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In-Depth Clinical Review

Novel solid phase-based ELISA assays contribute to an improved detection of anti-HLA antibodies and to an increased reliability of pre- and post-transplant crossmatching

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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, a standard crossmatch (CM) procedure, the complement-dependent cytotoxicity assay (CDC), was developed. This functional assay strongly depends on the availability of isolated vital lymphocytes of a given donor. However, the requirements of the donor's material may often not be fulfilled, so that the detection of the antibodies directed against HLA molecules is either impaired or becomes completely impossible. To circumvent the disadvantages of the CDC procedure, enzyme-linked immunosorbent assay (ELISA)-based and other solid phase-based ELISA-related techniques have been designed to reliably detect anti-HLA antibodies in 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.

Keywords: allograft; complement-dependent cytotoxicity assay; crossmatch; human leukocyte antigen; rejection

Introduction

More than 30 years ago, Patel and Terasaki described for the first time that antibodies, which are directed against antigens of donor tissues, are clearly associated with hyper-acute 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 hyper-acute 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 1960s. 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 cells/organs of the donor. Due to its low sensitivity, the CDC assay also fails to detect low antibody concentrations resulting in its modification by using secondary anti-human immuno-globulin antibodies [anti-human globulin (AHG)-enhanced CDC crossmatch] to recognize the primary donor-specific antibodies [4,5]. However, all variants of CDC assays depend on a high vitality of the donor cells and are hardly interpretable with cells exhibiting a vitality rate lower than 90%. As a consequence of these methodical disadvantages, novel methods independent of 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.

Methods established and modified for the identification of anti-HLA antibodies: their technical designs, advantages, disadvantages and limitations

Prior to transplantation, the CDC procedure is performed to (i) elucidate the general degree of pre-sensitization (antibody monitoring) or (ii) to identify actual donor-specific

antibodies against HLA phenotypes of a given donor (crossmatching). The general degree of pre-sensitization was originally 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 as well as the recipient 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, the PRA value does not unequivocally represent the identification of so-called donor-specific antibodies, which may occur also in patients with a low degree of PRA or antibodies directed against rare HLA phenotypes, which are not included in the panel. These donor-specific antibodies 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 that had been stored for several days to weeks.

The conventional complement-derived cytotoxicity 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. Despite 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 pro-

cedure is initiated with the isolation of 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 IgG/IgM 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 number of dead cells using a scoring 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. 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 centre. For these foreign donations, the 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 donations, and especially as a consequence of insufficient 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 weak 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 valuable results, the background of CDC-based assays should generally not exceed 10%.

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 only about 30% of PBL. Thus,
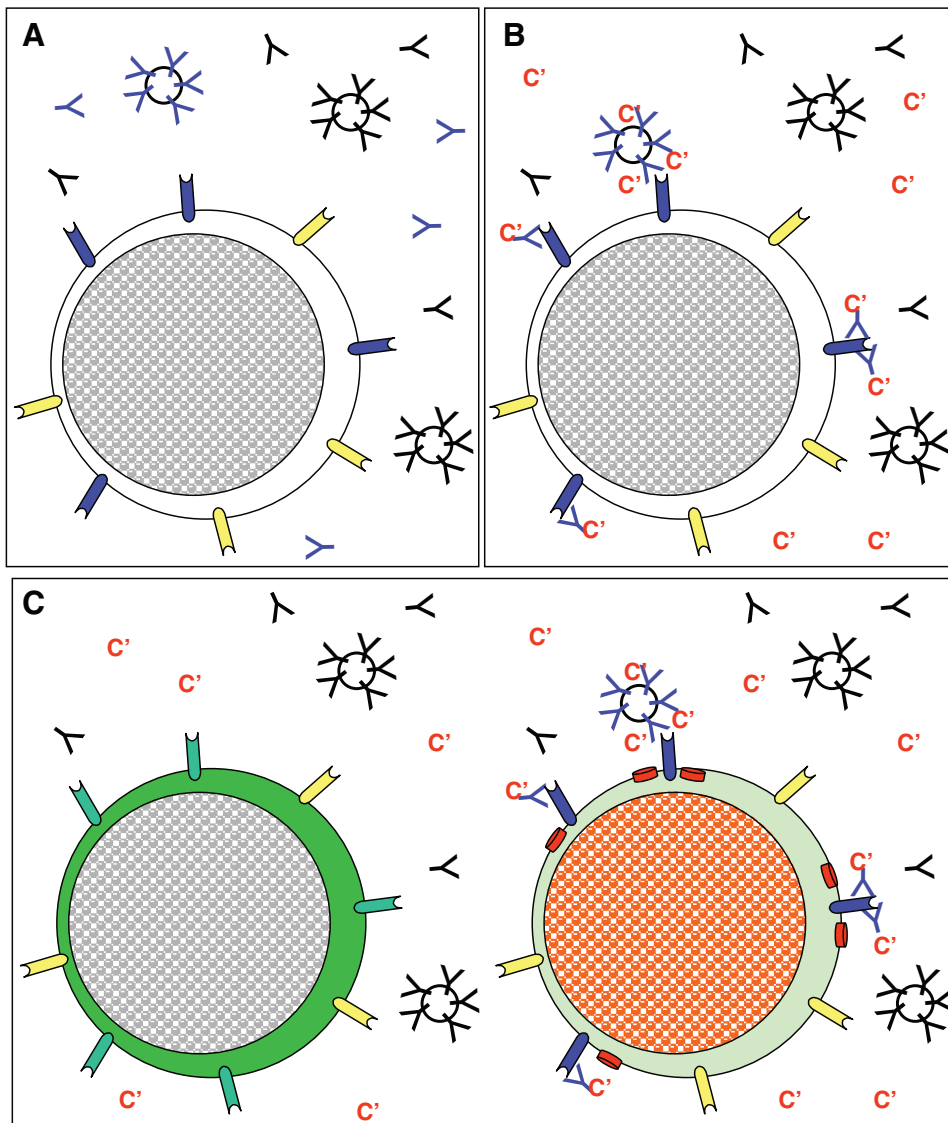
Fig. 1. Scheme of the classical CDC crossmatch as the current standard procedure for the detection of donor-specific antibodies. (**A**) Antibodies (monomeric IgG and pentameric IgM) are part of a recipient's serum and may recognize HLA molecules (blue and yellow) on donor's lymphocytes. (**B**) Activation 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 had not been lysed by the complement added. The red cylinders symbolize the Membrane Attack Complexes (MAC) as final complement activation products.

employing unseparated PBL, a strong anti-HLA class II reaction leading to the red (positive) staining of all class II antigen-bearing PBL never exceeds a weak positive reaction (score 4). 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-enhanced CDC-CM'. However, it is noteworthy that, in particular, this procedure is character-

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	Doubtful positive
4 = ++	20–40	Weakly positive
6 = +++	40–80	Positive
8 = ++++	80–100	Strongly positive

B-cell CM

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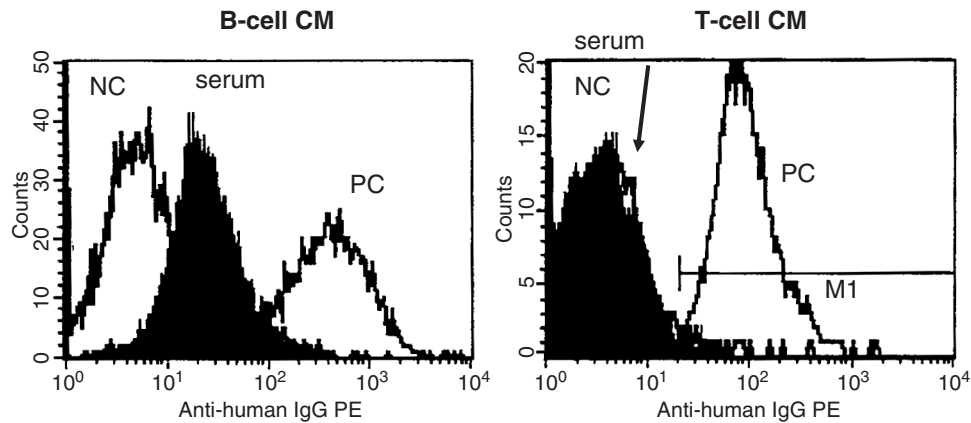
Fig. 2. FACS crossmatch of B cells and T cells as shown by their histograms. The FACS crossmatch of T cells is unequivocally negative, whereas the B cells provide a histogram of weakly increased intensity. Due to the faint intensity it does not lead to an interpretable result especially in the context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

ized 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.

No breakthrough of the flow cytometry crossmatch 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. even by the detection of 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 Fc receptors, which are highly expressed on B-lymphocytes 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 our own investigations illustrate a 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 with the negative control serum. However, the anti-HLA class II antibodies detected by this FACS-CM were not confirmed by the anti-HLA class II antibody screening ELISA using solid phase-coated HLA class II antigens (B-Screen-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 a common event rather than a rare one [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 [14–16]. 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. Very recently, a pronase-free B-cell flow cytometry crossmatch has been proposed using heat-inactivated rabbit serum [17], highly reducing the background caused by non-specific reactions but without the disadvantage of digesting additional cell surface proteins. This method, well known for immunohistochemical applications to block FCγ receptors, may have the capacity to overcome the problem of unspecific binding of antibodies through their Fc parts. Another striking disadvantage of this method in accordance with the CDC-CM is its dependence on the cell quality. Therefore, the development of novel methods which are partially or completely independent of 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.

Implementation of solid phase-based immunoassays for the detection of anti-HLA antibodies and their specificities (anti-HLA antibody screening ELISA)

In order to avoid problems which depend on the quality of cells, a solid phase immunoassay named FlowPRA™ was developed. In this assay, purified HLA class I or

class II molecules were immobilized on the surface of microparticles/beads. Besides 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 hyper-acute or acute rejections, general episodes of graft rejection, which required therapeutic interventions, occurred more frequently in the first group than in patients without detectable pre-transplant antibodies [19]. Furthermore, patients with anti-bodies 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 (0% PRA) defined by the AHG-CDC (cell panel tray) and 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 high 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 as well as the FACS-CM never reached a breakthrough in HLA antibody monitoring due to the alternative ELISA-based techniques, which are easy 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 15 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 microtitre 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 assays chosen by most manufacturers for the detection of 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 [13] 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 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 some inconsistencies exist 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 with the previously described results [13]. A third study of Uboldi de 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 ELISA/Sang Stat). Despite 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 with the conventional CDC-based procedure. The most expensive LAT-M kit, however, ascertained described to lead to the best results [32]. It is noteworthy that all ELISA assays are at least 10-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-CM presented by Uboldi de Capei and co-workers [32] are most probably to be regarded as non-realistic since the high storing costs for cells in liquid nitrogen have not been taken into consideration appropriately. Further-

more, 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 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 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, was 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.

Recently, the Luminex[TM] technology was successfully adapted to be used for the identification of HLA-specific antibodies. This microbead-based multiplex assay primarily employed for HLA typing by sequence-specific oligonucleotide (SSO) PCR allows the simultaneous detection of maximally 100 different analytes from one tube and apparently represents a valuable and reliable tool for kidney transplant risk stratification [48,49]. The evident advantages of the Luminex technology used for anti-HLA antibody screening have been reviewed in detail 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 differentiate between positive and negative patients (lowest level of resolution), followed by the level of single cells with each bead carrying two molecules derived from two alleles of each locus (HLA-A, -B and -C for class I) and HLA-DR and -DQ in the case of class II antigens. The highest level of resolution consists of beads with only one molecule immobilized (single-antigen Luminex assay). Patient 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 which in combination defines the specificity of the antibody 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 technology. Luminex technique identified 53 patients with anti-HLA antibodies in addition to 14 patients defined as positive (PRA > 5%) and 5 exhibiting donor-specific antibodies by CDC assays. 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 screening as the method chosen for the so-called virtual crossmatch [53]. In that study, solid phase-based assays, including the Luminex tech-

nique, exhibited a better prediction for a positive CDC-based crossmatch than CDC-based antibody screening 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 speculated to be 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 with the 'single antigen level of resolution' 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 crossmatch results and of 79% and 68% for CDC-based T- and B-cell flow crossmatch results, respectively, were reached. In their study [55], Vaidya and co-workers correlated anti-HLA antibodies 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 evidence was provided that patients who suffered from graft failures had higher titres of antibodies than patients with continuing graft functions. Thus, this method of data interpretation may result in the definition of clinically relevant titres 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 methodologies beyond CDC-based analyses.

However, not only the bead-based but also the solid phase-based technology has been improved during the last few years. In this context, the miniaturized chip technology was established for the analysis of anti-HLA antibodies (Invitrogen/Dynal, Bromborough, UK), named DynaChip[TM] HLA antibody analysis. This method combines the manageability of solid phase-based systems with the advantage of microarrays (high throughput because of multiple simultaneous measurements) and represents a completely automated system, which may be easily performed overnight (about 4 h/run). At the bottom of each well of a standard format 96-well plate, a glass microchip consisting of 138 incubation positions is fixed. Ninety-six of these positions are 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 serum is used per chip and well (i.e. per patient). Although the DynaChip [TM] assay is not a single-antigen assay, the combination of single-donor immobilized HLA class I ($n = 8$) or class II ($n = 10$) antigens, respectively, allows, in about 70% of the analyses, the identification of the specificity of a recipient's anti-HLA antibody. As a small study performed in our tissue typing laboratory, the quarterly screening of the kidney waiting list patients has been 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 using a cell panel composed in our laboratory was 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 screenings exhibiting an overall concordance of about 84% for anti-HLA class I (78% 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). Although these comparative investigations are still in progress, the DynaChip system appears to be superior to the old combined procedure because of its reliability and 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 allows the identification of non-complement-binding antibodies also relevant for graft failures. The concordance between both solid phase-based antibody detection systems Screening ELISA and DynaChip analysis was 88.2% for anti-HLA class I antibodies and 91.3% for anti-class II antibodies, respectively. Of special interest is that 6.5% of the sera were positive for anti-HLA class II antibodies only in the CDC- but not in the ELISA-based assays. First data provide evidence that the great majority of these patients (>75%) are characterized by autoimmune diseases but not by anti-HLA antibodies, although the analyses of the underlying diseases are ongoing. Thus, strong evidence is provided also by our laboratory that the CDC assay is artificially influenced by autoimmune diseases, which is discussed later in the context of the CDC-based crossmatch procedure.

Novel ELISA-based HLA crossmatch procedures developed for the direct detection of donor-specific antibodies

Recently, two ELISA-based assays have been established, which allow the detection of donor-specific antibodies independently from the specificity of the identified antibodies. These are the AbCross HLA class I/II ELISA (Biotest, Dreieich, Germany) and the Antibody Monitoring System (AMS) HLA class I/II ELISA (GTI diagnostics, Waukesha, USA) [58,59]. Both technologies allow the direct detection of donor-specific antibodies by immobilizing extracted donors' HLA molecules to pre-coated capture antibodies which are directed against a monomorphic structure 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). 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 pre-
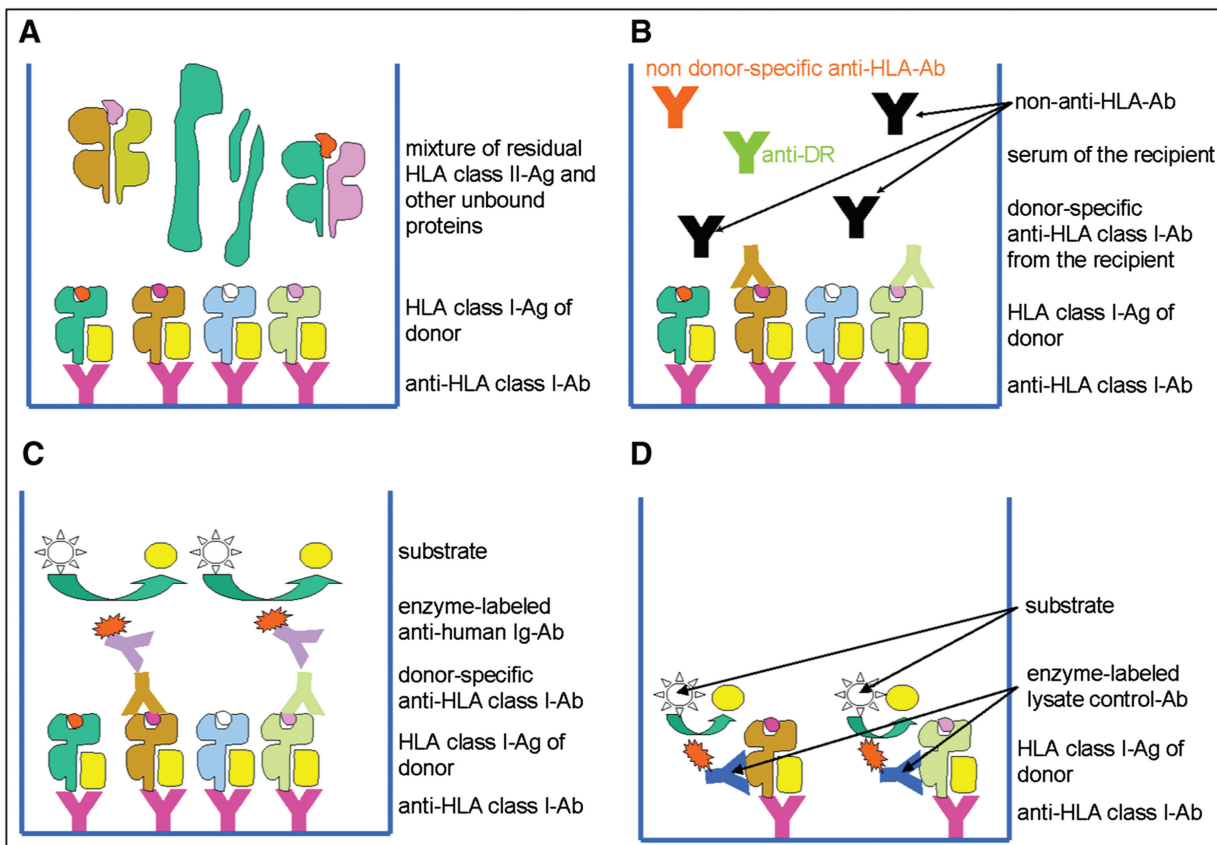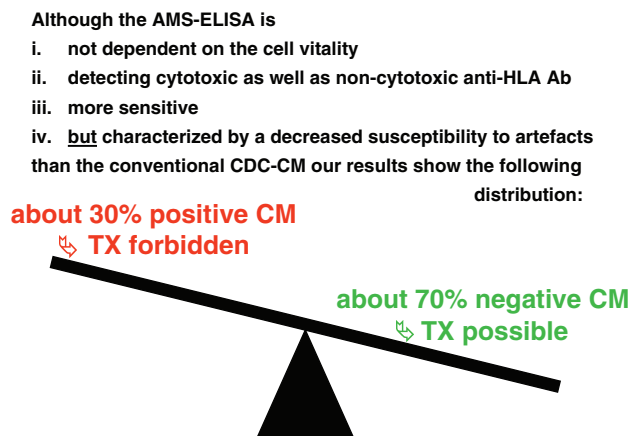
Fig. 3. Flow diagram of the AMS-ELISA for the detection of HLA class I molecules. (**A**) Binding of the donor's solubilized HLA class I molecules by monoclonal capture antibodies recognizing a monomorphic epitope on HLA class I molecules. (**B**) Binding of the donor-specific anti-HLA antibodies out 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.

coated 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 the 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 3 years ago and has been employed for more than 230 samples for which no alternative methods were available or which were characterized by special problems arising from the conventional CDC-CM procedure. Three main areas of crossmatching exist for which this assay is currently used. It is first used for retrospective cross-

matches after the transplantation of heart, lung and vessel allografts where no pre-transplant crossmatching is performed mainly due to the lack of time. As the material available from the donor's blood or spleen for these post-transplant crossmatches for logistical and organizational reasons which are not under the influence of the tissue typing laboratories is generally old (in many cases even older than 4 days), no 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, a crossmatch assay not requiring vital cells represents the only method to identify donor-specific antibodies from non-vital material. Second, the AMS-ELISA has been employed for prospective crossmatches of kidney living 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

Although the AMS-ELISA is

i.   not dependent on the cell vitality

ii. detecting cytotoxic as well as non-cytotoxic anti-HLA Ab

iii. more sensitive

iv. <u>but</u> characterized by a decreased susceptibility to artefacts

than the conventional CDC-CM our results show the following distribution:

about 30% positive CM
 ↳ **TX forbidden**

about 70% negative CM
 ↳ **TX possible**

Fig. 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 unequivocally do not exhibit donor-specific anti-HLA antibodies demonstrating its higher reliability and lower susceptibility to various sources of irritation.

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 with the conventional CDC-CM, patients appear to be devoid of donor-specific antibodies, thereby not providing a contra-indication for the prospective living donation. Third, for retrospective investigations, the AMS-ELISA was performed to confirm/falsify CDC-crossmatch results of the emergency duties. For different reasons, 33 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, only 27% ($n = 9$) 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 ($n = 7$ out of 9, $>70\%$) between the loss of kidney function due to clinically proven rejection episodes and a positive signal in the AMS-ELISA pointing to the importance of this group for the validation of the AMS-ELISA. From two patients positive in the AMS-ELISA, no clinical data were available. Only one of the patients lacking donor-specific anti-HLA antibodies using the AMS-ELISA ($n = 24$) suffered from a biopsy-proven rejection, suggesting that this reaction may have been HLA-independent. Apart from this patient, no final graft failure due to a hyper-acute or acute biopsy-proven rejection was observable in the AMS-ELISA-negative group, although the recipients observations are ongoing. 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. 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

10 years. Ozturk and Terasaki reported that autoantibodies and immune complexes such as rheumatoid factors may lead to false-positive results using the CDC-CM [60]. In analogy, cytotoxic autoantibodies were detectable in patients suffering from autoimmune diseases like systemic lupus erythematosus (SLE) even without previous alloimmunization. In this context, 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 alloantibodies of the IgM isotype on the survival of kidney and heart allografts have recently been published [61,62].

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). Thus, 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 [63–65], 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 [66,67]. (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 [67]. Thus; the detection of the deposited components of complement activation C3d and C4d in the capillaries of allografts [68–73] 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 [74,75]. 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 [76]. 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 destruction 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 [77]. 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 1-year graft survival of 20%. The graft survival in patients with non-complement-fixing DSA which was 54% and 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, in 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.

Conclusions 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 crossmatching developed as the 'prototype technique' for the identification of anti-HLA antibodies in a given recipient was introduced into transplant clinics in the late 1960s [1,78–80]. During the last 30 years, this diagnostic procedure has strongly improved the quality of life for the transplant patients as hyper-acute 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 when 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 been successfully introduced by many tissue typing laboratories for the regular screening of sera of patients on of the waiting list, which are stored in the laboratories. For the reasons discussed above, 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 certain specificity is not possible by any 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 immobilization 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, are, in comparison with the alternative solid phase-based assays, much more harmful for the patients when compared with the disadvantages of the novel assays.

The problems described for the diagnostic disadvantages using CDC-based screening cell trays to identify antigen specificities exist as well 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 [50]. 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 on the waiting list in many cases does not exceed 50 µL. Using the current variant of the AMS-ELISA with miniaturized wells, at least 30 µL of serum is required for the detection of both anti-HLA class I and class II antibodies. However, its adaptation to a novel 'micro format' would allow this problem to be overcome 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 was the time span of about 5 h required for the AMS-ELISA in contrast to the conventional CDC-CM which takes about 3 h. However, this initial problem has been solved by shortening the incubation steps and optimizing the procedure which can be completed now in about 3.5 h. 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 receive 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, we here postulate to comprehensively establish and legitimize the novel solid phase-based crossmatch method by the certifying societies.

Conflict of interest statement. None declared.

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