In: Organ Donation and Organ Donors
Editor: Reza F. Saidi
ISBN: 978-1-62618-853-2
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Chapter 5

Improved Detection of Donor-Specific Anti-HLA Antibodies and Increased Reliability of Pre- and Post-Transplant Cross-Matching Relevant for Organ Allograft Survival Through the Use of Novel Solid Phase-Based Assays

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Abstract

Antibodies directed against HLA antigens of a given organ donor represent the dominating reason for hyper-acute or acute allograft rejections. In order to select recipients without donor-specific antibodies, the complement-dependent cytotoxicity assay (CDC) was developed more than forty years ago and established as standard crossmatch procedure. This functional assay is characterized by the detection of allo-antibodies which exert their detrimental function by their complement-activating features. However, this technique fails to identify those donor-specific antibodies which lack complement-fixing activity although these may be as well detrimental for donors' organs. Furthermore, as a functional assay its outcome strongly depends on the availability of isolated vital lymphocytes of a given donor. These requirements of the donor's material

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may often not be fulfilled, leading to the situation that the detection of the antibodies directed against HLA molecules is either impaired or sometimes completely impossible. In addition, several diseases or pharmacological treatment of a given recipient may lead to "false-positive" results demonstrating the general insufficiency of this assay under certain circumstances. As a consequence of these methodical disadvantages novel procedures which function independently of the cell quality were generated. As a result solid phase-based enzyme-linked immunoassays (ELISA or Bead arrays) were designed in order to reliably detect general and/or donor-specific anti-HLA antibodies in prospective organ recipients. Due to the obvious advantages of these novel technologies, when compared with the classical CDC assay, there is an urgent need to implement them as complementary methods or even as a substitution for the conventional CDC crossmatch that is currently being applied by all tissue typing laboratories. This review provides an overview of the different crossmatch assays with their advantages and limits. Additionally, pros and cons of the so-called virtual crossmatch based on the identification of the recipents' anti-HLA antibody specificities in comparison with the HLA-types of given donors using different antibody detection/identification assays are discussed.

Keywords: Allograft; complement-dependent cytotoxicity assay; crossmatch; human leukocyte antigen; post-transplantation monitoring; rejection

Introduction

More than thirty years ago Patel and Terasaki described for the first time that antibodies, which are directed against antigens of donor tissues, are clearly associated with hyperacute rejections in recipients of renal allografts and allografts of other organs [1]. In subsequent studies evidence was provided that antibodies against human Major Histocompatibility Complex (MHC) antigens, the so-called Human Leukocyte Antigens (HLA), are the dominating reason for hyperacute rejections of allografts [2, 3]. Thus, a crossmatch (CM) procedure allowing the detection of antibodies in the recipient's serum against lymphocytes isolated from the donor's blood was developed as an effective predictor of short-term survival of renal allografts. As standard technology for the detection of donor-specific antibodies against HLA class I and/or class II molecules the complement-dependent lymphocytotoxicity (CDC-) assay was established in the late sixties. This functional assay is based on the detection of alloantibodies, which exert their detrimental function by their complement-fixing and activating features finally leading to the lysis of donor cells. However, this technique fails to identify those donor-specific antibodies, which lack complement-fixing activity although these may be as well detrimental for tissues/organs of the donor. Due to its low sensitivity the CDC assay furthermore fails to detect low antibody concentrations resulting in its modification by using secondary anti-human immunoglobulin antibodies [anti-human globulin (AHG)-enhanced CDC-crossmatch] recognizing the primary donor-specific antibodies and increasing the activation of complement [4, 5]. Anyway, all variants of the CDC assays depend on a high vitality of the donor cells and are hardly interpretable with cells exhibiting a vitality rate lower than 80-90 %. As a consequence of these methodical disadvantages novel methods independent from the cell quality were generated resulting in the design of solid phase-based enzyme-linked immunoassays (ELISA or Bead arrays) for the detection of anti-HLA class I/II antibodies. This review article provides an overview about the methods currently used for the detection of general and/or donor-specific anti-HLA

antibodies thereby suggesting at least the complementary implementation of novel solid phase-based methods.

Technical Designs of Procedures Established and Modified for the Identification of Anti-HLA Antibodies: Discussion of Their Advantages, Disadvantages and Limitations

Prior to transplantation the CDC procedure is performed to (i) elucidate the general degree of presensitization (antibody-monitoring) or (ii) to identify actual donor-specific antibodies (DSA) against HLA phenotypes of a given donor (crossmatching). The general degree of presensitization has originally been defined as conventional CDC-derived reactivity against either a panel of peripheral blood lymphocytes (PBL) or against a selected cell panel from chronic lymphatic leukaemia patients (CLL) and has thus been termed "Panel-Reactive-Antibodies" (PRA). The value is expressed as percentage of cells recognized by the recipient's serum out of the complete cell panel selected for this diagnosis. It is self-evident that the chosen cell panel has to comprise the HLA phenotypes of the donor's as well as the recipient's population. The statistical PRA value (%) determined for all patients on the kidney waiting list thus only represents a value indicating the relative risk for an antibody-mediated graft rejection. It allows the identification of patients who have to be monitored very carefully due to their pre-immunization status when they are selected as recipients. However, to determine the PRA value does not at all represent the identification of so-called donor-specific antibodies (DSA), which may occur also in patients with a low degree of PRA. Additionally these DSA may be directed against rare HLA phenotypes, which are not included in the panel. They have to be detected by crossmatching. In contrast to the crossmatch procedure performed prior to transplantations the retrospective post-transplant crossmatch may be used for the diagnoses of (hyper-) acute post-transplantation rejections. So far the dominating method is the invasive needle biopsy with its well-known risks. Consequently alternative approaches to circumvent the biopsy e.g. by the identification and monitoring of various biomarkers as well as the detection of occurring donor-specific immune responses have been developed and employed.

The pre- and the post-transplant crossmatch applications are summarized in order to point out that the different crossmatch techniques for methodological reasons are of completely different value for both applications. Methods requiring freshly isolated, vital cells like the conventional CDC or the flow cytometry-based crossmatch procedures exhibit only a very limited value for the post-transplantation monitoring of a donor-specific alloresponse since this alloresponse may occur several days to weeks after the transplantation. This leads to the requirement to conserve vital donor cells over this time period, which is an expensive and laborious method as the cells have to be stored in liquid nitrogen. The methodological strategies described in this article will in particular be discussed in the context of their post-transplant practicability using cellular or tissue-derived material after its storing for several weeks to months.

The Conventional Complement-Derived Cytotoxicity (CDC) Crossmatch Standard Assay and its AHG-Enhanced Modification

The conventional CDC crossmatch assay represents the current standard method for the detection of donor-specific anti-HLA antibodies in potential recipients. In spite of the existence of several disadvantages this method has generally been accepted for years for the selection of recipients. A scheme of the work flow is illustrated in Figure 1. The procedure is initiated with the isolation of peripheral blood lymphocytes (PBL) by sucrose polymer-based density gradient centrifugation and their subsequent incubation with serum of the selected recipient (Figure 1A) before complement proteins from rabbit are added. Activation of the complement system (Figure 1B) is mediated via the classical activation pathway by cytotoxic antibodies, which have been bound to the cells in the first incubation step. Only bound antibodies of the complement system-activating IgM/IgG3/IgG1 isotypes against cellular antigens of the donor lead to a positive result which is determined by fluorescence microscopy. Dead cells are consequently stained red by the DNA-intercalating agent ethidium bromide (Figure 1C), whereas vital cells exhibit a green staining pattern due to the active uptake of acridine orange (Figure 1C). The intensity of the complement reaction is categorized as the amount of dead cells using a score system according to a standard protocol of the National Institute of Health (Washington, USA) (Table 1). Due to various difficulties methodical modifications of this assay have been implemented, which in many cases are essential for the reliable outcome of the CDC crossmatch and its valid interpretation. In this context isolated T- or B-lymphocytes employing antibody-coated magnetic beads for their direct purification (System Dynal, Oslo, Norway) or the tetrameric antibody complex technology crosslinking unwanted cells to red blood cells followed by their elimination using density gradient centrifugation (System RosetteSep, Stem Cell Technologies via CellSystems® Biotechnology GmbH, St. Katharinen, Germany) have been implemented. However, the conventional CDC-based crossmatch without previous isolation of B- or T-lymphocytes does not lead to clearly interpretable crossmatch results in about 20 % of the assays performed during emergency duties. The number of uninterpretable crossmatch results increases to about 30 % for the foreign donations of kidneys during the emergency duties, for which only the second delayed crossmatch assay comprising also the historical sera of selected recipients has to be performed in the laboratory of the recipient's transplant center. For these foreign donations, CDC crossmatches have to be performed with lymphocytes from a blood sample or in most cases (> 90 %) with a piece of splenic tissue delivered together with the organ to be transplanted. Due to the enhanced delivery times of these foreign organ offers and especially as a consequence of unsufficient cooling of blood or tissue samples the background of the crossmatch caused by dead PBL may rapidly increase up to 30 % (score 4), which highly impairs the identification of weakly positive reactions (score 2-4). This is mainly due to the decreased survival rate of B-cells which are more sensitive to environmental factors than T-cells. Furthermore, the loss of a high blood volume of the donor accompanied by the release of CDC assay-irritating lymphoblasts from the bone marrow as well as the treatment of the donors with drugs often leads to high backgrounds in the CDC-CM mediated by dead PBL. To obtain valid results the background of CDC-based assays should generally not exceed 10 %.

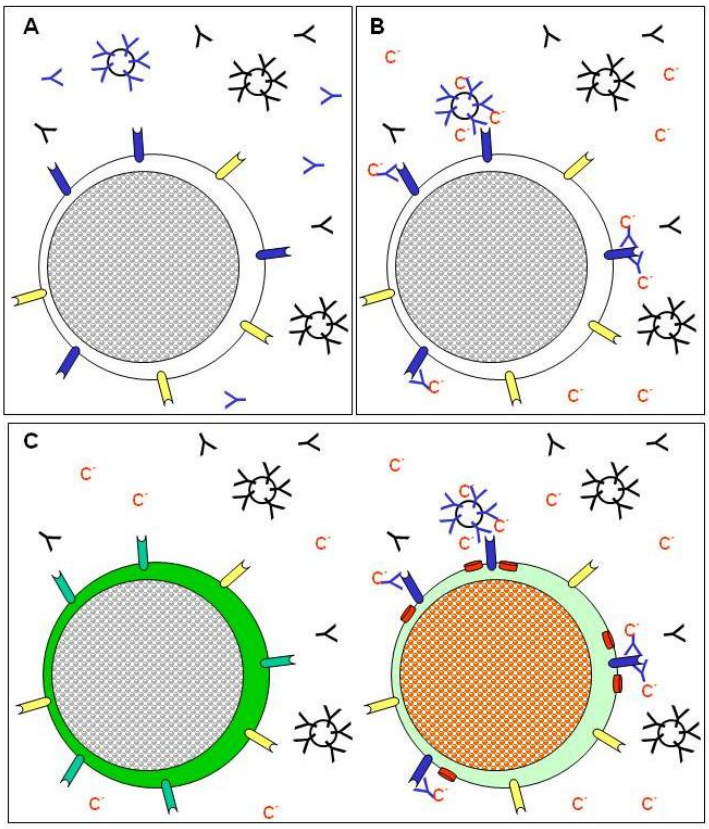

Figure 1. Scheme of the classical CDC-crossmatch as the current standard procedure for the detection of donor-specific antibodies. A Antibodies (monomeric IgG and pentameric IgM) are part of a recipient's serum and may recognize HLA molecules (blue and yellow) on donor's lymphocytes. B Activation of the complement cascade from added rabbit complement (C') by the antibodies (blue) bound to the corresponding HLA-molecules. C Positive reaction by ethidium bromide staining (red colour) of the nuclei of lethal cells which have been lysed by the complement system (right) and negative reaction detectable by acridine orange staining (green colour) of vital cells to which no antibodies had bound and which have not been lysed by the complement added. The red cylinders symbolize the Membrane Attack Complexes (MAC) as final complement activation products.

Table 1. Score system of the standard CDC-crossmatch assay as percent of dead/positive cells (red coloured) which are the result of the complement-mediated lysis

SCORE	DEAD CELLS (%)	DESCRIPTION/INTENSITY OF THE REACTION
1	≤ 10	negative
2 = +	10 - 20	doubtfully positive
4 = ++	20 - 40	weakly positive
6 = +++	40 - 80	positive
8 = ++++	80 - 100strongly positive	

Additional problems arise when recipients' antibodies are only directed against HLA class II antigens of the donor since the HLA class II antigen-bearing monocytes and B-cells

comprise no more than 30 % of PBL. Thus, employing unseparated PBL a strong anti-HLA class II reaction leading to the red (positive) staining of all HLA class II antigen-bearing PBL never exceeds a weakly positive reaction (score 4). However, the use of isolated T-cells and B-cells is not obligatory. Thus, it is in the authority of each single laboratory to perform this additional but essential effort. Since PBL and splenic tissue samples delivered during emergency duties do not lead to unequivocally interpretable results without analysing isolated T-cells and B-cells in at least 20 % of the donations, it should be mandatory to implement this additional separation procedure prior to the CDC crossmatch.

In order to increase the low sensitivity of the classical CDC crossmatch this assay was modified by enhancing the complement activation through the incubation with secondary antibodies directed against the primary human donor-specific antibodies. The modification was termed "anti-human globulin (AHG)-enhanced CDC-CM. However, it is noteworthy that in particular this procedure is characterized by an increased number of damaged PBL or isolated lymphocytes, respectively, due to the elongated incubation period causing a higher background and accompanied by a higher number of uninterpretable results.

The Flow Cytometry Crossmatch (FACS-CM) Which Reached No General Breakthrough Due to Methodical Difficulties

To circumvent the problems described for the CDC-CM the flow cytometric crossmatch (FACS-CM) was initially developed by Garovoy and co-workers in 1983 [6]. It represents an indirect immunostaining procedure detecting HLA antigens using secondary fluorescence dye-labelled antibodies. Thus, both complement-activating and complement-independent donor-specific antibodies are detected. Although this procedure is characterized by a higher sensitivity than the conventional CDC-CM, i.e. by the detection of even low concentrations of antibodies, which is in the range of the AHG-enhanced CM [7, 8], the outcome of this assay is frequently influenced by artefacts. This is due to the "irrelevant" binding of the recipients' antibodies through their Fc parts to subgroups of Fc receptors expressed on B-lymphocytes which are isolated for the flow cytometric analyses to identify anti-HLA class II antibodies. Concerning FACS-CM assays controversial studies were published either demonstrating that a positive CM does not necessarily correlate with an impaired outcome concerning graft rejections [9, 10] or providing evidence that non-complement-activating alloantibodies detected by this method are indeed associated with an increased number of rejections despite corresponding negative CDC crossmatches [11, 12]. Representative histograms of own investigations illustrate the above mentioned common problem of the FACS-CM procedure possibly leading to wrong conclusions (Figure 2). In contrast to the T-cell CM (right side) demonstrating no binding of anti-HLA class I antibodies, the B-cell CM (left side) is characterized by an increased signal in comparison to the negative control serum. However, the anti-HLA class II antibodies potentially detected by this FACS-CM were not confirmed by the anti-HLA class II antibody screening/identification Elisa using solid phase-coated HLA class II antigens (B-Screen-ELISA/Quik-ID Class II-ELISA, GTI diagnostics, Waukesha, USA) [13]. Furthermore, the CDC-CM performed with isolated B-cells did not detect anti-HLA class II antibodies most probably indicating a false positive FACS signal. It is noteworthy that this drawback of the FACS-CM of B-cells indeed represents rather a common than a rare event [14]. To improve the outcome of the FACS-based B-cell CM the

assay was modified by the pre-treatment of the cells with the enzyme pronase considerably increasing both the sensitivity and especially the specificity of the FACS-CM procedure [14, 15, 16]. However, the reproducibility of the pronase pre-treatment was difficult due to the loss of Fc receptors as well as of HLA molecules most probably depending on distinct enzymatic activities of different pronase sources used by the different groups. Due to these methodical disadvantages a standardized protocol has not yet been developed for this procedure suggesting that it generally does not have the capacity to substitute or complement the CDC-CM. For this reason only few tissue typing laboratories actually employ this assay. Recently a pronase-free B-cell flow-cytometry crossmatch has been proposed using heat-inactivated rabbit serum [17]. The procedure adequately reduces the background caused by non-specific reactions without the disadvantage of digesting additional cell surface proteins. This method well known for immunohistochemical applications to block Fc-receptors may first provide the capacity to overcome the problem of unspecific binding of antibodies through their Fc parts. However, as another striking disadvantage of this method which holds also true for the CDC-CM its dependence on the cell quality remains as a problem. Therefore, the development of novel methods which are partially or completely independent from the cell quality was required leading to various solid phase-based enzyme-linked-immunoassays which were generated to detect anti-HLA class I/II antibodies.

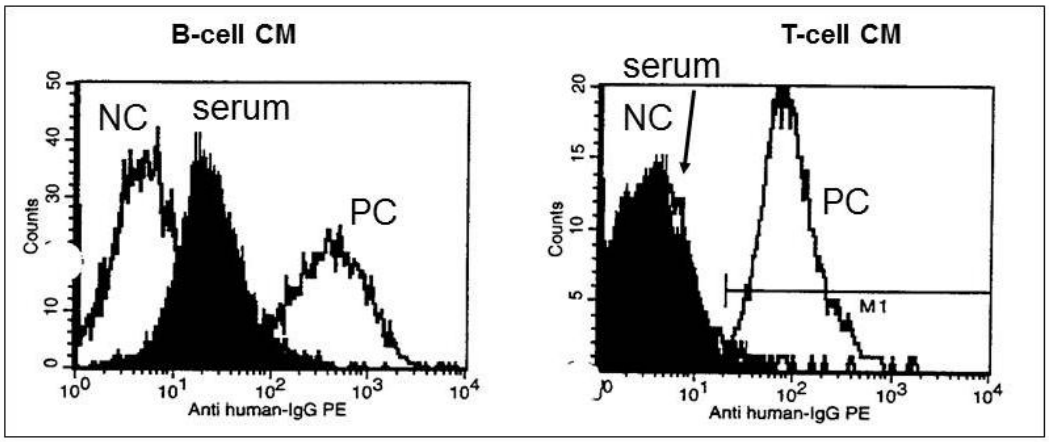

Figure 2. FACS-crossmatch of B-cells and T-cells as shown by their histograms. The FACS-crossmatch of T-cells is unequivocally negative, whereas the B-cells provide a histogram of weakly increased intensity. Due to the faint intensity it does not lead to an interpretable result especially in the context of a negative HLA class II-specific B Screen Elisa and a negative conventional CDC-crossmatch performed with isolated B-cells.

Solid Phase-Based Immunoassays for the Detection of Anti-HLA Antibodies and Determination of Their Specificities (Anti-HLA Antibody Screening/Identification ELISA)

In order to avoid problems which depend on the quality of cells a solid phase immunoassay named FlowPRA^{TM} was first developed. In this assay purified HLA class I or class II molecules were immobilized on the surface of latex beads. Beside its independence

from the cell vitality this bead-based flow cytometric procedure was characterized by its high sensitivity [4, 18-20]. This system allows the identification of antibody specificities against certain phenotypes immobilized on the beads. It has been described that 20 to 30 % of sera defined as antibody-negative in the AHG-augmented CDC-based antibody screening (cell panel tray) exhibited anti-HLA antibodies in solid-phase assays specific for HLA antigens such as Elisa and/or FlowPRA^{TM} [4]. Although patients positive in FlowPRA^{TM}-based and negative in the AHG-CDC-based screening did not show hyperacute or acute rejections, general episodes of graft rejection, which required therapeutic interventions, occured more frequently in the first group than in patients without detectable pretransplant antibodies [19]. Furthermore, patients with antibodies detectable by the FlowPRA^{TM} method exhibited a decreased mean time to a first rejection episode (after 100 days) in comparison with FlowPRA^{TM}-negative patients (after 250 days). These antibodies were associated with rejections even when they were not donor-specific although the underlying mechanisms about which different speculations exist have not yet been defined [21, 22]. Clinical relevance has also been claimed for antibodies detectable by the FlowPRA^{TM} method, but not by FACS-CM strengthening the clinical impact of non-donor-specific anti-HLA antibodies. In addition, the FlowPRA^{TM} procedure identified anti-HLA antibodies in about 10 % of HLA-negative sera defined by the AHG-CDC (cell panel tray with 0 % PRA) and ELISA assays. Furthermore, these were also negative in the AHG-augmented CDC crossmatch using donor material. Although the FlowPRA^{TM} method may be the most sensitive method among the techniques utilized, it is characterized by technical complexity associated with a higher financial investment. In contrast to a flow cytometer an Elisa reader belongs to the basal equipment of nearly all laboratories. Therefore the FlowPRA^{TM} antibody screening technique in accordance with the FACS-CM never reached a general breakthrough in HLA antibody monitoring due to the alternative Elisa-based techniques, which are easily to perform and exhibit a reduced level of technical complexity resulting in lower costs. In view of these apparent disadvantages of flow cytometrical methods Elisa-based strategies have alternatively been developed for the detection of anti-HLA antibodies during the last fifteen years [13, 23-26]. The first study concerning the identification of anti-HLA antibodies using an Elisa was published by Kao and co-workers in 1993 [27]. HLA class I antigens were purified from a pool of platelets and directly immobilized on the surface of microtiter plates. Based on the principle of solid phase-immobilized HLA class I or II antigens several other manufacturers developed Elisa for antibody screening such as the LAT-M (One Lambda, Canoga Park, CA, USA), the AbScreen (Biotest, Dreieich, Germany) or the QuikScreen (GTI, Waukesha, USA) [13, 26, 28, 29]. The antigen source of these Elisa assays chosen by most manufacturers for the detection of anti-HLA class I antibodies consists of a pool of more than 100 donations of platelets (AbScreen/Biotest, QuikScreen/GTI), whereas the LAT-M (One Lambda) uses Epstein Barr Virus (EBV)-transformed human lymphoma cells as antigen source. The HLA class II antigens (HLA-DR and –DQ) are generally purified from selected EBV-transformed cell lines and are immobilized as the HLA class I antigens [30]. For this purpose the HLA molecules are purified by immunoaffinity column chromatography. However, the loss of some epitopes of HLA molecules due to their solid-phase coating represents a theoretical disadvantage. Particular difficulties have been reported for HLA class II DQ antigens, since the HLA class II-specific B-Screen Elisa (GTI) could result in false-negative data for their detection [13]. Worthington and co-workers (2001) speculated that this phenomenon might be due to the low-avidity binding of HLA-DQ molecules and their possible removal during the washing

steps. This apparent inability of the B-Screen class II Elisa to identify antibodies specific for HLA-DQ represents the major disadvantage due to the relevance of anti-HLA-DQ antibodies for the graft survival [31]. Despite this drawback of the QuikScreen class II Elisa (B-Screen) to detect anti-HLA-DQ antibodies the comparative study of Worthington and co-workers (2001) certified a high degree of concordance ranging from 83% to 91%, respectively, for the three HLA class I-specific solid phase assays FlowPra I, LAT-M class I and QuikScreen class I and the corresponding HLA class II-specific assays (FlowPra II, LAT-M class II and B-Screen) [13]. The comparison of two different Elisa systems, the AbScreen and LAT-M, published by Monien and co-workers [26] is hardly interpretable since there exist several inconsistencies concerning tables, data in the text and statistical analyses. However, using the raw data of this publication the degree of concordance between both assays is about 89 % (548 out of 612 samples) for the detection/non-detection of antibodies against HLA class I and about 92 % (566 out of 612 samples) for antibodies recognizing HLA class II molecules, which is comparable to the previously described results [13]. A third study of Uboldi di Capei and co-workers [32] was published dealing with the comparison of three different commercially available Elisa kits (LAT-M/One Lambda, QuikScreen/GTI, PRA STAT/Sang Stat). In spite of the easier and faster handling of the Elisa-based techniques some disadvantages of single kits were reported such as a decreased sensitivity of the QuikScreen, a decreased sensitivity/reliability of the PRA STAT Elisa as well as the identification of HLA-specific antibodies of only the IgG isotype in comparison to the conventional CDC-based procedure. The most expensive LAT-M kit, however, was described to lead to the best results [32]. Due to the authors' valuation the ELISA assays are at least ten-fold more expensive than the CDC-based screening using a cell panel if the initial "positive versus negative" pre-screening step is followed by a secondary specificity assessment. The PRA STAT Elisa as the only system in which both procedures, pre-screening and specificity assessment, are contemporaneous exhibited the lowest reliability of the three Elisa systems under investigation. However, the costs for the antibody determination by the conventional CDC-cell panel procedure presented by Uboldi de Capei and co-workers [32] have to be regarded as non-realistic since the high storing costs for cells in liquid nitrogen and a high personal effort to compose the respective cell trays have not accordingly been taken into consideration. Furthermore, it is more than doubtful to get reliable specificity analyses for the majority of patients using panels between 30 and 60 cells as described by these authors. The disadvantage attributed to Elisa-based screening assays to identify only anti-HLA antibodies of the IgG isotype [32] may easily be overcome by the alternative use of secondary antibodies specific for both the IgG and the IgM isotypes. The dominating aspect for favouring the usage of Elisa-based systems is that they do not require vital cells and allow the identification of complement-activating as well as non-complement activating antibodies. However, any of the three Elisa assays and the conventional CDC-CM under investigation exhibited unique positive reactions, which were not confirmed by a second assay. In this context about 10 % of all samples were concerned when the three Elisa assays and the CDC-CM were included. The exclusion of the PRA STAT Elisa decreased the degree of discordance between the CDC-CM, LAT-M Elisa and GTI-Elisa to about 5 % thus representing a remarkable analogy of the three remaining assays. With the exception of the groups of Uboldi de Capei [32] and Pierquin [33] for reasons which mainly depend on specific insufficiencies of the PRA STAT-Elisa, nearly all studies [13, 23-25, 27-31, 34-39] highlight the advantages of the Elisa-based screening systems to detect anti-HLA antibodies with higher sensitivity and specificity.

In further studies the clinical relevance of antibodies directed against HLA antigens, which are undetectable by the standard complement-dependent cytotoxicity test but detectable using FACS-CM and/or Elisa-based procedures has been demonstrated [40, 41]. Thus the implementation of FACS- and/or Elisa-based methods is recommended to (i) predict immunological complications, (ii) to avoid their clinical manifestation through the adjustment of the consecutive immunosuppressive therapies and (iii) to discriminate between anti-HLA class I and class II antibodies thereby achieving information about the impact of anti-HLA class II antibodies. Apart from the study of Christiaans and co-workers [42] the great majority of all studies provides evidence for the necessity of this discrimination due to the existence of detrimental effects as a result of anti-HLA class II antibodies concerning kidney as well as heart graft failure and/or rejection [13, 32, 42, 44, 45]. In this context evidence has been provided that anti-HLA class II antibodies are more strongly associated with long term chronic rejection whereas antibodies against both classes of HLA molecules are clearly associated with early graft rejection [45-47].

During the last decade the specificities of latex bead flow cytometric (FlowPRA) and the Elisa assays were extended using an increased number of immobilized HLA antigens. The first flow cytometric bead assay developed in 1998 comprised a total of 60 HLA class I and class II specificities which were detectable as antigen panels on the surface of beads either bearing HLA class I or class II antigens. The introduction of the recombinant generation of HLA antigens in eukaryotic expression systems, however, allowed the generation of single antigens comprising all common and some rare antigens. The anti-HLA antibody Elisa using solid phase-immobilized HLA antigens were further processed using immobilized single HLA class I or class II molecules instead of antigen pools, respectively (Single-Antigen Class I Elisa/One Lambda), thereby allowing the identification of single antigens which had not been detectable using an antibody screening Elisa with immobilized mixtures of HLA antigens.

Additionally, the Luminex^{TM} technology was adapted to be used for the identification of HLA-specific antibodies. This microbead-based multiplex assay primarily employed for HLA typing by SSO (Sequence-Specific Oligonucleotide) PCR allows the simultaneous detection of maximally 100 different analytes from one tube and currently represents the dominating tool for kidney transplant risk stratification as a consequence of identifiable anti-HLA antibodies [48, 49].The technical features of the Luminex technology used for anti-HLA antibody screening have in detail been reviewed and discussed in the publications of Colombo and co-workers [50] and Tait and co-workers [51]. This technology used for antibody screening is composed of a series of polystyrene microspheres on which one or a group of HLA molecules are attached. The beads contain embedded fluorochromes of different intensities which gives this bead a unique signal. Depending on the number of immobilized HLA molecules the Luminex assay can be used for the screening to differ between positive and negative patients (screening level = lowest level of resolution), followed by the level of single cells (so-called ID-level) with each bead carrying two molecules derived from two alleles of each locus i.e. of HLA-A,-B and –C for class I and of HLA-DR and –DQ for class II antigens. The highest level of resolution consists of beads with only one molecule immobilized (single antigen Luminex bead/SAB-level). Patients' sera are added to the mixture of beads out of which the anti-HLA antibodies bind to the respective antigens. The bound antibodies are recognized by secondary anti-human IgG or IgG/IgM antibodies labelled with phycoerythrin. In the detection machinery one laser excites the fluorochrome of

the bead whereas the other detects the phycoerythrin-labelled secondary antibody. Both signals in combination define the specificity of the antibody out of the serum to be analysed. Evidence could be provided by several studies that the Luminex technology, which is the most sensitive antibody detection technique, is able to identify clinically relevant antibodies in addition to the antibodies identified by CDC-based cell trays. Smith and co-workers [52] reported data on cardiac transplant recipients [n=565] whose pre-transplant sera were retrospectively tested for anti-HLA antibodies using CDC and Luminex technique. Luminex procedure identified 53 patients with anti-HLA antibodies in addition to 14 patients defined as positive (PRA>5 %) by CDC assays and five to exhibit donor-specific antibodies by the same procedure. A comparison of the graft survival of patients with CDC-positive and Luminex-positive (40 %) in contrast to patients with CDC-negative and Luminex-positive donor-specific antibodies (42 %) indicated a subset of patients with donor-specific antibodies which are at high risk of graft rejection and are detectable only by Luminex technique. Further studies exist which deal with the approach to predict positive CDC crossmatch results by Luminex-based antibody identification as the method chosen for the so-called virtual crossmatch [53]. In that study solid phase-based assays including the Luminex technique exhibited a better prediction for a positive CDC-based crossmatch than CDC-based antibody differentiation using selected cell panel trays. A quite low rate of concordance of CDC-based antibody identification and prediction of the CDC-based crossmatch result is most probably due to the apparent failure of the CDC-based antibody identification to accurately determine the specificities in highly sensitized individuals. The antibody specificities of highly sensitized patients are in most cases only identifiable using systems providing the resolution of the single antigen bead (SAB) level which generally cannot be reached using cell trays but only by solid phase-based assays. Further studies were performed by Vaidya and co-workers [54, 55] to assess antibodies detected by Luminex assays for the prediction of CDC- and Flow crossmatch assays. Correct prediction rates of 93 % for T- and B-Cell Flow cross matching and of 79 % and 68 % for CDC-based T- and B-cell crossmatch results, respectively, were reached. In their study [55] Vaidya and co-workers correlated anti-HLA antibody specificities detected by Luminex technique using the measuring unit "Molecules of Equivalent Soluble Fluorophore values" (MESF) defined in 2002 by Schwartz et al. [56]. The approach to use MESF values was also chosen by Mizutani and co-workers [57] in a subsequent study, and there provided evidence that patients who suffered from graft failures had higher titres of antibodies than patients with continuing graft functions. Thus, this way of data interpretation may result in the definition of clinically relevant titers of anti-HLA antibodies and in the reliable identification of patients with high or increased risks of graft failure. At the moment this aspect is under highly controversial discussion in the field of antibody analysis relevant for graft survival [50, 51] and provides the main basis of arguments for the objectors of the Luminex-based method in general and especially the Luminex-based technique of single antigen bead (SAB) arrays. In terms of their plausibility these Luminex-based data have unfortunately to be assessed as unreliable in many cases. In spite of the doubtlessly finer specification of anti-HLA antibodies using Luminex-based SAB arrays the question concerning the clinical impact of these antibodies and especially concerning their definition as DSA by virtual cross-matching has not been resolved during the last six years and is still point of controversial discussions. Data on the clinical correlation of SAB-defined DSA with an impaired outcome of graft survival have been published [58-61] but were not confirmed by other groups [62, 63]. Furthermore, as nearly all laboratories use the measuring unit "Mean

Fluorescence Intensity" (MFI) instead of MESF no consensus has been found to define a cut off value between irrelevant background values and clearly positive antibody specificities leading to highly varying threshold MFI-values between all laboratories. The point to accept clinically irrelevant specificities for prospective recipients in order to avoid the refusal of an adequate organ has accordingly not been cleared for the whole time of this SAB-array's commercial availability. Recently the discussion came up whether the immobilization of the single antigens on the bead surfaces leads to novel linear epitopes which are not part of cell surface-expressed HLA molecules and thus are irrelevant for transplantation diagnostics. These de-novo epitopes would explain up to 20 % antibody specificities of certain recipients which would be directed against self HLA molecules thus representing auto-antibodies although humoral anti-HLA auto-immunity is a very rare diagnosis. These sources of irritation led to the approach to classify antibodies detected only by solid-phase based procedures as risk factors but not as contra-indications for projected transplantations which is indeed rather confusing than helpful as the result for the individual patient will always be completely unclear. Furthermore, ongoing tendencies to implement the CDC-based procedures as the leading procedure in view of these Luminex-technique-derived drawbacks by Doxiadis and co-workers [64] are more than doubtful since the authors do not consider that the various diagnostic solid phase tools (Luminex- or Elisa-based assays) which they solely refer to under the collective term "solid phase assay" are characterized by completely different features, drawbacks and resulting outcomes. Additionally, the severe drawbacks of the CDC-assay exhaustively discussed by us in this article are completely denied by Doxiadis et al. [64].

Generally the drawbacks of the Luminex technology do not hold true for another helpful solid-phase based technique established in 2006, thus demonstrating that not only the bead-based, but also other solid-phase-based technologies have been improved during the last few years. In this context the miniaturized chip technology named "DynaChip™ HLA antibody analysis" had been established for the analysis of anti-HLA antibodies (Invitrogen/Dynal, Bromborough, United Kingdom). Unfortunately after only five years' commercial availability this valuable system was discontinued by the manufacturer in 2011 for economical reasons. This method combined the manageability of solid phase-based systems with the advantage of microarrays (high throughput because of multiple simultaneous measurements) and in contrast to the Luminex-based bead technique represented a completely automated system, which could be easily run overnight (about 4 hours/run). At the bottom of each well of a standard format 96-well-plate a glass microchip consisting of 138 incubation positions was fixed. Ninety six (96) of these positions were covered with a mixture of HLA class I and 42 with HLA class II molecules. A small volume of only 8 µl of undiluted patient's serum was used per chip and well (i.e. per patient). Although the DynaChip ™ assay was not a single antigen assay the combination of single donors' immobilized HLA class I (n=8) or class II (n=10) antigens, respectively, allowed in about 70 % of the analyses the identification of the specificities of the recipients' anti-HLA antibodies. As a small study performed in our tissue typing laboratory the quarterly screening of the kidney waiting list patients was adapted to this system. Formerly a combined procedure of the QuikScreen/B-Screen Elisa and, for the sera positive in this first step, a second CDC-screening/identification using a cell panel composed in our laboratory had been employed. Both procedures, the historical combined Elisa- and CDC/cell tray-based methods and the novel DynaChip assay were used in parallel for two consecutive quarterly screening runs exhibiting an overall concordance of about 84 %

for anti-HLA class I (78.1 % negative and 5.9 % positive in all three assays) as well as for anti-HLA class II antibodies (78.9 % negative and 5.6 % positive in all three assays). However, the DynaChip system appeared to be superior to the old combined procedure because of its reliability and a highly decreased laboratory work due to the simultaneous identification of anti-HLA antibody specificities in about 70 % of the antibody-positive sera investigated. Furthermore, the DynaChip assay allowed the identification of non complement-binding antibodies also relevant for graft failures. The concordance between both solid phase-based antibody detection systems Screening Elisa (Quik-Screen/B-Screen) and DynaChip analysis was 88.2 % for anti-HLA class I antibodies and 91.3 % for anti-class II antibodies, respectively. Of special interest were 6.5 % of the sera positive for anti HLA class II antibodies only in the CDC but not in any of the Elisa-based assays. The great majority of these patients (>75 %) was found out to suffer from autoimmune/immune complex diseases but not to be characterised by anti-HLA antibodies. Thus, strong evidence is provided also by our laboratory that the CDC assay is artificially influenced by autoimmune diseases which will also be discussed subsequently in the context of the CDC-based crossmatch procedure.

Novel Elisa-Based HLA Crossmatch Procedures Developed for the Direct Detection of Donor-Specific Antibodies

Two Elisa-based assays have been established in the past, which allow the detection of donor-specific antibodies independently from the specificity of the identified antibodies (cross-matching). These are the AbCross HLA class I/II Elisa (Biotest/BioRad, Dreieich, Germany) and the Antibody Monitoring System (AMS) HLA class I/II Elisa (GTI diagnostics, Waukesha, USA) [65, 66]. Both technologies allow the direct detection of donor-specific antibodies by immobilizing extracted donors' HLA molecules to pre-coated capture antibodies. These are directed against a monomorphic structure/epitope of HLA class I or II molecules, respectively. Consequently these crossmatch assays allow the detection of antibodies directed against rare phenotypes of a given donor, which are not part of a selected panel of solid phase-coated antigens of screening Elisa (from thrombocytes or EBV-transformed cells) and for this reason may not be detectable by virtual cross-matching. Due to its first commercial availability the AMS-Elisa (GTI diagnostics) was established in our tissue typing laboratory despite results of comparable quality obtained with the AbCross Elisa (Biotest) (personal communication). However, a direct comparison of both assays in parallel has not yet been performed. Regarding the workflow of the AMS-Elisa detergent lysate of a given donor's material has to be pipetted into the wells of Elisa-strips precoated with monoclonal capture antibodies (Figure 3A). After the incubation of the strips with the donors' cell/tissue lysate, they are washed and then incubated with recipients' sera. Their antibodies serve as detection antibodies in case of recognizing the immobilized HLA molecules (Figure 3B). Upon several washing steps the samples are incubated with alkaline phosphatase-conjugated secondary anti-human IgG antibodies. This last step was modified in our laboratory by using secondary antibodies directed against the IgG/M/A isotypes of the primary human antibodies thus allowing the additional detection of anti-HLA antibodies, which are not of the IgG isotype (Figure 3C). The lysate control consisting of a second enzyme-labelled mAb for the detection of the bound HLA molecules recognizing a second monomorphic epitope provides evidence that a sufficient amount of the donors' HLA

molecules has been immobilized to get a signal (Figure 3D). This modified assay was established in our typing laboratory about seven years ago and has been employed for more than 400 samples for which no alternative methods were available or which were characterized by special problems arising from the conventional CDC-CM procedure. There exist three main areas of cross-matching for which this assay is currently used. It is first used for retrospective crossmatches after the transplantation of heart, lung and vessel allografts where no pre-transplant cross-matching is performed mainly due to the lack of time or the lack of statutory regulations. As the material available from donor's blood or spleen for these post-transplant crossmatches for logistical and organisational reasons which are not under the influence of the tissue typing laboratories is generally old (in many cases even older than three days) no retrospective crossmatch procedure requiring living cells is possible. However, also the post-transplant determination of donor-specific antibodies is important to monitor the immune reaction after transplantation and to adequately adjust the immunosuppression in these patients. In these cases an assay not requiring vital cells but capable to identify donor-specific antibodies from non-vital material represents the only method for cross-matching. Second, the AMS-Elisa has been employed for prospective crossmatches of live kidney donations with doubtful results of the conventional CDC crossmatch. Since the donor additionally bears a high burden by the donation of a functional organ it was our initial aim to verify the outcome of any CDC crossmatch procedure not providing a strikingly clear result. This occurs at a frequency of about 20 % of all living donations. However, of these patients characterized by doubtful CDC crossmatches, only 29 % exhibited a positive reaction in the AMS-Elisa, whereas more than 70 % of the patients lacked donor-specific anti-HLA antibodies (Figure 4). Due to the high reliability of the AMS-Elisa in comparison to the conventional CDC-CM most of these patients appear to be devoid of donor-specific antibodies thereby not providing any contra-indication for the prospective living donation. Third, the AMS-Elisa was performed for retrospective investigations to confirm/falsify CDC-crossmatch results of the emergency duties. For different reasons 61 patients with these doubtful negative or false positive pre-transplant CDC-based crossmatch results were reinvestigated using the pre-transplant serum for the AMS-Elisa. From these 61 patients only 23 % (n=14) exhibited donor-specific antibodies in this system. Although the number of patients is not sufficient for statistical analyses there was a high degree of concordance (n=10 out of 14, > 70 %) between severe rejection episodes and/or loss of the kidney function due to clinically proven situations in accordance with a positive signal in the AMS-Elisa pointing to the importance of this group for the validation of the AMS-Elisa. Only two of the 47 patients lacking donor-specific anti-HLA antibodies using the AMS-Elisa suffered from a biopsy-proven rejection suggesting that these rejections may have been HLA independent. Apart from these two patients no biopsy-proven rejection was observable among the patients of the AMS-Elisa –negative group. Taken together the implementation of the AMS-crossmatch Elisa despite its considerably increased sensitivity does not necessarily lead to a decreased number of patients acceptable for transplantation. Quite in contrast due to its highly decreased susceptibility to sources of irritation more than 70 % of the patients with doubtful results in the conventional CDC-CM did not exhibit donor-specific anti-HLA antibodies (Figure 4). Thus, the use of novel crossmatch assays to substitute or at least to complement the standard CDC assay has increasingly been discussed during the last ten years. More than thirty years ago Ozturk and Terasaki reported that autoantibodies and immune complexes such as rheumatoid factors may lead to false positive results using the CDC-CM [67]. In analogy

cytotoxic autoantibodies were detectable in patients suffering from autoimmune diseases like systemic lupus erythematosus even without any previous alloimmunization. In this context twenty years later Sumitran-Holgersson [40] described false-positive CDC crossmatch reactions caused by autoantibodies and immune complexes as a frequent event. It is noteworthy that these autoantibodies do not necessarily belong to the IgM isotype class but represent lymphocytotoxic antibodies of the IgG isotype, which may also be generated during autoimmune-mediated diseases such as SLE. Generally dithioerythritol (DTE) as a reducing agent is used in HLA diagnostics to destroy antibodies of the IgM isotype with the aim to avoid the detection of autoantibodies. However, the selective destruction of antibodies of the IgM isotype does not necessarily exclude the detection of autoantibodies in CDC-based crossmatching although indeed autoantibodies often belong to the IgM class [40]. In parallel some studies concerning detrimental effects of anti-HLA alloantibodies of the IgM isotype on the survival of kidney and heart allografts have been published [68,69] demonstrating the need to detect and not to destroy alloantibodies of this isotype.

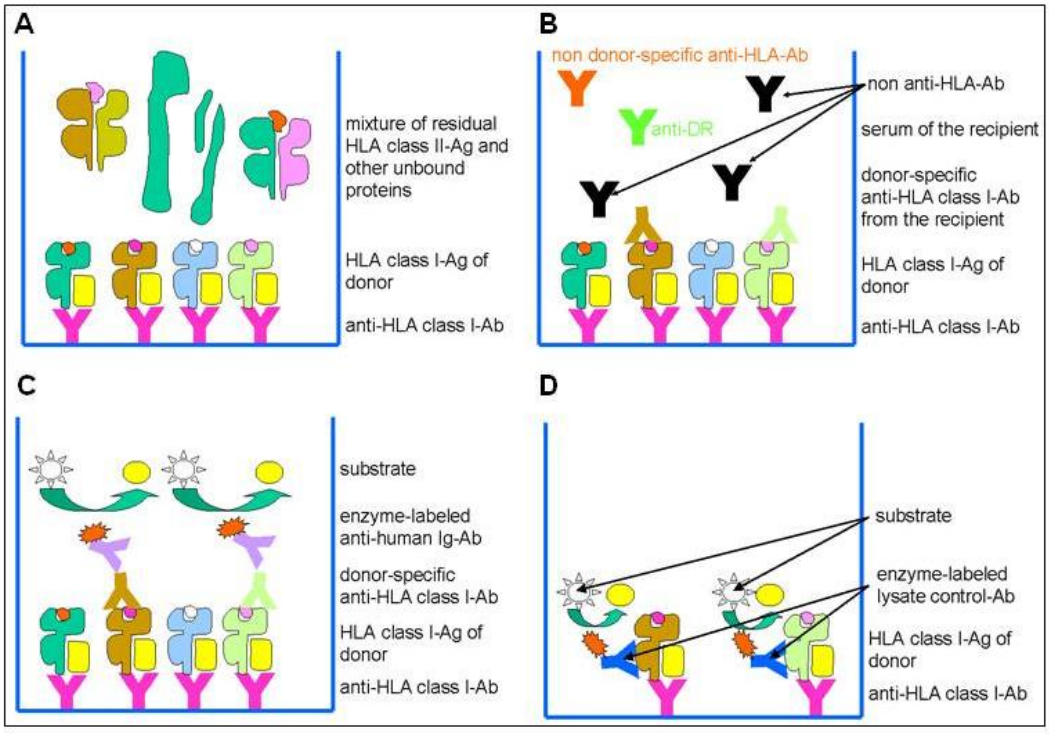

Figure 3. Flow diagram of the AMS-Elisa for the detection of HLA class I molecules. A Binding of the donor's solubilized HLA class I molecules by monoclonal capture antibodies recognizing a monomorphic epitope on HLA class I molecules. B Binding of the donor-specific anti-HLA antibodies out of the recipient's serum to the HLA molecules of the donor. C Binding of alkaline phosphatase-conjugated secondary antibodies to the recipient's bound donor-specific anti-HLA class I antibodies and subsequent colour reaction. The original protocol was modified by substituting the human IgG-specific by a human IgG/M/A-specific secondary antibody. D Lysate control using an alkaline phosphatase-conjugated monoclonal antibody directed against a second monomorphic epitope for detection to confirm the immobilization of a sufficient amount of HLA molecules by the solid phase-bound capture antibody. The AMS-Elisa variant for the identification of donor-specific antibodies directed against HLA class II molecules is designed correspondingly.

Mechanisms of Alloantibody-Mediated Rejections

The conventional CDC-CM exclusively identifies antibodies, which directly act against their target structures by means of complement-dependent cytotoxicity (cytotoxic antibodies). This test does not only identify antibodies specific for HLA target molecules, but also for other possible target structures. Although the main target of anti-HLA antibodies is the endothelium [70-72], the pathological processes leading to its destruction are known to be different: (i) The activation of the complement system via the classical pathway of activation by antigen-antibody complexes is involved which finally leads to the destruction of the endothelium by the membrane attack complex [73, 74]. (ii) Fragments as cleavage products of the complement system, the anaphylatoxins C5a and C3a, recruit inflammatory cells and mediate inflammatory reactions leading to vascular leakage, chemotaxis and activation of anaphylatoxin receptor-bearing leukocytes on the surface of the endothelial cells. (iii) Bound C3b and its degradation products iC3b and C3d are covalently bound to the membrane and act as opsonins which are recognized by the complement receptors CR1-CR3 differently expressed on leukocytes [74]. Thus; the detection of the deposited components of complement activation C3d and C4d in the capillaries of allografts [75-80] provides strong evidence for the involvement of this system after its activation/recruitment by an allogeneic humoral immune response. (iv) However, alternative mechanisms such as the antibody-dependent cell cytotoxicity (ADCC) exist, which may also lead to the damage of the endothelial target without any involvement of the complement system [81, 82]. In this context a pathological mechanism leading to artherosclerosis through an anti-HLA antibody-mediated induction of fibroblast growth factor receptor and the consecutive *in-vitro* proliferation of cultivated endothelial cells has been proposed [83]. Thus, detrimental secondary effects which are mediated through the binding of alloantibodies but act independently of the complement system can generally not be excluded. These complement-independent mechanisms of graft destructions are supported by Heinemann and co-workers who identified non-complement fixing antibodies of the IgG2 and IgG4 sub-isotypes in the eluates of about 28 % of 58 rejected kidneys [84]. In the same context the study of Smith et al. [52] investigating 565 cardiac transplant recipients demonstrated a pivotal effect of complement fixing donor-specific antibodies (DSA) resulting in a one-year graft survival of 20 %. The graft survival in patients with non-complement fixing DSA which was 54 % in contrast to 91 % in patients without DSA demonstrates that non-complement fixing antibodies also exert a negative effect by leading to a reduction in graft survival. It is noteworthy that Smith and co-workers for their investigations modified a Luminex-based assay additionally using human serum as source of C4d and a murine monoclonal anti-human C4d antibody for the detection of C4d covalently bound to the beads only in the presence of complement fixing antibodies.

Conclusion and Perspective

The screening of antibodies, directed against HLA molecules, is highly important for patients prior to or after allograft transplantations. The traditional CDC-based antibody screening or cross-matching developed as the “prototype technique” for the identification of anti-HLA antibodies in a given recipient was introduced into transplant clinics in the late

sixties [1, 85-87]. During the last 40 years this diagnostic procedure has strongly improved the quality of life for the transplant patients as hyperacute and acute rejections were efficiently reduced. In spite of additional major improvements in the field of immunosuppressive treatment allograft rejections remain a serious problem after the transplantation of kidneys and other solid organs if pre-formed donor-specific antibodies are not recognized by the CDC-based detection system. The Elisa techniques utilizing solid-phase-immobilized groups of HLA antigens or single antigens as well as microsphere-based assays have successfully been introduced by many tissue typing laboratories for the regular screening of patients' sera of the waiting list, which are stored in the laboratories. For the reasons discussed in this article any effort of the laboratories to complement or even exchange any CDC-based cell tray system by the novel systems (Elisa- , HLA-chip- or microsphere-based) should be supported. The general drawback that the identification of an antibody with a rare specificity is not possible by the one or other of the different technologies due to different sources of antigens and/or the proportions of single antigens as part of their immobilized groups must be accepted. Some of these differences may result from the masking of certain epitopes due to the immobilisation of HLA antigens to which the antibodies may have no access. However, diagnostic assays like the CDC-based screening trays which are completely unable to detect non-complement binding antibodies, which are characterized by low sensitivity and which are highly susceptible to false positive reactions in particular in the presence of certain accompanying diseases or medical treatment [65,66,88] are, in comparison to the alternative solid phase-based assays, much more harmful for the patients when compared to the disadvantages of the novel solid phase assays.

The problems described for the diagnostic disadvantages using CDC-based screening cell trays to identify antigen-specificities hold as well true for the pre-transplant crossmatch assays performed to identify donor-specific antibodies by using cellular material of the prospective donor. With the availability of the novel CM-Elisa assays in 2004 it was for the first time feasible to adequately substitute these CDC-based crossmatch assays. For the reasons discussed above the AMS crossmatch Elisa represents a sensitive and reliable tool with striking advantages over the classical CDC-crossmatch thereby clearly improving the diagnostic outcome for the recipients as recently highlighted for three groups of patients under medical treatment or suffering from underlying diseases [88]. At the moment the general substitution of the CDC-crossmatch is limited for technical reasons because the stored amount of serum of a potential recipient of the waiting list in many cases does not exceed 50 µl. Using the current variant of the Micro-AMS-Elisa with miniaturized wells, at least 30 µl of serum are required for the detection of both anti-HLA class I and class II antibodies. However, its adaptation to a novel "micro format" would allow to overcome this problem as the volume needed for the AMS-Elisa would be in the range of that required for the conventional CDC-based assay with separated lymphocytes (3 µl of each serum). Another disadvantage is the time span of about 4 to 5 hours required for the AMS-Elisa in contrast to the conventional CDC-CM which needs about 2.5 to 3 hours. However, this initial problem has been solved by optimizing the procedure which can be finished now in about 3.5 hours. Most probably the minimization of the assay by the implementation of the "Terasaki plate format" would lead to a further shortening of the incubation times which would be congruent or even shorter than that of the conventional CDC-CM. In conclusion the data reviewed here strengthen the urgent requirement for a novel Elisa-based crossmatch procedure to complement the "classical" CDC-crossmatch which will not necessarily result in a decreased

number of acceptable grafts. About 70 % of the recipients with doubtful i.e. in most cases weakly positive CDC-CM results did not produce donor-specific antibodies. Thus, the patients may be deprived of organs they could get since the current standard method is much more susceptible to disruptive factors than the alternative Elisa-based procedure (Figure 4). Although further studies are required to finally evaluate the superiority of the Elisa-based crossmatch over the CDC-based method, based on more than 400 diagnostic runs we here postulate to comprehensively establish and legitimize the novel solid phase-based crossmatch method by the certifying societies.

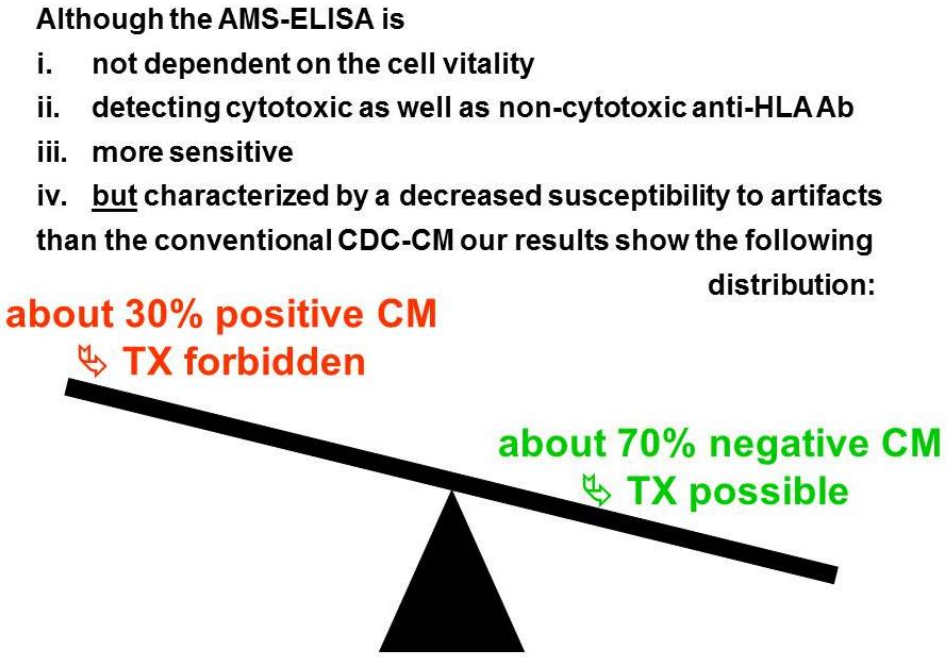

Figure 4. Diagram characterizing the outcome of doubtful results of the conventional CDC-crossmatch after their re-evaluation using the AMS-crossmatch Elisa. Using the AMS-Elisa more than 70 % of the doubtful conventional CDC-crossmatch results do unequivocally not exhibit donor-specific anti-HLA antibodies demonstrating its higher reliability and lower susceptibility to various sources of irritation.

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