronic ABMR and proteinuria, and they have shown mixed results[144,145].

An interim analysis of a very recent study[146] of eculizumab therapy in chronic ABMR documented an apparent stabilization of renal function.

Taken together, these results indicate that any treatment for chronic ABMR using drugs with potentially high toxicity should only be performed in the context of a randomized controlled trial.

A recent recognized context that should be distinguished from acute or chronic ABMR is the negative impact of *de novo* DSAs after transplantation on the transplant outcome.

Several authors have reviewed the incidence and impact of *de novo* donor DSAs, in both adult[77] and pediatric recipients[147].

The actual 5-year post-transplantation cumulative incidence of *de novo* DSAs in a low-risk population is 20% (Figure 5). Once DSAs appear, the probability of graft loss within the 3 years of the appearance of DSAs is 24% (Figure 6). In patients without DSAs, the relative risk of graft loss is 9-fold higher at 1 year after the appearance of DSAs. In a multivariate analysis[77], the primary causes of *de novo* DSAs were DQ locus mismatches, a younger age at transplantation and transplants from deceased donors. Others claim prior non-adherence or a history of a clinical acute cellular rejection as being causes of *de novo* DSAs[82].

If the appearance of DSAs is associated with the clinical signs of acute ABMR, the treatments used have already been discussed. The primary issue is how to treat when the appearance of DSAs is not associated with acute rejection.

To date, prophylactic treatments, such as rituximab and splenectomy[148] or eculizumab[109], do not appear to induce any effect on the appearance of DSAs.

Monitoring DSAs after transplantation appears to be essential because the appearance of DSAs is associated with a poor prognosis. Because procedures, such as antibody removal by PP or IA and the down regulation of antibody production by B cell- or plasma cell-targeting or complement cascade inhibition show very limited success when employed during the advanced phase of chronic ABMR[142,149,150], the prompt removal of *de novo* DSAs appears to be essential. However, no SOC exist for this issue. To date, only a multicenter antibody removal trial study in Italy is ongoing; it is using a randomized, prospective PP and low-dose CMV-IVIGs[151].

NEW AND FUTURE THERAPIES

Some of the drugs mentioned above that have been used to prevent or treat acute ABMR remain in pre-marketing clinical trials or have been approved for other diseases.

Drugs already known to control T cells also appear to be active in the long-term control of B cells.

Belatacept, a fusion receptor protein that blocks the co-stimulation pathway CD80/CD86-CD28, was recently approved for the prevention of acute rejection. Belatacept inhibited DSAs in phase 3 trials[152].

Another co-stimulation pathway is the CD40/CD40L pathway. Previous studies with antibodies directed against CD40L failed due to severe episodes of thrombosis. Indeed, CD40L is also expressed on the platelet surface, and its inhibition may induce thrombosis. More recently, the inhibition of the CD40/CD40L pathway by directly targeting CD40 has drawn interest from investigators particularly because CD40 is not expressed on the platelet surface. Humanized anti-CD40 antibodies prevented acute rejection and prolonged renal graft in non-human primates. In addition, these anti-CD40 antibodies appear safe and effective as maintenance immunosuppressive therapies[153-155]. To date, five monoclonal antibodies di-

rected against CD40 have been studied for different diseases including kidney transplantation (Clinical Trial.gov NCT01780844).

Newer drugs that target B cell have been described. The most exciting are likely those that target survival factors and are part of the tumor necrosis factor super family: BAFF, Blys and the proliferation-inducing ligand (APRIL)[103].

Belimumab has already been discussed: it is a fully human antibody that neutralizes BAFF and deprives B cells of this important survival factor. The FDA approved belimumab in March 2011 for systemic lupus erythematosus (SLE). A group from Pennsylvania has enrolled patients in a phase II clinical trial of desensitization in sensitized patients awaiting clinical transplantation (clinicaltrials.gov NCT01025193). In this context, the study was unable to demonstrate the efficacy of belimumab.

Atacicept is a fusion receptor protein that neutralizes both BAFF or Blys and APRIL. In allo-sensitized nonhuman primates, atacicept reduced T-cell and B-cell alloantibodies by 36% and 24%, respectively[156].

A further possibility is complement inhibition by C1 esterase inhibitors, a plasma-derived human C1 esterase inhibitor. Initially used in allotransplantation to protect against ischemia/reperfusion injury[157], it is now under investigation for solid organ transplantations and approved by the FDA for use in other disease states. A trial studying the safety and tolerability of C1 inhibitor therapy in the context of the prevention of acute rejection (clinicaltrial.gov NCT01134510) is now ongoing. However, thus far no patients have been recruited.

CONCLUSION

The relevant graft injury is now well recognized to be caused by alloantibodies. Both acute and chronic graft injury may be caused by alloantibodies, and the most recent Banff classifications have been modified to introduce acute and chronic ABMR. The latter appears to be the most relevant cause of long-term graft injury rather than CNIs nephrotoxicity and "chronic allograft nephropathy".

In addition to the major histocompatibility antigens, a large number of minor antigens have been recognized as possible antibody targets. The most important and the most widely studied antibodies responsible for graft injury are the HLA-DSAs.

The availability of new techniques for detecting circulating antibodies has enabled better understanding in recent years of the presence and role of antibodies in determining graft injury.

From a clinical point of view, we must distinguish between acute ABMR and chronic ABMR. In addition, we now recognize indolent ABMR and C4d-negative acute ABMR. Indolent ABMR develops sub-clinically. It often manifests in patients with *de novo* DSAs and causes slowly progressive microvascular abnormalities that lead to chronic ABMR. C4d-negative ABMR is cause for great discussion among scholars. It may be caused by an injury that is non-complement-mediated; however it may also be due to defective techniques. The Banff group is still working to improve understanding of this entity.

Recently, evidence has accumulated on the significant role of HLA-DSAs in the pathogenesis of slowly progressive graft injury and dysfunction. Several studies have shown that circulating DSAs (class Ⅰ or class Ⅱ) are found in a substantial fraction of renal allograft recipients and are associated with long-term graft loss.

The primary therapeutic approach comprises antibody removal, B-cells and plasma cells- targeting and inhibition of the complement pathway. The therapeutic approach used is based on the clinical conditions.

In patients waiting for transplantation who show positive XM-CDC, the removal of antibodies with or without B- or plasma cell-inhibition remains the best approach.

Patients with acute ABMR should be treated with a heavy regimen of T/B cell-targeting drugs (pulse corticosteroids and ATG), by removing antibodies, and using specific B- or plasma cell-inhibition or by complement inhibition.

No SOC exists for chronic ABMR, and only randomized controlled trials will indicate the best therapeutic option.

What may we hope for in the future? Unfortunately, the pipeline is almost empty.

Essentially, we may consider two types of drugs that are either already on the market or remain in premarket trials: (1) drugs targeting both T and B cells; Belatacept has already been approved by the FDA for the prevention of acute rejection. In a 3-year follow-up study[152], it proved to be effective on DSAs also in CNIs free protocols. The blockade of CD40-CD154 with humanized anti-CD 40 antibodies has prevented acute rejection[154]. In addition, these antibodies appear safe and effective in maintenance therapy; and (2) drugs targeting B cells or the complement pathway.

BAFF-blocking drugs: Represent new interesting drugs that target B lymphocyte stimulators. Belimumab, a fully human recombinant IgG monoclonal antibody to BAFF, was approved in 2011 for the treatment of SLE; however the above-mentioned phase Ⅱ trial for desensitization failed. Atacicept was evaluated in diseases including rheumatoid arthritis, SLE, multiple sclerosis and B-cell malignancies. It awaits evaluation in human transplant patients.

While waiting for the approval of eculizumab, the C1 esterase inhibitor is being studied. This drug has been FDA-approved for treating hereditary angioedema; however it appears to be far from approval for use in transplantation.

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REVIEW

ABO incompatible renal transplants: Good or bad?

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Abstract

ABO incompatible kidney transplantation (ABOi-KT) was previously considered to be an absolute contraindication for patients with end-stage kidney disease (ESKD) due to hyperacute rejection related to blood type barrier. Since the first successful series of ABOi-KT was reported, ABOi-KT is performed increasingly all over the world. ABOi-KT has led to an expanded donor pool and reduced the number of patients with ESKD awaiting deceased kidney transplantation (KT). Intensified immunosuppression and immunological understanding has helped to shape current desensitization protocols. Consequently, in recent years, ABOi-KT outcome is comparable to ABO compatible KT (ABOc-KT). However, many questions still remain unanswered. In ABOi-KT, there is an additional residual immunological risk that may lead to allograft damage, despite using current diverse but usually intensified immunosuppressive protocols at the expense of increasing risk of infection and possibly malignancy. Notably, in ABOi-KT, desensitization and antibody reduction therapies have increased the cost of KT. Reassuringly, there has been an evolution in ABOi-KT leading to a simplification of protocols over the last decade. This review provides an overview of the history, outcome, protocol, advantages and disadvantages in ABOi-KT, and focuses on whether ABOi-KT should be recommended as a therapeutic option of KT in the future.

Key words: Kidney transplantation; ABO incompatible; Antibody depletion; Immunosuppression; Desensitization protocols; Living donor transplantation

Core tip: This article demonstrates merits and demerits of ABO incompatible kidney transplantation (ABOi-KT). Although the excellent outcome of ABOi-KT has been achieved, unresolved matters still remain. We review the role of ABOi-KT for patients with end-stage kidney disease and considered validity whether ABOi-KT should be recommended as a therapeutic option of KT in the future.

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INTRODUCTION

Kidney transplantation (KT) is known as a standard therapy for patients with end-stage kidney disease (ESKD) and has been adopted widely in the world. However, the

living and deceased kidney donor pool does not resolve the shortage of transplantable organs. Different ways have been proposed to increase the donor pool and ABO incompatible KT (ABOi-KT) represents a valid source of organs to decrease the donor waiting list. ABOi-KT requires extra strategies and suffers extra risks across ABO blood type barrier compared to ABO compatible KT (ABOc-KT). ABOi-KT was previously considered to be contraindicated for many years. Presently, ABOi-KT has been accepted as a valid alternative therapy for ESKD and the outcome of ABOi-KT has become equivalent to ABOc-KT in adult and pediatric recipients[1-4]. When a patient with ESKD requires KT and an acceptable living donor is ABO incompatible with the recipient, the patient can currently chose one of three options: (1) stay on the waiting list for deceased donor KT; (2) have paired kidney donor exchange (PKDE); or (3) undergo ABOi-KT.

According to the Organ Procurement and Transplantation Network (OPTN) report 2011, 86500 patients on the deceased donor waiting list, and almost 28000 were added to the list annually in the United States. Ten thousand patients received deceased donor KT, and 4900 patients received living KT. Almost 5000 patients died while waiting for a kidney. The median waiting time depended on the blood type of patients, but it is reported to be around 4 years for all patients on the OPTN report[5]. Various reports analysing graft and patient survival related to the waiting time showed that 6 mo or more of dialysis negatively affect the outcome[6,7]. PKDE is an innovative method whereby 2 or more incompatible donor-recipient pairs exchange donors to create 2 or more compatible pairs. It is a very reasonable idea for human leukocyte antigen (HLA) sensitized and/or ABO incompatible patients. This primary idea was reported first by Rapaport in 1980s[8]. There are currently several variations of exchange such as three-way, four-way and domino paired donation[9]. PKDE provides a recipient with an incompatible donor the chance to receive a compatible kidney, which is available by expanding the donor source and reducing the waiting time for deceased donor KT. Advantages of PKDE are low immunological risk, avoidance of intensified immunosuppression due to desensitization, and cost effectiveness[10].

Alexandre *et al*[11] demonstrated the ABOi-KT strategy using plasmapheresis and splenectomy to break the ABO barrier. This has been used as a desensitization strategy for ABOi-KT for 20 years. ABOi-KT has become common in Japan due to the lack of deceased donors, and ABOi-KT has accounted for approximately 30% of all living-donor KT in that country.[12]. On the contrary, a tiny proportion, only 738 cases (0.94%) of ABOi-KT were performed between 1995 and 2010 in the United States[4], but this number is increasing annually. The same trend continues in the United Kingdom: over the last decade, there has been an increase of ABOi-KT from less than 10 per year to 100 per year representing 1.0% of living donor transplants performed[13]. This increase is possibly due to the fact that protocols have been simplified over the years from complex surgical and pharmacological processes that variably may have involved splenectomy, rituximab (RIT), plasmapheresis and antibodies titration.

Although the use of ABOi-KT has increased worldwide, there are arguments against ABOi-KT as a universal treatment. To consider whether ABOi-KT is viable a therapeutic option for patients with ESKD, this review will focus on the transitional outcomes alongside current and future prospects in ABOi-KT.

Table 1 Combination of blood type and compatibility

		Donor			
		A	B	O	AB
Recipient	A	-	+	-	+
	B	+	-	-	+
	O	+	+	-	+
	AB	-	-	-	-

+: ABO incompatible transplantation; -: ABO compatible transplantation.

ABO ANTIGENS AND ANTIBODIES

The concept of blood groups A, B and O (H) was established by Nobel laureate Karl Landsteiner in the early 1900s. These are polysaccharide antigens which are found in red cell, platelets, and other tissues such as endothelium[14]. The antibodies to blood group antigen are isohemagglutinins and can be of either immunoglobulin M (IgM) or immunoglobulin G (IgG) type antibodies. However, in the context of transplantation it is IgG that is functionally significant.Blood type A develops anti-B antibody, and blood type B has anti-A antibody. Blood type AB with A and B antigen has both antibodies, while blood type O with both antibodies does not have any antigen. Blood type incompatibility means the exposure of A or B antigen to a person who has antibodies against these antigens. Therefore, these antigen expressions of an organ have been obstacles for ABOi-KT (Table 1). All blood type recipients accept a blood type O donor as a universal donor, and a blood type AB accepts all blood type donors as a universal recipient. Blood group type A, however, carries A1 or A2. The expression of A2 antigen is weaker than that of A1 antigen[15]. The A2 subtype constitutes approximately 20% of blood type A in white races, while it is only 0.15% in Japanese population[16]. A2 kidney may be less likely to suffer antibody rejection in the presence of anti-A antibody. In fact, non-A recipients receiving kidneys from A2 donors[17], can universally and safely accept the transplantation without preconditioning at times of KT.

HISTORY

Splenectomy, rituximab and no B-cell depletion

Previous clinical studies related to ABOi-KT are summarized in Table 2[1-4,11,18-42]. The first successful report of ABOi-KT is dated back to 1987 when authors achieved long-term allograft survival in a series of 23 patients[11]. Plasmapheresis and splenectomy were performed to re-

Table 2 Historical clinical reports in ABO incompatible kidney transplantation

Ref.	Type of study	Study population	ABOi population	Desensitization	Outcome
Hume *et al*[18]	Observational	9	1	No treatment	Graft nephrectomy day 17
Starzl *et al*[19]	Observational	3	2	SPx (1 case)	Graft survival 74 d (1 case), patient death day 24 (1 case)
Sheil *et al*[20]	Observational	2	2	No treatment	Graft nephrectomy day 14
Alexandre *et al*[11]	Observational	23	23	PE/SPx	2-yr graft survival: 88% (related donor), 50% (unrelated donor)
Ota *et al*[21]	Observational, comparative	51	51	DFPP and/or IAs/SPx	2-yr graft survival: 87% *vs* 84.6% *vs* 50% (A- *vs* B- *vs* ABO-incompatible)
Tanabe *et al*[22]	Observational, comparative	433	67	DFPP and IAs/SPx	8-yr graft survival: 73% *vs* 80 % (ABOi *vs* ABOc)
Ishida *et al*[23]	Observational	93	93	DFPP/SPx	5-yr graft survival: 73%
Ohta *et al*[24]	Observational, pediatric	10	10	DFPP or PE or IAs/SPx	5.4-yr graft survival: 100%
Shishido *et al*[25]	Observational, pediatric	16	16	PE and IAs/SPx	5-yr graft survival: 85%
Takahashi *et al*[2]	Observational, comparative	1496	441	DFPP or PE or IAs/SPx	9-yr graft survival: 59% *vs* 57% (ABOi *vs* ABOc)
Shimmura *et al*[26]	Observational, comparative	167	167	DFPP and/or IAs/SPx	5-yr graft survival: 74.3% *vs* 78.5% (CYA with AZ or MZ *vs* TAC or MMF)
Futagawa *et al*[27]	Observational, comparative	37803	191	NA	5-yr graft survival: 66.2% *vs* 79.5% (ABOi *vs* ABOc)
Ishida *et al*[28]	Observational, comparative	222	222	DFPP/SPx	5-yr graft survival: 73% *vs* 90% (CYA with AZ *vs* TAC with MMF)
Tyden *et al*[29]	Observational, comparative	334	60	IAs/RIT/IVIG	Graft survival: ABOi 97% (1.5-yr) *vs* ABOc 95% (1.8-yr)
Galliford *et al*[30]	Observational	10	10	PE/RIT/IVIG	1-yr graft survival: 100%
Genberg *et al*[31]	Observational, comparative	45	15	IAs/RIT/IVIG	Graft survival: ABOi 86.7% (3.4-yr) *vs* ABOc 86.7% (4.0-yr)
Oettl *et al*[32]	Observational	10	10	IAs/RIT/IVIG	1.3-yr graft survival: 100%
Toki *et al*[33]	Observational, comparative	57	57	DFPP/SPx	8-yr graft survival: 49% *vs* 95% (AAMR *vs* non-AAMR)
Wilpert *et al*[34]	Observational, comparative	83	40	IAs/RIT/IVIG	Graft survival: ABOi 100% (3.3-yr) *vs* ABOc 93% (1.5-yr)
Tyden *et al*[1]	Observational, comparative, pediatric	38	10	IAs/RIT/IVIG	Graft loss within 3 years: ABOi 1 case, ABOc 2 cases
Flint *et al*[35]	Observational, comparative	89	37	PE/IVIG	1-yr graft survival: 100% (ABOi *vs* ABOc)
Fichinoue *et al*[36]	Observational, comparative	393	113	DFPP or PE/SPx or RIT	5-yr graft survival: 88.4% *vs* 90.3% *vs* 100% (ABOc vs ABOi-SPx *vs* ABOi-RIT)
Habicht *et al*[37]	Observational, comparative	68	21	IAs/RIT/IVIG	1-yr graft survival : 100% (ABOi *vs* ABOc)
Lipshutz *et al*[38]	Observational	18	18	PE/RIT/IVIG	1-yr graft survival: 94.4%
Shirakawa *et al*[39]	Observational, comparative	74	74	DFPP/RIT	1-yr graft survival: 95.7% *vs* 98.% (RIT 500mg *vs* RIT 200 mg)
Shishido *et al*[3]	Observational, comparative, pediatric	323	52	PE/SPx or RIT	15-yr graft survival: 86% *vs* 78% (ABOi *vs* ABOc)
Montgomery *et al*[4]	Observational, comparative	78193	738	NA	10-yr cumulative incidence of graft loss: 27.1% *vs* 23.9% (ABOi *vs* ABOc)
Morath *et al*[40]	Observational, comparative	19	19	IAs or IAns/RIT/IVIG	1-yr graft survival: 100% (IAs *vs* IAns)
Uchida *et al*[41]	Observational	25	25	DFPP or PE/SPx or RIT	4.5-yr graft survival: 100%
Ashimine *et al*[42]	Observational, comparative	320	92	DFPP/SPx or RIT or none	5-yr graft survival: 87% *vs* 97.7% (ABOi *vs* ABOc)

ABOi: ABO incompatible; SPx: Splenectomy; PE: Plasma exchange; DFPP: Double-filtration plasmapheresis; IAs: Antigen-specific immunoadsorption; ABOc: ABO compatible; CYA: Cyclosporine; AZ: Azathioprine; MZ: Mizoribine; TAC: Tacrolimus; MMF: Mycophenolate mofetil; NA: Not available; RIT: Rituximab; IVIG: Intravenous immunoglobulin; AAMR: Acute antibody-mediated rejection; IAns: Non-antigen-specific immunoadsorption.

duce anti-blood type A or B (anti-A/B) antibody and to minimize the risk of hyperacute humoral rejection. Most of the modern desensitization protocols of ABOi-KT have been derived from their procedure and have since evolved. Their work was further greatly expanded in Japan due to the shortage of deceased donors with successful outcomes in ABOi-KT[2].

Nowadays, splenectomy has been totally abandoned and the various desensitization protocols in use are combinations of antibody removal by plasmapheresis or immunoadsorption (IA), intravenous immunoglobulin (IVIG) to neutralize preformed antibodies, B lymphocyte depletion by anti-CD20 monoclonal antibody (RIT) and standard triple immunosuppression (calcineurin inhibitor, CNI; mycophenolate mofetil, MMF; and steroid). Recently, some authors reported successful outcomes of ABOi-KT without RIT and splenectomy[35,42,43].

ABOI-KT PREOPERATIVE MANAGEMENT

Current strategies of ABOi-KT compose three common principles: (1) antibody measurement; (2) B-Cell depletion; and (3) antibody depletion.

Antibody measurement

Assessment of anti-A/B antibody titer is crucial in ABOi-KT. It guides the effectiveness of operative preconditioning and determines the period to permit transplantation. In addition, posttransplant monitoring helps early detection of antibody-mediated rejection (AMR) by antibody rebound.

There are various measurement methods of anti-A/

B titer, the most common used are tube technique, gel technique and flow cytometry[44-48]. Although each center uses their familiar technique, there is a discrepancy of measured titer level. Kobayashi *et al*[46] surveyed the differences of anti-A/B titers from the same blood samples which were measured by tube test in 29 Japanese centers. It was revealed that inter-institutional differences were 1:8 to 1:32 in IgM and 1:16 to 1:256 in IgG, because of low reproducibility by visual observation. Therefore, they concluded standardized measurement should be necessary. Kumlien *et al*[47] analyzed the same blood samples in three centers. They also pointed out an inter-center variation of titer level using tube technique and suggested that gel technique is more reproducible than tube technique. Flow cytometry showed excellent reproducible compared with other techniques and would be suitable for the accurate measurement[48]. However, this technique is not available in all centers due to the expensive equipment required.

High preoperative anti-A/B IgG titers are associated with poor long-term allograft survival in ABOi-KT[49]. Gloor *et al*[50] showed preoperative high anti-A/B IgG titers is a predictor for AMR, and the rapid increasing of titers is also associated with AMR and graft loss. In addition, Tobian *et al*[51] also demonstrated that AMR was also associated with high titer at 1-2 wk posttransplant. Chung *et al*[52] described there was no statistically significant difference between high- (> 1:256) and low-titer (< 1:128) at the baseline in allograft function at 6 mo after transplantation. Therefore, appropriate monitoring of anti-A/B titer is essential before and after ABOi-KT. Although anti-A/B antibody titer has to be measured during the early period after ABOi-KT due to the risk of AMR, but how long the monitoring should be continued remains unclear. Preoperative titer should be low in ABOi-KT, but the acceptable titer of anti-A/B antibody at the time of transplant has varied between 1:4 and 1:32 in line with the protocol of individual centers[1,30-43,53-55]. After the ABO incompatible transplant necessitating initiation of antibody-depletion procedures, the level of anti-ABO antibody titer must be monitored to detect rebound in the serum antibody production.

B-cell depletion

Splenectomy: Splenectomy was considered a prerequisite for desensitization protocol in ABOi-KT after Alexandre *et al*[11] reported that it reduced the risk of AMR. The principle of splenectomy was based on the concept that spleen is reservoir of antibody producing B-cells and antibody-producing plasma cells in the body. However, the efficacy of splenectomy in ABOi-KT is debatable, because severe AMR sometimes still occurs after splenectomy. The effect of splenectomy on the immune system is permanent. Following splenectomy the patients are at risk for the development of life-threatening sepsis, especially from encapsulated bacteria and they require life-long antibiotic prophylaxis. Splenectomy can lead to surgical complications such as hemorrhage, pancreatic injury, pancreatic leakage , and portal vein thrombosis[56].

A comparative analysis of splenectomized recipients compared with RIT treated but without splenectomy, showed no statistically significant difference in the anti-A/B titer of KT and liver transplantation[57,58]. It was concluded that splenectomy was not an essential prerequisite treatment in ABOi-KT. Although splenectomy has been replaced with RIT, Locke *et al*[59] reported that splenectomy could be useful as salvage treatment for severe AMR secondary to anti-HLA antibody. Current consensus states that splenectomy is not necessary for the induction of ABOi-KT.

Rituximab: Splenectomy has been largely replaced by RIT in ABOi-KT protocols to remove B-cell. RIT is an anti-CD20 monoclonal antibody, which binds to CD20 on immature and mature B-cell resulting in depletion of B-cell. RIT was originally developed for the treatment of non-Hodgkin's lymphoma[60]. RIT has been used extensively in the treatment of patients with autoimmune diseases and KT besides hematological malignancies[61]. Adverse events related to B-cell depletion by RIT include fever, chill, headache, and nausea[60], whilst serious cardiovascular and pulmonary events are rare[61].

In the field of KT, RIT has been used as part of desensitization protocols in ABO- and HLA-incompatible KT, treatment of AMR, post-transplant lymphoproliferative disorder, and recurrent nephrotic syndrome[62]. In the first experience of RIT use in ABOi-KT recipients, Sawada *et al*[63] tried RIT, splenectomy, and double-filtration plasmapheresis (DFPP) for A1 to O ABOi-KT with persistent high anti-A antibody titer. The dosage of RIT was 375 mg/m^2 per week for 4 wk pretransplant and there was no rebound of the titer after transplantation. Tydén *et al*[64] succeeded with 4 ABO incompatible recipients using RIT and antigen-specific IA (IAs) with standard immunosuppression, without splenectomy. In their protocol, RIT (375 mg/m^2) was administered once 10 d prior to transplant which was enough to deplete peripheral B-cell. Moreover, its effect was long-active for at least 12 mo without any serious side effects. After these successful reports were published, RIT has replaced splenectomy in desensitization protocol. Recently, some have tried low dose of RIT or even omitting it in ABOi-KT protocol to avoid over-immunosuppression without compromising excellent outcomes[35,42,43,55].

Twenty-seven recipients who were diagnosed with steroid-resistant cell-mediated rejection or AMR received a single dose of RIT (375 mg/m^2) as a salvage treatment[65]: twenty-four (88.9%) among these demonstrated improved renal function. Serum creatinine decreased from a mean of 5.6 mg/dL before the treatment to a mean of 0.95 mg/dL after the treatment. RIT is useful not only in AMR, but also in chronic antibody-mediated rejection (CAMR) prevention. Kohei *et al*[66] observed that ABOi-KT with RIT had a statistically significant lower rate of CAMR at 2 years posttransplant than living ABOc-KT (3.5% *vs* 28.9%). However, this beneficial effect of RIT

needs independent verification.

Antibody depletion

The antibody depletion treatments are the basis of ABOi-KT. In order to eliminate existing anti-A/B antibody, plasma exchange (PE), DFPP and IA[67] are available. They differ in their mechanisms of action, specificity, efficiency and cost.

In PE, plasma is removed and replaced by human albumin, colloid solutions, and/or fresh frozen plasma (FFP). It has been widely used around the world for antibody removal in ABOi-KT. This method is simple, but it has several disadvantages compared with more specific techniques. Because of non-selective apheresis, PE removes not only anti-A/B antibody, but also coagulation factors and anti-viral/-bacterial immunoglobulin. Consequently, the risk of bleeding and infection is increased. FFP is generally needed for the last session before KT to prevent these complications. Other complications were reported by Tobian *et al*[68]. In all PE sessions (n = 512), the total rate of complications was 15.4%. The most common complication was hypocalcemia (6.8%), followed by urticaria or pruritus (4.3%), hypotension (2.9%) and nausea or vomiting (1.2%).

DFPP is designed to remove selectively the immunoglobulin from plasma and requires less substitution fluid compared to PE. When plasma separated by a first filter is passed through a second filter, IgG and IgM are filtered out and discarded. By single DFPP, 70% of IgM and 60% of IgG were removed and a one-fold titer reduction of anti-A/B antibody was observed[69]. This technique also avoids the loss of coagulation factors and albumin unlike PE. However, significant amounts of albumin are lost by DFPP, and almost always albumin is needed as the replacement fluid. DFPP is also removes variable amount of fibrinogen[70], and its measurement is necessary to avoid bleeding complication.

IA can be A/B antigen IAs or A/B non-antigen IAns (non-specific/semi-selective immunoadsorption) respectively if it removes only a specific antibody such as anti-A/B antibody or removes non-antigen-specific immunoglobulin. Between the two techniques IAs is most utilized method in ABO incompatible setting. On the other hand, IAns is suitable for the elimination of HLA antigens and it is most used in HLA incompatible/ABOi KT recipients. In IAs, the plasma is processed through an ABO immunoadsorbent column, which is coated with either blood type A or B antigens and allow selective removal of anti-A or B antibody, and the processed plasma is re-infused into the patient. Volume replacement is not necessary. IAs is selective and free from side effects of PE and DFPP. Single IAs reduces 2- to 4-fold titer between pre- and post-IAs, and at least four preoperative IAs are usually needed to obtain an acceptable titer at the expense of increased cost compared to PE and DFPP[67]. IAs is generally safer and more effective, and therefore normally preferred. However, ultimate choice depends on each center's decision, based on the availability of infrastructure and skill mix of staff.

USE OF IVIG

IVIG's recognized immunomodulatory properties have been employed for the treatment of autoimmune diseases[71]. IVIG is believed to act through various mechanisms: (1) complement down-regulation; (2) interactions with the Fc receptors; (3) inhibit of B/T-cell proliferation; (4) inhibit of CD8 T-cell cytotoxicity; and (5) increased apoptosis of B-cell[71-73]. Mild and early adverse effects of IVIG include headache, chill, nausea, fatigue, myalgia, arthralgia, chest pain, back pain, and elevated blood pressure[74,75]. However, rare but serious delayed adverse effects include renal toxicity, thromboembolic events (cerebrovascular accident and deep venous thrombosis), neurological toxicity (aseptic meningitis), hematological toxicity (neutropenia), and dermatological toxicity[76]. The administration of high dose IVIG can cause hemolysis by anti-A/B antibody within the IVIG[77]. In ABOi-KT, it is preferable if possible to use IVIG with low anti-A/B titer in order to avoid not only hemolysis but also AMR after transplantation due to anti-A/B titer elevation.

There is no uniformity in the dose IVIG used in the desensitization protocols of ABOi-KT[1, 30-32,34,35,37,38,40,43,54,78]. IVIG is usually administered after plasmapheresis, to reconstitute the natural levels of IgG. In the absence of control data, the use of IVIG in ABOi-KT can best be described as empirical.

ACCOMMODATION

Without adequate anti-A/B antibody reduction and desensitization before KT, an incidence of AMR and irreversible damage cannot be avoided. Successful ABOi-KT requires the reduction of anti-A/B antibody titers against ABO antigens on the graft at the time of KT. However, anti-A/B antibody titer returns to the baseline level within almost 1 wk after KT[11,79,80], even if optimal desensitization is performed. Therefore, intense monitoring is necessary during critical first two weeks after ABOi-KT[12]. Paradoxically, a phenomenon of accommodation is acquired in this term.

Accommodation is defined as a phenomenon whereby graft rejection is avoided despite reemergence of incompatible antibody. The mechanism was originally discovered in the field of xenotransplantation[81], whereby endothelial cell posttransplant humoral injury was avoided, possibly due to changes of antibody specificity, avidity, affinity and alteration of the antigen structure. This phenomenon is allegedly responsible for normal graft function and structure despite reemergence of anti-A/B antibody against incompatible A or B antigen in the graft[82]. However, it is fair to accept that mechanism as well as the very existence of accommodation remains speculative.

CURRENT PROTOCOL OF ABOI-KT

In ABOi-KT, intensified immunosuppressive protocol usually starts before KT in order to deplete anti-A/B

antibody. Many centers have modified original successful protocol of ABOi-KT[11]. The splenectomy-free protocols published in the last decade are summarized in Table 3[1,30-32,34-43,53-55,78]. RIT has been adopted in the place of splenectomy by majorities of centers. However, the timing and dose of RIT administrated remains variable. RIT or splenectomy-free protocols have successfully, used low dose IVIG after plasmapheresis. The basis of the North Europe protocol is IAs followed by high dose IVIG. However, postoperative IAs is not performed routinely and its use is determined by antibody titers[83]. Maintenance immunosuppressive agents are mostly triple agents which are CNI, MMF and steroid. Tacrolimus is the CNI of choice in these ABOi-KT protocols. MMF was taken 7-14 d pretransplant in order to inhibit antibody production. Some centers use a protocol without daclizumab, basiliximab or antithymocyte globulin, and report excellent outcomes. Thus it is controversial whether these clonal antibodies should be introduced in ABOi-KT or not. All protocols of ABOi-KT have resulted in satisfactory outcome in the absence of randomized control trials. It is impossible to select an ideal protocol fit for all purpose.

MINIMIZE IMMUNOSUPPRESSION

Efforts have have been made to minimize immunosuppression in order to reduce the long-term risk of over-immunosuppression[84,85]. The long-term effect of steroid use remains unclear in ABOi-KT. Oettl *et al*[86] described 11 ABOi-KT recipients with late steroid withdrawal. Six recipients showed biopsy-proven acute rejection during or soon after steroid cessation. However, Galliford *et al*[30] tried early steroid sparing protocol in 10 recipients. Prednisolone was maintained at 1 mg/kg until 3 d posttransplant. It was reduced to 0.5 mg/kg at 4 d posttransplant, and discontinued after 1 wk posttransplant. In this study, patient and graft survival were 100% at 1 year posttransplant but 3 patients experienced acute rejection within 1 mo after transplantation.

HISTOLOGICAL FINDINGS IN ABOI-KT

In ABOi-KT, acute AMR by anti-A/B antibody is a well-recognized cause of early graft loss. Diagnosis of acute AMR needs C4d staining in the peritubular capillary (PTC) and the presence of anti-donor antibodies[87,88]. Morphologic changes include acute tubular necrosis, capillary and/or glomerular inflammation, and transmural arteritis and/or arterial fibrinoid change. C4d staining is the hallmark of humoral induced complement activation and like ABOc-KT was thought to be a useful indicator of AMR even in the setting of ABOi-KT[89]. However, C4d deposition without AMR was seen in 85.7% of ABOi-KT at 3 mo posttransplant[90]. Setoguchi *et al*[91] analyzed protocol biopsies of ABOc-KT and ABOi-KT. C4d expression of PTC was detected in 94% of ABOi-KT, whereas in only 11% of ABOc-KT. In protocol biopsies during stable allograft function, 80% of ABO incompatible grafts showed as C4d positive, while 74% of HLA incompatible grafts were C4d negative[92]. These histological studies indicate that the detection of C4d alone in ABO incompatible graft does not indicate AMR and support a concept of accommodation in ABOi-KT. Therefore, AMR after ABOi-KT can only be diagnosed on the basis of morphological evidence, serological evidence and the clinical course.

Morphologically transplant glomerulopathy (TG) at 1 year after transplantation was reported as an indicator of poor outcome[93]. ABOi-KT had more severe TG than ABOc-KT without HLA antibody at 1 year posttransplant[94]. However, there were no differences in interstitial fibrosis, tubular atrophy, chronic vasculopathy and allograft function between both groups. In the absence of prior AMR, histological change at 1 year posttransplant was mild irrespective of ABO compatibility. Moreover, prior AMR in ABOi-KT was associated with TG and interstitial fibrosis and not to arteriolar hyalinosis and chronic vasculopathy[91]. Consequently, ABO incompatible grafts with TG and/or interstitial fibrosis had lower GFR at 1 year after transplantation than those with normal histology.

THE INCIDENCE OF ACUTE CELLULAR AND ANTIBODY MEDIATED REJECTION IN ABOI-KT

As previously described, the outcome of graft survival in ABOi-KT has been similar to ABOc-KT. However, there is an increased risk of AMR in ABOi-KT due to anti-A/B antibody. Protocol biopsies at 3 mo posttransplant in ABOi-KT had a significantly higher incidence of AMR compared to ABOc-KT (17.9% *vs* 1.1%). However, there was no significant difference in the rate of acute cellular rejection between ABOi-KT and ABOc-KT (48.4% *vs* 35.7%)[90]. In the acute lesion score based on Banff classification[95], *t*2-3 and *g*2-3 following ABOi-KT was higher than that of ABOc-KT (*t*2-3: 42.9% *vs* 19.4%, *g*2-3: 28.6% *vs* 6.5%). Gloor *et al*[94] described in the study of protocol biopsies at 1 year posttransplant that there was a significant difference in the incidence of acute rejection between ABOi-KT and ABOc-KT without HLA antibody (50% *vs* 13.6%). Acute rejection in ABOi-KT was mainly AMR (73.3%) as compared to ABOc-KT without HLA antibody (12.5%). Setoguchi *et al*[91] also compared the histologic findings of protocol biopsies in 48 ABO incompatible and 133 compatible grafts. There was no difference in clinical and subclinical rejection between ABO incompatible and compatible grafts (clinical: 37.5% *vs* 25.6%, subclinical: 10.4% *vs* 15%). However, ABO incompatible grafts had a high incidence of AMR compared to ABO compatible grafts (27% *vs* 5.3%). Interestingly, rejection was detected in only 15.0% at 1 mo in ABOi-KT compared to 34.7% in ABOc-KT, but in 30.0% at 6-12 mo compared to 10.5%. Wilpert *et al*[34] demonstrated that the rejection rates in ABOi-KT were similar to that in ABOc-KT. Acute cellular rejection was

Table 3 Current protocols for ABO incompatible kidney transplantation

Author	Country, year	Rituximab dose	Pretransplant IS	Antibody depletion	IVIG	Target titer at the time of transplantation	Induction IS	Maintenance IS	Posttransplant antibody depletion
Adult recipients									
Rituximab protocol									
Saito *et al*[53]	Japan, 2006	375 mg/m^2 (twice) at -14 and -1 d	MMF/MP at -1 Mo	DFPP or PE	-	< 1:16	BAS (20 mg at 0 and 4 d)	CYA/MMF/MP	-
Tyden *et al*[54]	Sweden, 2006	375 mg/m^2 (once) at -1 mo	TAC/MMF/Pred at -13 d	IAs	0.5 g/kg after last IAs	< 1:8	-	TAC/MMF/Pred	IAs, 3 times
Chikaraishi *et al*[55]	Japan, 2008	100 mg/m^2 (twice) at -8 and -1 d	MMF/MP at -14 d, TAC at -3 d	DFPP and PE	-	< 1:8	BAS (20 mg at 0 and 4 d)	TAC/MMF/MP	-
Galliford *et al*[30]	United Kingdom, 2008	1000 mg (twice) at first day of PE and at the operative day	TAC/MMF at -14 d	PE	0.1 g/kg after each PE	< 1:4	DAC (2 mg/kg at 0 and 14 d)	TAC/MMF/Pred	PE at 1 and 3 d
Genberg *et al*[31]	Sweden, 2008	375 mg/m^2 (once) at -1 mo	TAC/MMF/Pred at -10 d	IAs	0.5 g/kg at -1 d	< 1:8	-	TAC/MMF/Pred	IAs, 3 times
Oettl *et al*[32]	Switzerland, 2009	375 mg/m^2 (once) at -1 mo	TAC/MMF/Pred at -14 d	IAs	0.5 g/kg after last IAs	< 1:8	BAS (20 mg at 0 and 4 d)	TAC/MMF/Pred	IAs or PE (not routinely)
Sivakumaran *et al*[78]	United States, 2009	375 mg/m^2 (once) at -3 wk	MMF at -1 mo	PE	2 g/kg after last PE	NA	ALE (1 mg/kg at 0 and 14 d)	TAC/MMF/Pred	-
Wilpert *et al*[34]	Germany, 2010	375 mg/m^2 (once) at -1 mo	TAC/MMF or MPS/Pred at -7 d	IAs	0.5 g/kg at -1 to -5 d	< 1:4	BAS (20 mg at 0 and 4 d)	TAC/MMF/Pred	IAs (not routinely)
Fuchinoue *et al*[36]	Japan, 2011	100-1000 mg, 1-3 times	CYA or TAC/MMF at -2 d	DFPP or PE	-	< 1:16	BAS (20 mg at 0 and 4 d)	CYA or TAC/MMF/steroid	-
Habicht *et al*[37]	Germany, 2011	375 mg/m^2 (once) at -1 mo	TAC/MMF/Pred at -1 mo	IAs	30 g at -1 to -2 d	< 1:8	-	TAC/MMF/MP	IAs (not routinely)
Lipshutz *et al*[38]	United States, 2011	375 mg/m^2 (once) at -1 mo	TAC/MMF at the first day of PE	PE	10 g after each PE	< 1:8	ATG (1.5 mg/kg for 4 d)	TAC/MMF/Pred	PE (not routinely)
Shirakawa *et al*[39]	Japan, 2011	500 or 200 mg/m^2 (once), at -5 to -7 d	TAC/MMF/MP at -7 d	DFPP	-	< 1:32	BAS (20 mg at 0 and 4 d)	TAC/MMF/MP	-
Morath *et al*[40]	Germany, 2012	375 mg/m^2 (once) at -1 mo	TAC/MMF/MP at the first day of IAs	IAs	0.5 g/kg after last IAs	< 1:16	BAS (20 mg at 0 and 4 d)	TAC/MMF/MP	IAs or PE (not routinely)
Uchida *et al*[41]	Japan, 2012	150 mg/m^2 (twice) at -14 and 0 d	MMF/MP at -1 Mo, CYA or TAC at -3 d	DFPP or PE	-	< 1:16	BAS (20 mg at 0 and 4 d)	CYA or TAC/MMF/MP	-
Rituximab-free protocol									
Montgomery *et al*[43]	United States, 2009	-	TAC/MMF at the first day of PE	PE	0.1 g/kg after each PE	< 1:16	DAC (2 mg/kg initial dose, 1 mg/kg every 2 wk for total 5 doses)	TAC/MMF/Pred	PE, at least twice (with IVIG 0.1 g/kg)
Flint *et al*[35]	Australia, 2011	-	MMF at -10 to -14 d	PE	0.1 g/kg after each PE	< 1:8	BAS (20 mg at 0 and 4 d)	TAC/MMF/Pred	PE (not routinely)
Ashimine *et al*[42]	Japan, 2013	-	MMF at -14 d	DFPP	-	< 1:8	BAS (20 mg at 0 and 4 d)	CYA or TAC/MMF/Pred	-
Pediatric recipients									
Genberg *et al*[31]	Sweden, 2008	375 mg/m^2 (once) at -1 mo	TAC/MMF/Pred at -10 d	IAs	0.5 g/kg at -1 d	< 1:8	-	TAC/MMF/Pred	IAs, 3 times
Tyden *et al*[1]	Sweden, 2011[1]	375 mg/m^2 (once) at -1 mo	TAC/MMF/Pred at -13 d	IAs	0.5 g/kg after last IAs	< 1:8	-	TAC/MMF/Pred	IAs, 3 times

IS: Immunosuppression; IVIG: Intravenous immunoglobulin; MMF: Mycophenolate mofetil; MP: Methylprednisolone; DFPP: Double-filtration plasmapheresis; PE: Plasma exchange; BAS: Basiliximab; CYA: Cyclosporine; TAC: Tacrolimus; Pred: Prednisolone; IAs: Antigen-specific immunoadsorption; DAC: Daclizumab; NA: Not available; ALE: Alemtuzamab; MPS: Mycophenolate sodium; ATG: Antithymocyte globlin.

Table 4 Pro and cons for ABO incompatible kidney transplantation

Pro ABOi-KT
Reducing waiting list and time
Expanding living donor pool
Improvement of patient's prognosis
Excellent graft survival (comparable with ABOc-KT)
Contra ABOi-KT
Comparative high immunological risk
Higher incidence of acute AMR
Intensified immunosuppression
Antibody depletion therapy
Increasing expenditure
Higher incidence of viral infection

ABOi-KT: ABO incompatible kidney transplantation; ABOc-KT: ABO compatible kidney transplantation; AMR: antibody-mediated rejection.

detected in 23.2% of ABOi-KT and in 22.5% of ABOc-KT. Acute AMR was shown in 4.7% of ABOi-KT, which was similar to ABOc-KT (5.0%).

ADVERSE EFFECT OF ABOI-KT

Infection

The improvement in ABOi-KT graft survival rate has come at the expense of increased posttransplant infection. The infection rate in ABOi-KT is significantly higher than in ABOc-KT (60% *vs* 29.8%)[37]. The rates of infection including cytomegalovirus (CMV), herpes simplex virus, varicella zoster virus and BK virus (BKV) in ABOi-KT were also significantly higher than in ABOc-KT. The most common viral infection was BKV in 25% of ABOi-KT compared to only 8.5% of ABOc-KT. However, the incidences of rejection, graft survival rate and function of ABOi-KT patients were compatible with these of ABOc-KT patients. On the contrary, Genberg *et al*[31] showed that there was no statistical difference in overall infection complications between ABOi-KT with RIT and living ABOc-KT (40% *vs* 63.3%). However, ABOi-KT patients who were treated with RIT, may have had different infection profiles. Grim *et al*[96] retrospectively analyzed the incidence of posttransplant infection in HLA sensitized KT or ABOi-KT treated with RIT and compared to HLA sensitized KT without RIT. The acute rejection rate in RIT treated KT was similar to KT without RIT (40% *vs* 33%). However, posttransplant infection rate was 48.0% RIT with KT, but only 11.1% without RIT. Kamar *et al*[97] reported that infection rate was 45.5% in KT with RIT which was similar to KT without RIT (53.9%). Bacterial, viral and fungal infection were observed in 36.3%, 18.2% and 16.9% in KT with RIT, against 31.6%, 34.3% and 5.32% in KT without RIT. Polyoma virus infection rate (64.3%) was relatively high in RIT. Moreover, infection related-death was significantly higher in RIT treated patients. This data ascertained that RIT was associated with severe infection which causes death rather than an increased risk of infection. Other report confirmed earlier observation showing that the incidence of posttransplant infection in RIT-treated recipients was similar to RIT-untreated recipients (52.2% *vs* 40.2%)[98]. However, as in earlier studies the incidences of CMV and BKV infection in RIT-treated recipients were higher than in non RIT-treated recipients (CMV: 16.4% *vs* 5.7%, BKV: 13.4% *vs* 8.0%).

Malignancy

It is generally accepted that immunosuppression is associated with an increased incidence of malignancy in KT recipients compared to the general population[99]. However, several studies have demonstrated that ABOi-KT did not increase the risk of posttransplant malignancy compared with ABOc-KT. Yamamoto *et al*[100] analyzed the risk of ABOi-KT compared to ABOc-KT retrospectively. ABOi-KT recipients were older than ABOc-KT recipients and all ABOi-KT recipients received splenectomy, in this study despite increased age and splenectomy[101,102], there was no significant difference in the incidence of malignancy between ABOi-KT and ABOc-KT (4.8% and 4.2%). Similarly, Hall *et al*[103] showed that 7 of 318 ABOi-KT recipients experienced posttransplant cancer. The incidence rate ratio (IRR) of cancer in ABOi-KT was identical to that in matched control ABOc-KT (IRR: 0.99). This limited data reassuringly indicates that ABOi-KT is not associated with an increasing incidence of malignancy after KT. Thus, a further analysis of long-term observations in ABOi-KT after RIT is needed.

COST OF ABOI-KT

It is recognized that KT is a cost-effective option over dialysis[104-106]. The estimated cost for ABOi-KT over 20 years was $315600, which was approximately 15% lower than dialysis[107]. ABOi-KT is more expensive than ABOc-KT because of requirement for desensitization and removal of anti-A/B antibody. The cost of ABOi-KT in the first 90 d posttransplant is $90300 compared to $52500 for ABOc-KT[108]. The additional cost of ABOi-KT amounts to €31948 for IAs, RIT, IVIG, and prolonged hospital stay[31]. The cost of single IA is approximately €4340-1433[40]. However, despite more expensive, ABOi-KT is still more cost-effective than dialysis in the long-term and delivers a better quality of life.

CONCLUSION

Since first performed over 50 years ago, ABOi-KT has become an accepted source of KT. Reassuringly, despite lack of control trials in ABOi-KT, more than satisfactory outcomes have been observed in adult and pediatric recipients, in many studies equivalent to living ABOc-KT. ABOi-KT also has disadvantages in spite of excellent outcomes (Table 4). Preconditioning treatment of ABOi-KT, such as antibody reduction and desensitization, is more intensified and complicated than that of ABOc-KT. With current protocols, the occurrence of early graft loss and AMR are not completely abolished. Preconditioning strategy in ABOi-KT has evolved over time. RIT has replaced splenectomy which was once thought a cru-

cial procedure for ABOi-KT, although this is increasingly abandoned in favor of IAs and IVIG. Overall, ABOi-KT is more expensive than ABOc-KT which may restrict its adoption in resource poor countries. We believe that a live donor ABOi-KT is a viable alternative to waiting on deceased donor list.

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REVIEW

Immune monitoring post liver transplant

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Abstract

Many of the causes of short and late morbidity following liver transplantation are associated with immunosuppression or immunosuppressive medications. Current care often involves close monitoring of liver biochemistry as well as therapeutic drug levels. However, the postoperative course following liver transplantation can often be associated with significant complications including infection and rejection, suggesting an inadequacy in current immune function monitoring. Many assays have been tested in the research setting to identify possible biomarkers that may be used to predict clinical events such as acute cellular rejection, and therefore allow modification of a patient's immunosuppressive regimen prior to a clinical event. However, these generally require significant laboratory processing and have had difficulty becoming established in common clinical use outside the research setting. One assay, Cylex ImmuKnow has been food and drug administration approved but has had variable results. In this review we discuss the assays that have been used to assess monitoring of immune function after liver transplantation and consider possible future directions.

Key words: Immune function monitoring; Review; Biomarkers; Liver transplantation

Core tip: Although many research assays have attempted to identify potential biomarkers that may be used to monitor immune function after liver transplantation, most require significant laboratory processing and are not clinically feasible. The rejection cascade is complex and not completely understood, with many likely interactions between innate and adaptive immune processes. Therefore, no single test is likely to provide a fool-proof window to the immune response and a combination of assays may be necessary. However, nothing can replace the clinical judgement of an expert transplant clinician for pooling together data to individualize immunosuppression therapy.

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INTRODUCTION

Although the use of modern immunosuppression has greatly increased the life expectancy of organ transplant recipients, they are not without problems. Mortality within the first year following liver transplantation (OLTx) usually occurs within the first three months with causes including infection, primary graft failure, rejection and technical complications[1]. Causes of late mortality include cardiovascular disease (9%-22%), *de novo* malignancy (16%-23%), infections (6%-19%), chronic rejection and graft failure (5%-19%) and chronic renal failure (5%-10%)[2-5]. Many of the causes of short and late mortality following OLTx are related to immunosuppression, with an estimated 40%-70% of all post-transplant mortality attributable to immunosuppression or immunosuppressants[5,6].

Table 1 Clinically available immune monitoring after adult liver transplantation

	Sensitivity	Specificity
Currently available		
Liver biochemistry	High	Low
Therapeutic drug levels	Low	Low
ImmuKnow	Low	High
Liver histology	Gold standard	Gold standard
Future possibilities		
PlexImmune	Only Paedeatric studies published	
? Combination assays		

To minimize side-effects, clinicians often empirically attempt to minimize dosages. Only very few patients are trialed or able to completely withdraw successfully from all immunosuppression. Tailored therapy for each patient, based on a functional measure of their individual immune response, would clearly be preferable to empiric reduction of therapy in all patients[7].

The challenge in balancing the risks of over and under immunosuppression is complicated by the lack of reliable means of predicting patients' immunosuppressive needs. OLTx in particular, presents unique challenges compared with other solid organ transplants. The liver is an immunotolerant organ but rejection rates remain at 30%-40%[8-10]. Despite this, some individuals have the potential for complete withdrawal of immunosuppression. Furthermore, the postoperative course after OLTx is often complicated, with biliary strictures and recurrent diseases shrouding the diagnosis of rejection and confusing the management of a patient's immune function post transplant. Therefore, it has long been suggested that we monitor transplant patients for their functional immunity to optimize therapy[11,12].

An ideal immune function assay would be based on whole blood, require minimal handling, be reproducible and standardized across laboratories, relatively cheap, and offer a rapid turn around that would allow interpretation of results and corresponding adjustments in immunosuppression early enough to prevent complications or drug related side-effects.

Currently available standard of care in most centres to monitor immune function involves liver biochemistry, drug levels and clinical events (Table 1). Several other potential bio-markers and diagnostic parameters have been suggested in order to confront the immune monitoring challenge and are summarized in Table 2. In this review, we examine the current available options for monitoring the immune system after liver transplantation.

LIVER BIOCHEMISTRY

Clinicians have traditionally relied on liver biochemistry (LFT) in making non-invasive assessments regarding graft function after OLTx. An increase in LFTs is seen during rejection but is non-specific and many other important aetiologies need to be considered. These include but are not limited to biliary strictures, hepatic artery thrombosis, cholangitis, recurrent viral hepatitis and drug induced injuries. There is often a delay between the first LFT abnormality being noted, and patients undergoing a liver biopsy for diagnosis of rejection. It is an imprecise and late marker of graft injury.

THERAPEUTIC DRUG MONITORING

Calcineurin inhibitors

Cyclosporine and tacrolimus are the two commonest drugs used in maintenance following OLTx and inhibit the phosphatase activity of calcineurin through binding of cyclosporine-cyclophilin and tacrolimus-FKBP12 complexes. This inhibits T-cell activation, but because calcineurin and the nuclear factor activated T-cell pathway are not T-cell specific, calcineurin inhibitors (CNIs) are often associated with significant toxicity[13]. In particular, tacrolimus has high rates of diabetes, while cyclosporin is associated with increased hypertension and dyslipidemia[14]. Furthermore, both drugs are associated with end-stage renal failure that can complicate up to 20% of patients following OLTx[15].

Tacrolimus (> 90%) and cyclosporine (> 50%) are concentrated in erythrocytes, and therefore whole blood is used to measure the therapeutic drug levels[16]. Most centres use an ELISA to measure trough levels of tacrolimus, while large clinical trials of OLTx patients treated with cyclosporine show lower rates of rejection and nephrotoxicity complications with monitoring based on either AUC_{0-4} or the concentration 2 h following administration[17-19]. Therefore many units (including our own) perform a level 2 h (C2) following the patient's morning dose.

Setting a therapeutic target for the CNIs has been difficult with standard protocols generalized to managing large number of recipients, but not specific to each patient's individual clinical situation[20]. CNIs also have a poor dose-level correlation, an unpredictable level-effect association, individual pharmacokinetic differences, and an unclear level-toxicity relationship[21,22]. Side-effects are seen even with CNI levels below the "therapeutic range"[23]. Further problems arise as the monoclonal antibodies used to detect certain metabolites may not capture all biologically active forms of the CNIs[24,25].

Given the level of drug determined by immunoassay is not correlated with immunosuppressive drug efficacy or the level of immunosuppression[22,26,27] the United States Food and Drug Administration (FDA) has gone so far as to reclassify assays for measuring tacrolimus and cyclosporin blood levels indicating that no suitable therapeutic ranges exist and these tests should not be used alone to adjust drug dosing[28].

Optimising CNI drug dosing

CNI dosing is impacted by the variable metabolism of the drugs. Tacrolimus is metabolised by CYP3A enzymes in the small intestine and the enzymatic activity can vary by a factor of 5 between patients[29]. Genetic polymorphisms of CYP3A have shown higher tacrolimus clearance and lower levels in some kidney transplant re-

Table 2 Summary of assays for immune function monitoring

		Advantages	Disadvantages
Antigen-specific assays:	Limiting dilution aAssays, mixed lymphocyte reactions, ELISPOT	Measure individual antigen specific response	Need donor cells, Laboratory intensive
Antigen non-specific:	ImmuKnow	Available, FDA approved	Inconsistent results
	Cytokine levels/polymorphisms		Inconsistent results
	Immune competence scores	Readily available	Lack of published validation studies
	Regulatory T cells (Tregs)	Associated with rejection	Laboratory intensive. Lack of published validation studies
	Soluble CD30		Lack of association with clinical outcomes in OLTx
Identifying operational tolerant recipients:	Tregs, Gene expression, dendritic cell types, delayed type hypersensitivity	Able to identify recipients in whom immunosuppression could be withdrawn	Laboratory intensive. Only few recipients suitable

FDA: Food and drug administration; ELISPOT: Enzyme-linked immunosorbent spots; OLTx: Liver transplantation.

cipients[30] while attempts to evaluate pharmacodynamics directly through monitoring of CNI biological activity have demonstrated correlation between peak levels of CNIs and residual gene expression (by nuclear factor of activated T-cells), but not clinical events[31].

High-performance liquid chromatography was developed for evaluating four cyclosporine degradation products and two related compounds (CyB and CyG)[32]. Initially developed to test quality control of generic formulations, future studies may consider evaluating whether these could have a closer association with outcomes than the cyclosporin blood level[20].

Other drugs

The CNIs are often used in combination with other immunosuppressants. Steroids and induction agents such as basiliximab (anti-IL2) have no specific monitoring mechanisms apart from side-effects, while the optimal dosing and levels of the mTOR inhibitors remain uncertain.

Even if the biological activity of each individual drug could be accurately determined, this would not provide an objective net biomarker of immune function as the cross-reactive effects of the drugs would remain uncertain. As such, therapeutic drug monitoring may continue to assist clinicians in managing patients, but is unlikely to be the dominant method of future immune system monitoring following OLTx.

Clinical events

One of the major influences on drug dosing and immunosuppression following liver transplantation is the presence of complications. In particular, patients who develop sepsis or malignancy following transplantation often have their immunosuppression empirically reduced. Correspondingly, patients undergoing rejection are treated with increased medication. Clearly this is a crude method of monitoring immunosuppression and the purpose of immune monitoring is to optimise immunosuppression prior to the occurrence of clinical events.

Biopsies

Acute cellular rejection is diagnosed on histology based on the commonly accepted Banff criteria[33]. Sampling graft tissue has the further advantage that it can reveal the local ongoing antidonor immune responses[34] and protocol biopsies provide a more accurate marker of graft function compared to liver biochemistry[13]. Surveillance biopsies of the transplanted organ may represent the gold standard for directly assessing the extent of immune activity within the allograft. However, serial biopsies are invasive and almost impractical outside of a research setting[35].

Immune monitoring assays

Although commonly used, the aforementioned tests have significant disadvantages and do not provide an accurate marker of a patient's immune system following OLTx. As a consequence, clinical events and side-effects remain common causes of morbidity and mortality. Many assays have been developed and evaluated with varying results but are yet to achieve use outside of research settings. In general, these assays can be broadly classified as antigen-specific or non-antigen specific and will be discussed below.

ANTIGEN-SPECIFIC ASSAYS

Donor specific assays

Functional donor specific assays may allow detection of immunological states favouring alloimmune quiescence over reactivity[36]. Functional or cytokine kinetics assays may then be applied to determine preemptively whether immunosuppression dosing should be altered.

Limiting dilution assays (LDA) are an example which can provide more precise quantification of immunity to a given stimulus and allow estimation of frequencies of antigen-specific cells participating in an immune response[37]. It requires recipient peripheral blood mononuclear cells (PBMCs) interacting with donor stimulator cells. This can then be used to determine production of different cytokines in the presence of supernatant cultures such as interferon-gamma, interleukin (IL)-5, IL-4, IL-10, IL-13 or TNF-α present in the well[37]. LDA has been employed to show a highly significant correlation between the donor-specific and third-party stimulated IL-4 and IL-10 produced from recipient PBMCs with

stable liver graft function compared with rejectors, independent to level of immunosuppression[38].

The main limiting step is availability of donor cells that can be difficult to obtain from cadaveric transplants unless cells are harvested at time of surgery from the spleen or lymph nodes and cryopreserved for future donor-specific assays[20]. Furthermore, the assays often require substantial laboratory work and may need significant amounts of blood and cells for repeated stimulations/experiments.

Mixed lymphocyte reaction

Mixed lymphocyte reaction (MLR) assays provide an estimate of the primary *in vitro* response to the direct recognition of allogenic molecules[37]. Their main value is in assessing tolerance - that is MLR responsiveness in the face of clinically evident donor-specific tolerance.

Studies with ^{3}H-thymidine mixed leukocyte responses (MLR) show that enhanced donor-specific alloreactivity persists longer among children with early rejection and is associated with early and late liver rejection[39,40]. To account for the significant variation that is often seen in donor-specific alloresponses, values are often expressed as a ratio to a third-party response known as the immunoreactivity index. A ratio under 1 suggests low rejection risk[40]. However, this assay is non-antigen specific, requires prolonged stimulation and larger amounts of blood than would be routinely feasible in transplant populations[41].

Further enhancements to MLR include combination of results with carboxyfluorescein diacetate succinimidyl ester (CFSE) labelling by flow cytometry[42]. CFSE is an intracellular fluorescent label that divides equally amongst daughter cells and can be used to study cell division[37]. It measures the proliferative response of recipient lymphocytes after culture or stimulation with donor cells. Unlike many other immune monitoring studies, this has been investigated in an interventional study of 51 adult OLTx recipients. Immunosuppression was increased, decreased or maintained depending on results from the MLR compared with 64 OLTx recipients who had standard of care with empirical based management. This showed trends towards improved rates of rejection and survival, but not sufficient to reach significance ($P < 0.05$)[42]. A MLR-CFSE assay has also been used to distinguish between rejection on suspicious biopsies[43].

To overcome the issues of prolonged stimulations and blood sample requirements common in MLR assays, Ashokkumar *et al*[41] evaluated a CD154^{+} (CD40L) T-helper and T-cytotoxic cells MLR as measures of rejection risk[41]. This requires < 24 h of stimulation and only 3 mL of blood. These authors identified pre OLTx CD154^{+} cytotoxic T memory cell responses were associated with significantly increased risk (HR = 7.355, $P = 0.02$) for rejection. This assay can be ordered as PlexImmune™ (Plexison, Pittsburgh, United States) with results in the United States available 2 d after obtaining blood samples. Only small studies have been published to date with PlexImmune in paediatric liver and small intestinal transplant recipients. The assay requires extraction of PBMCs not only from the recipient but also the donor. In some cases when donor cells have been insufficient or unavailable, "surrogate PBMCs" have been used[41] but their validity is uncertain in a clinical population.

Enzyme-linked immunosorbent spots

Enzyme-linked immunosorbent spots (ELISPOT) quantifies the frequency of previously activated (memory) T cells that respond to donor antigens by producing a selected cytokine *in vitro*. Recipient T cells are cultured with donor cells on tissue culture plates coated with a cytokine-specific antibody that is detected using labeled secondary antibodies. Each detected spot represents an effector or memory T cell which has been primed to the stimulating antigens[37].

ELISPOT has been proposed as a surrogate marker of allogenic responsiveness in renal transplantation[44-46]. Pretransplant IFN-γ ELISPOT has been associated with rejection risk following renal transplant[44,45,47] which suggests that IFN-γ-producing cells represent cells that have been sensitized to the graft antigens. Thus providing an *ex vivo* reflection of the evolving *in vivo*, donor-reactive immune response which may allow patients without a positive response to reduce or withdraw their immunosuppression[7]. Apart from IFN-γ, granzyme B (GrB) has been studied in a small number of paediatric OLTx recipients but failed to predict the occurrence of rejection[48].

The labor-intensiveness and time-consuming nature of these assays, the need for donor cells, the questionable reliability for stored cells along with some inconsistent correlations with clinical outcomes have prevented their broad acceptance as reliable immune monitoring tools[7,49].

Chimerism

After OLTx, haematopoietic donor cells are transferred with the graft from donor to recipient. These chimeric cells may persist in the recipient and be detectable even years post-transplant[50]. It has been hypothesised that developing chimerism may be desirable after OLTx and potentially associated with tolerance[51]. This could allow immunosuppression to be reduced in patients who have detectable chimerism. However, a meta-analysis has failed to demonstrate a significant association between microchimerism and rejection, but techniques of varying sensitivity were used to measure the degree of chimerism[52]. The value and role of chimerism after liver transplantation remains uncertain, and may also differ depending on the time post-transplant[53].

ANTIGEN NON-SPECIFIC

ImmuKnow

As immunosuppressive drugs ultimately target T-cell function, it would seem logical that assessing T-cell function would provide a potential biomarker for monitoring immune function after transplantation[54]. ImmuKnow (Cylex Ltd, United States) was developed as a biomarker

to guide immunosuppressant dosing following solid organ transplantation and was approved by the United States FDA in 2002. ImmuKnow measures adenosine triphosphate produced after stimulation of T-cells with plant lectin phytohemagglutinin (PHA) mitogen[54]. Whole blood is used to ensure that CNIs are maintained during incubation. After overnight incubation, CD4 cells are selected using paramagnetic particles coated with a monoclonal antibody to CD4[54]. ImmuKnow does not correlate with CD4 cell numbers, and the assay is theorized to provide an independent variable[54].

Studies in OLTx recipients have reported contradictory results for ImmuKnow in predicting acute rejection and infection[55-62]. Most of these studies are retrospective, have limited follow-up, heterogenous in study design, and often include multiple solid organ transplants in the analysis despite immunosuppression protocols and clinical event risks differing substantially amongst different transplant populations.

Further, many of these studies only employ single time point measurements and risk potential bias and the effect of confounders. For example, one study assessing ImmuKnow and infection risk declared lower values in patients who suffer an infection following transplant. However, one of the triggers to run the assay in this study was an event such as fever or raised liver biochemistry[63]. Furthermore, a single result cannot be expected to predict the long-term immune function of the patient. Ideally serial measures, correlated with changes in immunosuppressant dosing, would be needed to adequately assess the immune response post OLTx.

To coincide with the multiple studies demonstrating conflicting results, there have been two opposing meta-analyses published[64,65]. One recent meta-analysis by Ling *et al*[64] suggests a sensitivity of 0.43 (95%CI: 0.34-0.52) and specificity of 0.75 (95%CI: 0.72-0.78) of ImmuKnow for predicting rejection with a diagnostic odds ratio 1.19 (95%CI: 0.65-2.20). This study incorporated multiple organ transplants and when a sub-analysis of liver transplant patients was conducted, results suggested poor sensitivity but improved specificity (sensitivity 0.11 95%CI: 0.01-0.33, specificity 0.94 95%CI: 0.91-0.95).

A separate meta-analysis in liver transplant recipients identified 4 studies which assessed ImmuKnow for both infection risk and rejection, one further study assessing infection specifically, and a further study examining rejection risk alone. All but one study were retrospective, and in general had small patient numbers with short or undeclared periods of follow-up. In this meta-analysis, the ImmuKnow assay was identified as having a diagnostic odds ratio of 14.7 with sensitivity 83.8% and specificity 75.3% for diagnosing infection. When evaluating rejection, a diagnostic odds ratio of 8.8 (sensitivity 65.6%, specificity 80.4%) was noted alongside significant variation amongst studies included in analysis. In particular, the sensitivity ranged from 9.1%-85.7%[65].

A possible explanation for the perceived poor sensitivity of ImmuKnow in detecting rejection may be that it relies on T cell stimulation with PHA mitogen, which is a non-specific antigen that stimulates the adaptive immune system. With the renewed interest in Toll-like receptors, current evidence suggest that the innate immune system also plays a central role in rejection and allorecognition[66-69]. By only stimulating the adaptive immune system, we postulate that the poor sensitivity may reflect ImmuKnow failing to recognize and therefore measure the contribution made by innate immune mediators to rejection processes.

Clearly there have been issues with several studies that incorporate ImmuKnow. However, the assay is FDA approved and with few other options, the assay is employed in several centres. However, there are often no clear protocols and use varies even amongst individual clinicians in the same centre[35]. A large, formal, multi-centre randomized controlled trial would resolve many questions regarding ImmuKnow in regards to its ability to be an objective biomarker of immune function in OLTx patients.

Cytokine genetic polymorphisms

Productions of cytokines vary amongst individuals, and detecting possible polymorphisms in the responsible genes could help in stratifying patients for risk of clinical outcomes. However, in a meta-analysis studying the impact of cytokine gene polymorphism on graft acceptance in clinical transplantation, the only genetic risk factor associated with acute liver rejection was IL-10 polymorphism at position 1082[70] which is associated with low *in vitro* production of IL-10[71].

Circulating cytokine levels

Circulating cytokine levels have the benefit of being reasonably easy to determine. However, analysis of published clinical studies correlating circulating levels with immunological status after liver transplantation are confusing and often contradictory[49]. This probably reflects the multitude of confounding factors that impact this patient population, including surgical stress, the associated ischaemia-reperfusion injury, blood transfusions, hepatic regeneration and infectious complications[72].

Immune competence scores

Some have evaluated multiple factors such as complement and immunoglobulin levels in an attempt to determine an immune competence score to assist in determining risk of infection[73]. This scoring system assigned two points for each of the following: increased levels of baseline IgG, increased levels of baseline IgA, and decreased levels of pre-OLTx C3. This score was found to have a relative risk of infection of 1.99 ($P < 0.001$) and would be both relatively cheap and employs pathology tests already available in many labs[73]. However, to our knowledge it has not been validated in larger cohorts and would not take into account the multitude of other factors involved in a patient's immune function after the transplant operation.

Regulatory T Cells (Treg)

In adult allograft recipients there is evidence that Tregs

are involved in transplantation tolerance by directly inhibiting the proliferation of effector T cells. A substantial number of donor Tregs detach from the liver graft during perfusion and continue to migrate into the recipient after OLTx. These suppress the direct pathway alloresponses and are theorized to contribute to chimerism-associated tolerance *in vivo* in the early stage after transplantation[74].

Lower levels of these regulatory cells have been identified in patients undergoing acute rejection[75,76] while patients completely weaned off immunosuppression demonstrate higher numbers in their grafts and peripheral circulation[77-81]. Despite this, Treg analysis still requires significant laboratory work to isolate PBMCs and perform laboratory analysis and are not currently marketed or used in clinical settings that we are aware of.

Soluble CD30

Both CD4 and CD8 cells express CD30 after primary alloantigenic stimulation. Although there is some suggestion that soluble CD30 may be a useful marker in kidney transplantation[82,83], studies in adult[84] and paedeatric[85] liver transplantation have failed to reveal a role in predicting rejection outcomes.

Operational tolerance

The liver allograft can often be maintained after transplantation with low levels of immunosuppression and in some cases be withdrawn completely without histological damage from rejection - defined as operational tolerance (OT)[86]. It is estimated that OT rates after OLTx are as high as 20%-25%[87,88]. It appears that OT recipients have different cellular immunophenotypic or peripheral blood transcriptional profiles compared with healthy volunteers, recipients on immunosuppression or those experiencing rejection[80,86]. Several studies have sought to identify which patients are likely to achieve OT which could then facilitate drug withdrawal in this select group.

Gene expression

Martínez-Llordella *et al*[89] identified and validated a "tolerant genetic fingerprint" using transcriptional profiling from transplant PBMCs. This identified a modest number of genes capable of identifying tolerant liver recipients with good accuracy. In particular, NK and $\gamma\delta TCR^+$ T cells were the main PBMC subsets associated with tolerance-associated transcriptional patterns.

Although transcriptional profiling of peripheral blood may allow identification of some patients capable of completely weaning off immunosuppression, data directly supporting these assays and their ability to monitor the net immunosuppressive state are yet to be published and not available in clinical settings[20].

Dendritic cells

In humans, 2 major types of blood dendritic cells have been described[90]. Monocytoid DC ($CD11c^+$) can be derived from circulating monocytes in response to granulocyte-macrophage colony-stimulating factor and IL-4 and induce Th1 cell differentiation *in vitro* and may be specialized for induction of immunity. Plasmacytoid DC ($CD123^+$) develop after stimulation with IL-3 and $CD125^+$ (CD40L) and promote Th2 responses which can be for induction of tolerance[91]. The ratio of these cells may be important, with flow cytometry demonstrating operationally tolerant patients exhibiting higher incidence of plasmacytoid dendritic cells (theorised to induce tolerance) compared with myeloid dendritic cells[92,93].

Delayed-type hypersensitivity

In OLTx patients, the trans vivo delayed-type hypersensitivity (DTH) assay has been shown to be valuable in identifying OT recipients[94]. This technique involves transfer of PBMCs plus donor antigen in the footpads of naive, severe combined immunodeficiency mice and measuring for response[94]. This has the advantage of evaluating *in vivo* cell-mediated allogenic immunity without direct exposure of patients[95]. The logical limitation is the need to have immunodeficient mice available and this makes the assay unfeasible outside research.

Identifying patients who can achieve OT would prove valuable in reducing immunosuppression and related side-effects in these recipients. It would also reduce the ad hoc nature that is sometimes employed to withdraw immunosuppressants following OLTx. However, only a small proportion of patients are likely to have the potential to achieve full operational tolerance and other methods of immune monitoring are therefore needed for the majority of patients.

CONCLUSION

Immune function monitoring following OLTx remains a difficult area, but an area in which even small advances would likely result in significant improvements to morbidity and long-term mortality for patients following liver transplantation today. Many options for immune monitoring have been considered, and vary in methodology from predicting risk of clinical complications, varying dosing of immunosuppressants, and identifying those who may be able to develop operational tolerance.

No single method or assay has been able to meet the diagnostic requirements while answering the basic technical requirements: an assay that is standardized, reproducible, cost-effective, easy and intuitive to perform[35]. Most vary in degree of promise based on ease of execution, precision, specificity, reproducibility and cost, as well as the type of information they provide[96]. It is possible that multiple assays or a combination assay may be needed in the same patient at different times to distinguish an accurate immunological profile in the future[37]. In particular, combining assays from both arms of the immune system (innate and adaptive) may provide clinicians a more comprehensive net immune response of a patient.

Many antigen specific assays also suffer from being based on PBMC which excludes the red cells from. This can pose several issues. Firstly, both the CNIs and mTOR inhibitors are found in whole blood rather than extracted PBMCs, and whole blood has been considered the best

matrix for monitoring immune function[20,97]. Secondly, extraction of PBMCs is often a process that requires significant laboratory effort and its applicability outside research settings in commercial laboratories would likely be personnel and cost-prohibitive.

Without available objective markers of immune function, drug levels, liver biochemistry and clinical events are often used to guide immunotherapy. This approach is crude and drug side-effects and clinical complications remain common[63]. Although the ImmuKnow assay offered early promise and is FDA approved, some conflicting results have limited its widespread acceptance. A formal randomised controlled trial would help in answering many questions regarding the assay given the issues in many of the trials previously undertaken.

The rejection cascade is complex and not firmly understood, with many likely interactions between innate and adaptive immune processes. Therefore no single test is likely to provide a foolproof window to the immune response. As such, nothing can replace the clinical judgement of an expert transplant clinician for pooling together data to individualize immunosuppression therapy[20] but an unmet need exists to measure immune function and assess the risk of clinical complications objectively in OLTx patients[41].

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An informative, structured abstract should accompany each manuscript. Abstracts of original contributions should be structured into the following sections: AIM (no more than 20 words; Only the purpose of the study should be included. Please write the Aim in the form of "To investigate/study/…"), METHODS (no less than 140 words for Original Articles; and no less than 80 words for Brief Articles), RESULTS (no less than 150 words for Original Articles and no less than 120 words for Brief Articles; You should present *P* values where appropriate and must provide relevant data to illustrate how they were obtained, *e.g.*, 6.92 ± 3.86 *vs* 3.61 ± 1.67, $P < 0.001$), and CONCLUSION (no more than 26 words).

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Please list 5-10 key words, selected mainly from *Index Medicus*, which reflect the content of the study.

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For articles of these sections, original articles and brief articles, the main text should be structured into the following sections: INTRODUCTION, MATERIALS AND METHODS, RESULTS and DISCUSSION, and should include appropriate Figures and Tables. Data should be presented in the main text or in Figures and Tables, but not in both.

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Three-line tables should be numbered 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each table. Detailed legends should not be included under tables, but rather added into the text where applicable. The information should complement, but not duplicate the text. Use one horizontal line under the title, a second under column heads, and a third below the Table, above any footnotes. Vertical and italic lines should be omitted.

Notes in tables and illustrations

Data that are not statistically significant should not be noted. $^{a}P <$

0.05, $^{b}P < 0.01$ should be noted ($P > 0.05$ should not be noted). If there are other series of P values, $^{c}P < 0.05$ and $^{d}P < 0.01$ are used. A third series of P values can be expressed as $^{e}P < 0.05$ and $^{f}P < 0.01$. Other notes in tables or under illustrations should be expressed as ^{1}F, ^{2}F, ^{3}F; or sometimes as other symbols with a superscript (Arabic numerals) in the upper left corner. In a multi-curve illustration, each curve should be labeled with ●, ○, ■, □, ▲, △, *etc.*, in a certain sequence.

Acknowledgments

Brief acknowledgments of persons who have made genuine contributions to the manuscript and who endorse the data and conclusions should be included. Authors are responsible for obtaining written permission to use any copyrighted text and/or illustrations.

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Format

Journals

English journal article (list all authors and include the PMID where applicable)

1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

Chinese journal article (list all authors and include the PMID where applicable)

2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.
Genotypes: *gyrA*, *arg* 1, *c myc*, *c fos*, *etc.*
Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc.*
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