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Review

Solid phase-based cross-matching for solid organ transplantation: Currently out-of-stock but urgently required for improved allograft outcome

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Summary. Transplant recipients who have undergone sensitizing events, such as pregnancy, blood transfusion or previous transplants, frequently develop antibodies directed against the highly polymorphous human leukocyte antigen (HLA)-molecules. These pre-formed, donor-specific antibodies (DSA) present a high risk of causing organ failure or even complete loss of the grafted organ as a consequence of antibody-mediated, hyper-acute or acute allograft rejection. In order to detect DSA, the so-called functional complement-dependent lymphocytotoxicity assay (CDC-XM) was established about 50 years ago. Although effective in improving the outcome of solid organ allo-grafting, for the last ten years this assay has been controversially discussed due to its low sensitivity and especially because of its high susceptibility to various artificial factors, which generally do not yield reliable results. As a consequence, novel immunochemical test systems have been developed using ELISA- or bead-based solid phase assays as replacements for the traditional CDC-based assays. Because these assays are independent of single or vital cells, which are frequently not available, they have provided an additional and alternative diagnostic approach compared with the traditional CDC-based and flow-cytometric analyses. Unfortunately, however, the AMS-ELISA (Antibody Monitoring System), which was the first system to become commercially available, was recently discontinued by the manufacturer after seven years of successful use. Alternative procedures, such as the AbCross-ELISA, had to be either considerably modified, or did not yield reliable results, as in the case of the Luminex-based assay termed DSA. We draw the conclusion that due to the unique features and fields of application reviewed here, the implementation of solid phase cross-matching still represents an urgent requirement for any HLA-laboratory's routine tasks.

Key words: Allograft, Crossmatch (XM), Donor-specific antibodies (DSA), Human leukocyte antigen (HLA), Rejection

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Benefits and drawbacks of the conventional crossmatch procedures: Complement-dependent lymphocytotoxicity (CDC-) and flow-cytometric crossmatch assays

It has been known for about 50 years that antibodies which are directed against antigens of donor tissue represent the main reason for hyper-acute and acute rejections of renal allografts, as well as of other organ allografts (Patel and Terasaki, 1969). Numerous subsequent studies have shown that these deleterious antibodies were primarily directed against antigens of the human major histocompatibility complex (MHC), the so-called human leukocyte antigens (HLA) (Ahern et al., 1982; Chapman et al., 1986). These donor-specific anti-HLA antibodies (DSA) are thus regarded as a contra-indication for grafting as defined by the

transplantation guidelines of most countries and supranational societies (e.g. Eurotransplant Foundation), which supervise the allocation of kidneys and other solid organs.

The so-called crossmatch (XM-) procedure was developed in the late 1960s to detect antibodies in a given recipient's serum that reacted with lymphocytes isolated from the donor's blood (Patel and Terasaki, 1969). Even today, a negative crossmatch outcome in such a test is regarded as the best predictor for short-term survival of renal allografts. The standard technique established originally was the complement-dependent lymphocytotoxicity (CDC) assay and the work-flow of this assay has been described in detail previously (Altermann et al., 2006). Using this functional vitality assay, only those DSA were detectable that exert their detrimental function by their complement-activating features, ultimately leading to the lysis of donor lymphocytes. Additional DSA including isotypes other than IgM, IgG1 and IgG3 without complement-fixing activity are not detectable by this method, although these can be just as detrimental for donor tissues/organs. The second drawback of this assay is its low sensitivity leading to its general inability to detect low concentrations of DSA. Consequently, the CDC was modified by introducing secondary anti-human immunoglobulin (Ig) antibodies, which recognize the primary DSA, thereby supplementing this assay's technical design in order to enhance its complement-activating potency (Gebel and Bray, 2000; Karpinski et al., 2001; Altermann et al., 2006). The procedure was termed anti-human globulin- (AHG-) enhanced CDC-XM. The additional incubation time required in this assay was a "stress factor" that sometimes could lead to a higher background and possibly to uninterpretable results due to a high rate of artificially induced cell death. This was especially a problem when using donors' cells pre-damaged during inadequate storage conditions and extended storage times prior to the CDC-XM.

In order to circumvent some of the CDC-XM-specific problems the flow-cytometric crossmatch (FACS-XM) was established, which allowed the detection of complement-activating as well as complement-independent DSA (Lobo et al., 1981; Garovoy et al., 1983; Scornik et al., 1994, 1997; Bittencourt et al., 1998; Christiaans et al., 1998; Karpinski et al., 2001; Altermann et al., 2006). Its sensitivity is in the range of the AHG-enhanced CDC-crossmatch. Frequently, controversial results have been published concerning the significance of FACS-based cross-matching. For example, it was demonstrated that a positive outcome does not necessarily correlate with an increased number of rejection episodes (Kerman et al., 1999; Lobashevsky et al., 2000) or it was also shown that alloantibodies, which do not activate the complement system, were actually associated with an increased number of rejections, despite the corresponding CDC-XM results showing negative outcomes (Scornik et al., 1994; Scornik, 1995). These discrepancies, however, were most probably due to a pivotal problem regarding signal-specificity, which, even today is still a problem with this assay. In particular, the FACS-XM of isolated B-cells is frequently influenced by artefacts due to the "irrelevant/unspecific" binding of antibodies through their Fc-domains to the Fc-receptors expressed on this cell type ultimately leading to false positive results. In contrast to T-cells to which no binding of DSA was demonstrable, our own investigations have shown that the B-cell crossmatch is often characterized by a positive signal due to a shift in the FACS-histogram (Altermann et al., 2006). It is noteworthy, however, that most of these positive flow-cytometric B-cell crossmatch outcomes were neither confirmed virtually, i.e. by identifying the respective antibodies through the use of corresponding anti-HLA class II screen and specification assays, nor were they confirmed by conventional CDC-cross-matching using isolated B-cells, suggesting that the problem represents a common rather than a rare event (Altermann et al., 2006; Delgado and Eckels, 2008).

The approach to pre-digest lymphocytes with the enzyme-mixture pronase in order to increase the specificity of DSA binding was introduced by some groups (Lobo et al., 1995; Vaidya et al., 2001a) but the specificity-enhancing effects were not shown to be reproducible by several other groups, including our own laboratory, and did not perform as well as the FACS-based cross-matching approach. The theoretical benefit of using pronase was doubtful from the beginning, as this is not a single protease with defined proteolytic cleavage sites, such as pepsin, but a complex mixture of non-specific bacterial proteases, which is isolated from the extracellular fluid of *Streptomyces griseus*. Its activity extends to both denatured and native proteins leading to almost complete digestion to the single amino acid level. Due to this lack of specificity, the general reproducibility of this procedure was strongly challenged, particularly as a consequence of the pronase-mediated loss of Fc-receptors and of HLA-molecules. Indeed, the findings indicated that Fc-receptors are not the primary target of pronase treatment, as was recently summarized (Brown et al., 2017). Furthermore, Fc-receptors and HLA molecules both belong to the immunoglobulin superfamily, which indicates structural homologies. This was contrary to the claims of initial publications, all of which originated from two groups (Lobo et al., 1995, 1997, 2002; Vaidya et al., 2001a,b; Bearden et al., 2004) and highlighted the benefit of using pronase. Meanwhile, nearly all of the more recently published studies have clearly demonstrated the limits, including frequent false-positive outcomes, as a consequence of this enzymatic pre-treatment (Hetrick et al., 2011; Park et al., 2012; Hart et al., 2015; Szewczyk et al., 2016; Brown et al., 2017; Alheim et al., 2018). It must be concluded, therefore, that a standardized protocol has not been developed for this procedure to date, suggesting that flow-cytometry-based cross-matching has not yet obtained the general

acceptance necessary to substitute for, or routinely supplement, the CDC-XM. For this reason, only a few tissue-typing laboratories (especially among those of the Eurotransplant community) actually employ this procedure.

About ten years ago, an alternative blocking procedure was adapted to flow-cytometry-based cross-matching which already had gained broad recognition by avoiding artefacts and consequently led to improved outcomes in immunohistochemical applications (Hajeer et al., 2009). Hajeer and co-workers used heat-denatured rabbit serum in order to reduce the background caused by non-specific IgG-binding through its Fc-domain. In this context it is noteworthy that several suppliers of flow-cytometric diagnostic equipment have already for a number of years offered commercial kits in order to block Fc-receptors expressed on target cells or target tissues using the same functional principle as in the context of cross-matching first published by Hajeer and co-workers. Generally, it is puzzling that this otherwise well-established procedure was not introduced earlier into this field of transplantation diagnostics in order to replace the use of pronase, which for the reasons discussed above seems to be rather a pseudo-scientific approach.

In spite of this technical advance, a striking disadvantage of the FACS-XM, which is also in complete accord with the CDC-XM, is the general dependence on a high cell quality, i.e. on a high degree of vital cells, in both assays. As these requirements are often not fulfilled by the donors' samples, novel solid-phase-based crossmatch systems were developed, which, in addition to other benefits, worked independently of cell quality. Furthermore, we could show that, based on their technical design, solid-phase-based crossmatch assays extend the fields of applications, which are generally not feasible through the use of both classical cellular crossmatch assays, CDC-XM and FACS-XM.

Technical benefits lead to valid and reliable diagnostic outcomes of solid-phase cross-matching not attainable by CDC-based standard procedure

In the last 15 years, four different solid-phase-based crossmatch assays have become commercially available, three of which are based on the technical design of an ELISA and one as a Luminex-based assay. They all have been exhaustively tested in our laboratory between 2005 and 2019. As shown below, the first highly efficient ELISA-based assay, which served as a valuable tool in order to find adequate solutions for many patients, was replaced by consecutive assay-based systems of increasing levels of inadequacy. This has led to the current unsatisfactory situation that no solid-phase crossmatch system of sufficient validity is available. Thus, many of the diagnostic approaches discussed below are generally no longer feasible and cannot be carried out.

Solid-phase-based cross-matching as a solution for allograft recipients pretreated with therapeutic antibodies or cytostatic agents

Solid-phase-based cross-matching was first described in the year 2005 in the context of CDC-based crossmatch interferences through the use of therapeutic humanized monoclonal antibodies (Book et al., 2005). Book and co-workers investigated crossmatch outcomes using CDC- and flow-cytometry-based techniques, which were either completely or partially influenced by the recipients' sera after application of rituximab (anti-CD20), basiliximab/Simulect (anti-CD25) and alemtuzumab/Campath (anti-CD52). The authors confirmed previous observations regarding the artificially positive outcomes of CDC- as well as flow-cytometric cross-matching after the administration of alemtuzumab/Campath (Lyon et al., 2001; Wagenknecht et al., 2004). Book and co-workers in their ground-breaking study first systematically described an ELISA-based crossmatch assay as a suitable tool to circumvent the falsifying influence of applied therapeutic antibodies. As the assay was termed Transplant Monitoring System-(TMS-) ELISA (formerly GTI, Waukesha, USA) we were unaware of this group's investigations until 2013 when we serendipitously discovered their publication. Thus, using the down-scaled second-generation system termed Micro Antibody Monitoring System-(MicroAMS-) ELISA (GTI, Waukesha, USA; FDA-No. BK060038 awarded on 26th of June, 2006; later Immucor, Stamford, USA) the idea to implement a solid-phase-based crossmatch assay in the context of rituximab-induced AB0-bloodgroup incompatible living kidney donations arose independently in our laboratory and was initiated in 2006 (Schlaf et al., 2012a,b, 2014a). A scheme of this assay's workflow is given in Fig. 1. Briefly, a detergent-treated lysate of a given donor's tissue sample is pipetted into the wells of ELISA strips pre-coated with mAb, which are directed against monomorphic epitopes of HLA-class I or class II molecules (Fig. 1A). After consecutive washing steps the wells are incubated with the sera of the recipients under investigation. These sera may contain the donor-specific antibodies to be detected (red arrow) as they serve as detection antibodies in this sandwich assay by recognizing the immobilized donors' HLA molecules (Fig. 1B). After additional washing steps, the samples in the wells are then incubated with secondary alkaline phosphatase-conjugated anti-human IgG antibodies in order to recognize the immobilized donor-specific antibodies (Fig. 1C). Early in the development of the procedure we modified this final incubation step by using secondary antibodies directed against IgG/M/A isotypes of the primary DSA, as these isotypes are known to be relevant in the context of allograft rejection (Arnold et al., 2008, 2013). Furthermore, due to their complement-activating feature DSA of the IgM isotype are particularly readily detected using the CDC-based crossmatch but not using standard commercial IgG-

specific antibody detection or identification assays. In order to validate the data three controls are included: i) the Lysate Control Reagents (LCR) consisting of a second enzyme-labelled mAb for the detection of immobilized HLA class I or class II molecules by recognizing a second monomorphic epitope on the bound HLA-molecules. It is important to note that a sufficient amount of immobilized HLA-molecules is a prerequisite to obtain a clear positive signal and this is provided by this approach (Fig. 1D). ii) An additional positive control in which the reagents used are tested (not shown), consisted of adding freeze-dried control lymphocytes and serum samples that are positive for anti-HLA class I or class II antibodies, and these are components of the kit. After rehydration of the cell pellet and its application as antigen source this control demonstrates the functionality of the reagents provided by the supplier, even if the preparation of the individual donors' tissues is inadequate. iii) A negative control, which corresponds to the positive reagent control, with the difference that an irrelevant serum negative for HLA-antigens is used. The value of the recipient's serum under investigation had to exceed two-fold the value of the negative control to be classified as positive. Taken together, the network of controls in all cases under investigation allowed categorization of the raw data as valid or not and additionally pointed to the sources of any errors.

Until 2012, all four kidney transplant centers for which we put the prior-to-transplant crossmatches into practice participated in AB0-bloodgroup incompatible living kidney donations. Consequently, all of the recipients were pre-conditioned with anti-CD20 rituximab always leading to highly positive NIH-derived scores of 6 to 8 for CDC-cross-matching with isolated B-cells and scores between 2 and 4 with PBL, depending on individually varying fractions of B-cells. Thus, in all cases described the complement-activating potency of rituximab, which belongs to the IgG1 isotype finally leading to its B-cell-depleting activity, was always monitored instead of donor-specific antibodies. Gatault and co-workers confirmed these investigations by their observation that rituximab, even at low concentrations (i.e. inferior to 1μg/ml), has the potential to falsify B-

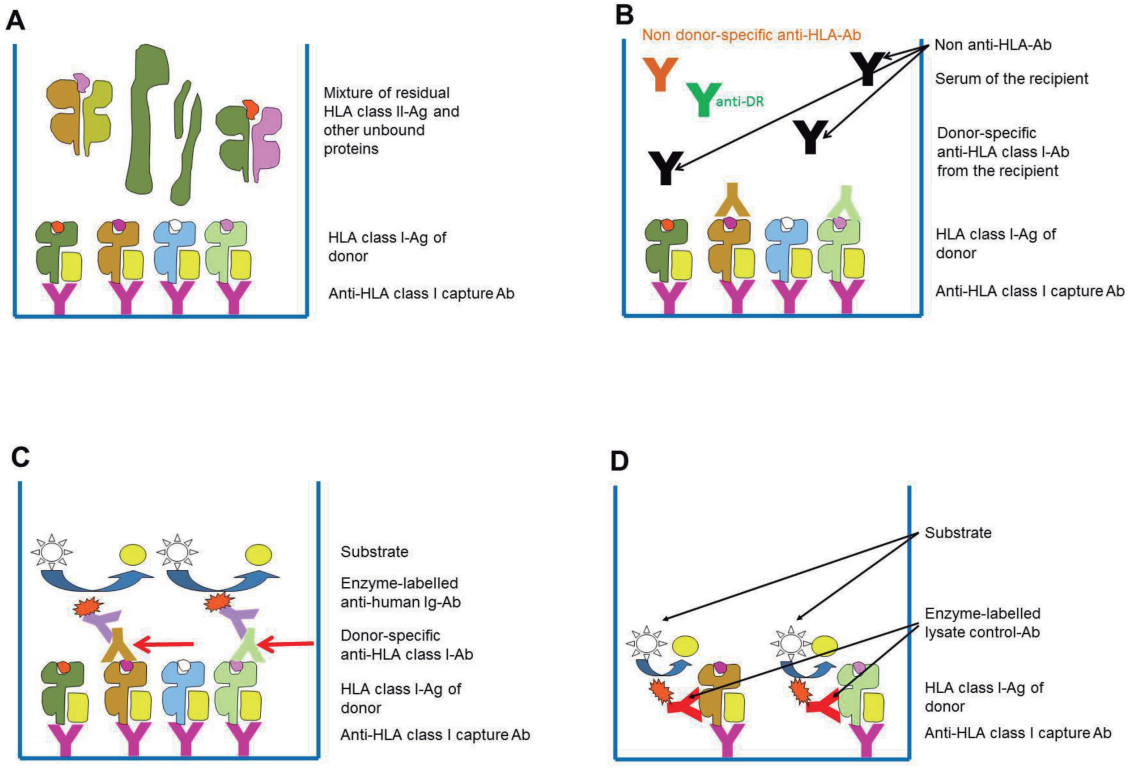

Fig. 1. Flow-chart of the AMS-ELISA for the detection of donor-specific HLA class I molecules (lysate procedure). **A.** Binding of the donor's solubilized HLA class I molecules by monoclonal capture antibodies that recognize a monomorphic epitope on HLA class I molecules. **B.** Binding of the donor-specific anti-HLA antibodies (red arrows) to be detected from 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 color 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 as detection antibody in order to confirm the immobilization of a sufficient amount of HLA molecules by the solid phase-bound capture antibody. The AMS-ELISA variant for the detection of donor-specific antibodies directed against HLA class II molecules is designed in a similar manner.

cell cross-matching over the broad window of 30-120 days after treatment prior to transplantation. In two case reports data were first provided on a dose-effect relationship leading to positive B-cell crossmatch outcomes up to about 80 days after the last application of this antibody (Gatault et al., 2013).

Furthermore, an unexpected positive B-cell crossmatch was reported as a consequence of rituximab treatment in the context of severe idiopathic thrombocytopenic purpura of a given donor 12 days prior to organ harvesting (Desoutter et al., 2016). Thus, the authors presented a case allowing no other conclusion than the treatment of a given donor with rituximab antibodies also can lead to artificial CDC-based crossmatch outcomes; these data were not supported by any other reference investigation, such as the identification of DSA by virtual cross-matching.

This aspect of false-positive cross-matching was also observed for the therapeutic anti-CD25 antibody basiliximab/Simulect. Basiliximab is directed against the alpha-chain of the interleukin 2 receptor (CD25) and was found to influence the results of CDC-based cross-matching more or less evenly in all three cell populations under investigation leading to an artificial CDC-XM score of about 4 (Schlaf et al., 2012a,b, 2014a; Schlaf and Altermann, 2017).

It is noteworthy that false-positive crossmatch outcomes, even though usually at higher concentrations, also are observed for flow-cytometric cross-matching when performed after the application of therapeutic antibodies, as was shown for alemtuzumab (campath) (anti-CD52), rituximab (anti-CD20) and daclizumab (basiliximab) (anti-CD25) (Book et al., 2005; Guillaume, 2018). In all cases, however, the alternative ELISA-based crossmatch assay (TMS-ELISA) was never affected by any therapeutic antibody, clearly indicating the general advantage of using cell-free solid-phase systems over any cellular, i.e. CDC- or FACS-based, crossmatch procedure (Book et al., 2005).

Patients suffering from various forms of leukemia, and who are, therefore, destined for allogeneic stem cell transplantation, generally do not have to fulfill a negative crossmatch as a given recipient and her/his chosen donor must be HLA-identical at the high resolution/four-digit level of resolution. However, in the past few years we have increasingly been receiving recipients who: i) had been treated by thrombocyte donations as a consequence of their anti-leukemia therapy; and ii) due to a lack of HLA-compatible stem cell donors were destined for so-called haploidentical stem cell donations e.g. arranged between parents and their children or siblings exhibiting this decreased degree of HLA-compatibility. Such configurations may lead to the detection of DSA representing a contra-indication for the intended stem cell transfer and the requirement of a crossmatch to exclude them. In nearly all of these situations, CDC-cross-matching did not allow the exclusion of DSA, as all results (PBL, separated T- and B-cells) were doubtfully (score of 2) or weakly (score of 4) positive. We concluded that the positive reactions were due to an unspecific cell death induced by the cytostatic agent 6-mercaptopurine used for the anti-leukemia therapy. The findings of the alternatively performed AMS-ELISA, however, were in complete accord with the corresponding virtual cross-matching and in no case exhibited DSA (Schlaf et al., 2012a).

Solid-phase-based cross-matching as a solution for allograft recipients suffering from underlying autoimmune (immune-complex) diseases

In addition to the invalid outcomes of tests that can occur in the presence of therapeutic antibodies or certain drugs, the requirement to substitute, or at least to complement, the CDC-based standard crossmatch has increasingly been discussed over the last 10-12 years. In particular, false-positive or -negative results using this assay can be induced by the presence of immune complexes in prospective recipients. These are particularly readily detectable in patients who suffer from underlying autoimmune diseases, mainly of the immune-complex type (type III). Immune complexes represent the first known sources of interference that frequently lead to invalid CDC-crossmatch outcomes. Notably, in an early study Ozturk and Terasaki reported false-positive CDC-crossmatch outcomes as a result of auto-antibodies and immune complexes such as rheumatic factors (Ozturk and Terasaki, 1980). These cytotoxic factors were at that time detected in patients suffering from autoimmune diseases such as Systemic Lupus Erythematosus (SLE) without any previous allo-immunization. Subsequently, Sumitran-Holgersson described the frequent occurrence of artificially positive outcomes of CDC-based cross-matching as a consequence of auto-antibodies and immune complexes (cytotoxic factors) (Sumitran-Holgersson, 2001). The approach to avoid these diagnostic artefacts through the use of reducing agents such as dithiothreitol/dithioerythritol (DTT/DTE), although widely accepted in the Eurotransplant community due to their selective destruction of auto-antibodies of the IgM-isotype, has been regarded for many years as ineffective. Sumitran-Holgersson first described that autoantibodies generated during autoimmune diseases such as SLE do not necessarily belong to the IgM isotype but may also belong to the complement-fixing (sub-) isotypes IgG1 and IgG3 (Sumitran-Holgersson, 2001). Additionally, studies exist which refer to detrimental effects of HLA-specific alloantibodies of the IgM-isotype, thus clearly highlighting the diagnostic value not to destroy them using reducing agents but to detect them (Vaidya and Ruth, 1989; Stastny et al., 2009). Unfortunately, these IgM alloantibodies, along with so-called weak (low titer) IgG alloantibodies, can be eliminated by DTE/DTT, despite still being readily detectable using solid-phase crossmatch techniques modified with secondary antibodies recognizing IgG, IgM and IgA isotypes of the

primary DSA. Thus, as mentioned above we also introduced this modification into the workflow of the AMS-ELISA as early as 2006 (Altermann et al., 2006; Schlaf et al., 2012a). Based on the above arguments, the general diagnostic approach of using reducing agents to selectively eliminate autoantibodies and to specify HLA-specific alloantibodies has been challenged for several years. It must be concluded that the application of reducing agents, as with the use of pronase in order to selectively digest Fc-receptors in the context of flow cytometry cross-matching, must be regarded as a non-scientific practice rather than an approach based on the current state of scientific and technical knowledge.

To date, autoimmune diseases, and especially those of the immune complex type (type III), represent the most prominent disruptive factor for CDC-based cross-matching leading to artificially positive outcomes. Consequently, these artefacts lead to increasing numbers of patients on the waiting lists for kidney allografts. On the one hand, immune complex diseases are a frequent reason for end-stage renal failure, but on the other hand, the underlying disease-based artificially positive outcomes of CDC-based cross-matching result in this test system being a major hindrance to the allocation of a kidney allograft. In this context, we published the findings of several investigations that displayed ELISA-based cross-matching as an adequate alternative in order to validly indicate DSA and not disease-based interfering factors (Schlaf et al., 2012a, 2013, 2014b, 2016). In spite of this long-standing knowledge and our experience, which we have gained over several years up to 2010, the approach to circumvent artificial CDC-based crossmatch results by considering the alternative valid solid-phase-based crossmatch results was only followed twice in the years 2009 and 2010 for legal reasons (Schlaf et al., 2013, 2016). Strongly influenced by some Eurotransplant authorities at that time, the German Federal Medical Association in December 2010 defined the CDC-based crossmatch as the only procedure accredited for the allocation of post-mortem kidney donations, whereas prior to this amendment the former guidelines only claimed to "exclude the existence of cytotoxic donor-specific anti-HLA antibodies" without any methodical determination. Thus, the AMS-ELISA, which was successfully used in order to exclude/detect both cytotoxic and non-cytotoxic DSA (Schlaf et al., 2013, 2016) and despite validly indicating the correct grafting in these two reported cases, is no longer permitted. Consequently, we immediately stopped using this approach and worked in accordance with the updated guidelines, even though they are ineffective in terms of their basis of the current state of knowledge in immunology and may even be potentially harmful to the patients. The increase in the number of positive CDC-based crossmatch assays led to the consequent accumulation of patients with the respective underlying diseases from 6.5% in 2008 to currently at least double this value (Altermann and Schlaf, 2010; Schlaf et al., 2014c). The increasing number of patients who fail CDC-based crossmatches and consequently do not receive offered kidney allografts merely because of their underlying autoimmune diseases, and not due to DSA, should be the rationale to stop the erroneous methodical determination imposed in 2010 and to accept additional solid-phase-based cross-matching in this context. In view of this drawback of CDC-based cross-matching, coupled with the CDC-based antibody specifications using cell tray analyses, both the decision of the German Federal Medical Association and the corresponding publication of a Eurotransplant group highlighting the CDC as the leading procedure to define highly sensitized patients must be regarded as wrong and completely ineffective (Doxiadis et al., 2010).

Over the last nine years we have used solid-phase-based cross-matching, several times and highly successfully, as a diagnostic approach for living kidney donations in addition to the CDC-based crossmatch outcomes, which were mainly due to underlying autoimmune diseases. Until now this has been possible because the CDC-based crossmatch restrictions do not hold true for accepting or refusing living kidney allografts. New guidelines are currently being prepared by the German Federal Medical Association, which hopefully will not lead to the prohibition of solid-phase cross-matching in this additional context. Furthermore, we have used it as a reference procedure in order to identify false-positive CDC-outcomes, which may be the result of temporary autoimmune attacks. These attacks in some cases ease after their initial clinical appearance and thus lead to some attack-accompanying sera, which should be identified and rejected for upcoming CDC-crossmatches. Thus, by defining the false-positive sera to be rejected we were frequently able to accept kidney offers for patients that would otherwise have been refused due to exclusive artificially false CDC-based outcomes (Schlaf et al., 2014b). The problem of adequate kidney allocations, however, under the current guidelines remains irresolvable for prospective recipients who have consistently, or over a long period, generated false-positive CDC-XM sensitive sera and for patients on waiting lists who present for false-positive cytotoxic factors in addition to anti-HLA antibodies. Without doubt, these numbers on waiting lists will continue to increase if solid-phase cross-matching does not become available as a legitimate procedure (Schlaf et al., 2016).

Solid-phase cross-matching using acellular donor tissue such as corneal material, arterial vessel allografts or stored donors' cell lysates lacking single vital cells

Due to the fact that for corneal allografting neither single nor vital cells are available for preparing detergent lysates of donor tissue, we used the outer scleral rim of cornea donors, which is generally available as a retained sample after the excision of the inner part used for allografting. Although this tissue is very poor in cells, and these are self-evidently not isolatable as a

suspension of vital cells, the outer scleral rims actually serve as adequate material for the Micro-AMS-ELISA in order to explain preceding or to predict forthcoming corneal rejections due to detected DSA (Altermann et al., 2006; Sel et al., 2012). The same argument proved valid for arterial vessel allografts as their tissue fragments used as donor material are characterized by very similar features. Thus, the Micro AMS-ELISA was also used by us in order to detect DSA directed against HLA-antigens of fresh or frozen arterial allografts (unpublished data).

Furthermore, we provided the first evidence that solid-phase-based cross-matching is suitable to demonstrate an upcoming humoral immune reaction as indicated by DSA. Deep-frozen leukocyte pellets from blood or, due to its increased portion of HLA class II-expressing B cells superior to blood-derived leukocytes, especially spleen-derived leukocyte pellets were successfully used as donor materials (Schlaf et al., 2015a,b). This approach was followed using leukocyte pellets, which had been deep-frozen up to 4.5 years and most probably provides the feasibility to use cellular material of post mortem organ donors stored for more than 10 years. Thus, this ELISA-based procedure provides the option to routinely perform *de facto* cross-matching using deep-frozen samples from deceased donors in addition to virtual cross-matching; in other words, the comparison of the recipients' anti-HLA antibody specificities with the donors' historically identified HLA antigens.

To exclude, or to define, highly harmful DSA not only virtually but additionally by *de facto* cross-matching may be of special relevance in cases of insufficiently documented historical typing results of deceased donors, e.g. when the donor's so-called split antigens have not been adequately resolved or when so-called allele-specific antibodies are present, as have been increasingly described to have arisen in an organ recipient (Proust et al., 2009; Arnold et al., 2010; Schlaf et al., 2012b; Pandey and Harville, 2019). These antibodies are not directed against the complete antigen (e.g. HLA-A25) which genetically comprises a group of alleles (e.g. HLA-A*25). Allele-specific antibodies belonging to the same group of alleles, which a given donor shares with her or his recipient (i.e. who both have been typed HLA-A*25) virtually appear as auto-antibodies (e.g. recipient's anti-HLA-A25 directed against donor's HLA-A25) if exclusively defined at the two-digit (one field) level, which currently represents the guideline-compliant requirement for solid organ transplantations. Thus, allele-specific DSA are virtually undefinable at this level of resolution. In spite of such a given low resolution-matched combination a recipient characterized by the rare allele HLA-A*2514 may develop antibodies against the common allele HLA-A*2501 shared by the vast majority of the HLA-A25 bearing patients, as both alleles present different epitopes (Schlaf et al., 2012b). These antibodies, however, were clearly demonstrable through the use of the donor's leukocyte pellet for retrospective solid-phase-based *de facto* cross-matching. It is noteworthy that this positive crossmatch result was subsequently confirmed by virtual cross-matching at the high (four digit / two field) level of resolution of typing and corresponding high resolution antibody specifications. These were performed using the so-called single antigen level comprising the majority of well-documented recombinantly generated and immobilized allelic antigens.

Of course, this specialized application requires that donor material is available and thus it would be highly advantageous to establish systematically something akin to a deceased donors' tissue bank. Historically, the extremely useful approach of implementing a *de facto* crossmatch using deceased donors' materials has only once been followed in the context of corneal transplantations of high risk patients and this was over two decades ago. The ophthalmologists and immunologists involved used retinal pigment epithelial cells isolated from explanted eyes and afterwards stored them in liquid nitrogen. After thawing the cells they had to be re-cultured and stimulated with IFN-γ in order to upregulate the surface expression of HLA molecules for the subsequent flow-cytometric crossmatch analysis (Baumgartner et al., 1992; Zavazava et al., 1996).

However, this historical approach requires the use of vital cells and is characterized as time-consuming, expensive and extremely challenging in terms of technical demands. In contrast to the procedure of Zavazava and co-workers, which is inappropriate as a routine method in most tissue-typing laboratories, the ELISA-based technique presented here is easily implementable in any laboratory without complex or expensive technical equipment.

Discontinuation of the AMS-crossmatch ELISA and its subsequent replacement by diagnostic systems characterized by increasing insufficiencies, including complete diagnostic failure

Various fields of diagnostic application all have clearly demonstrated the superiority of solid phase-based cross-matching over the CDC-based procedure and it was therefore a signficant setback when this highly reliable solid-phase assay was suddenly discontinued by the manufacturer for commercial reasons in 2013. After the announcement, there was a limited time-period of only four weeks to establish and optimize the alternative AbCross ELISA (manufactured by MicroCoat, Bernried, Germany and distributed by Biorad, München, Germany) in a technically modified manner, which deviated significantly from the original protocol. According to the original AbCross protocol (presented in Fig. 2) the binding of the recipient's DSA had to be fulfilled using intact and detached lymphocytes of the respective donors (Fig. 2A). Subsequently, the resulting complexes of HLA-antigens and DSA were isolated by a detergent-mediated lysis step followed by immobilization of immune complexes to the pre-coated

monoclonal capture antibodies specific for either HLA class I or class II molecules (Fig. 2B). Afterwards, in complete accordance with the AMS-ELISA, the immobilized immune complexes were recognized by DSA if present in the recipient's serum. These were finally visualized using an enzyme-conjugated secondary anti-human IgG antibody (Fig. 2C). Also in accordance with the AMS-ELISA, and as positive control, a second monoclonal antibody directed against a second monomorphic epitope provided evidence that a sufficient quantity of immune complexes had been extracted from the donor's tissue sample and immobilized to generate a clear signal (Fig. 2D). This control was termed "Lysate Control" using the scheme of the Micro-AMS ELISA. The resulting rather laborious procedure, including the initial density-gradient centrifugation step in order to isolate lymphocytes, took at least six hours. Furthermore, a large volume (20 ml) of donor blood, which is seldom available from any given live donor and is an even more significant problem when dealing with deceased donors, was required in order to isolate a sufficient number of HLA-class II antigen-expressing cells (mainly B-cells). Despite these drawbacks Süsal and co-workers used this original protocol and highlighted the superiority of the AbCross method, especially in comparison to CDC-based B-cell cross-matching (Gombos et al., 2013). However, the conclusions drawn by the authors that the better predictive value for a 2-years post-transplant graft loss is due to a higher sensitivity of the AbCross technique must be critically challenged because the majority of the CDC-based crossmatch artefacts also hold true for B-cell cross-matching (Schlaf et al., 2014b, c). Thus, it is likely that the increased specificity of HLA-class II in AbCross cross-matching is the reason for this assay's superiority.

Apart from circumventing CDC-crossmatch-specific artefacts, which resulted from the artificial activation of

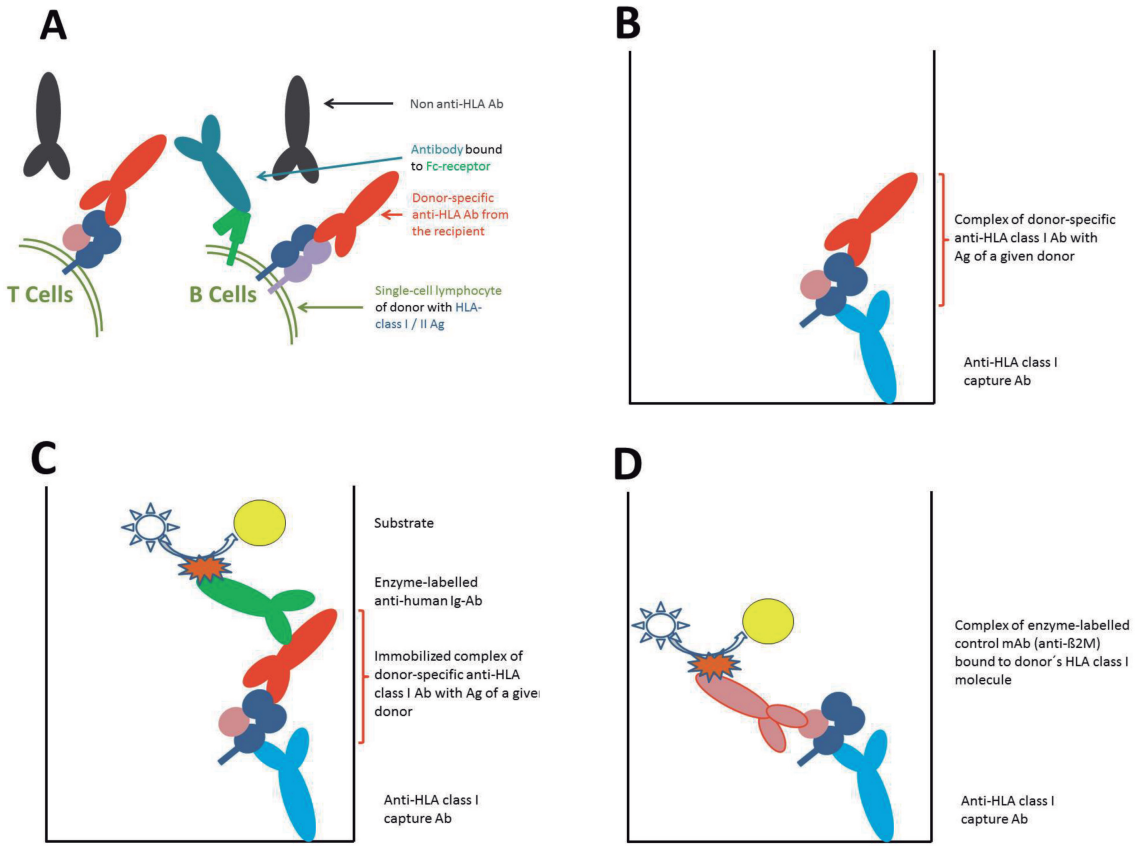

Fig. 2. Flow-chart of the original AbCross-ELISA for the detection of donor-specific HLA class I molecules (immune-complex procedure). **A.** Binding of the donor-specific anti-HLA antibodies (red) to their target HLA-antigens on the surfaces of detached T-cells and B-cells, respectively, in a first step. **B.** Immobilization of the detergent-solubilized HLA-class I immune-complex to the monoclonal capture antibody recognizing a monomorphic epitope on HLA-class I molecules. **C.** Binding of the enzyme-conjugated secondary anti-human IgG (alternatively anti-IgG/M/A) antibody to the bound donor-specific anti-HLA antibodies of the recipient and subsequent color reaction. **D.** Positive control consisting of an enzyme-conjugated second monoclonal control antibody bound to a second monomorphic epitope (here anti-ß2 microglobulin in order to form immune complexes with all HLA-class I antigens, which are then immobilized by the capture antibodies). The AbCross-ELISA variant for the detection of donor-specific antibodies directed against HLA class II molecules is designed accordingly.

the complement system, the original AbCross protocol, as well requiring detached cells in suspension, did not provide any additional advantage over the cellular crossmatch technique. Thus, all of the application fields dealing with donor materials lacking single or intact cells, but which were nevertheless successfully handled using the AMS-ELISA (Altermann et al., 2006; Sel et al., 2012; Schlaf et al., 2015a,b), were not practicable using the original AbCross-protocol provided by MicroCoat/Biorad. In particular, since using "non-cellular" samples from donors represents a unique feature of the application, the technical design of the AbCross-ELISA had to be completely changed and adapted before use to be in full compliance with the workflow of the AMS-ELISA.

However, surprisingly, and again for commercial reasons, the AbCross-ELISA was discontinued by MicroCoat/Biorad at the end of 2016. This forced us rapidly to find an alternative procedure. The possibility of a relaunch of the AMS-ELISA was received early in 2017 and raised our hopes to resolve this problem. Based on using the same set of diagnostic antibodies as the Micro-AMS, the novel diagnostic system, now termed Donor-Specific Antibodies/DSA (Immucor Transplant Diagnostics, Stamford, USA), was manufactured again as a microbead-based assay using the Luminex platform, which is well known from various anti-HLA antibody-screening and specification assays of two commercial suppliers. Given the aim of establishing the DSA-assay as the only remaining solid-phase-based crossmatch system, it was systematically evaluated in our laboratory. The resulting data, however, were rather disappointing because the accordance between virtual crossmatch results used as reference data and those of the DSA assay were far too low (Bau et al., 2019). Of the overall results, comprising 212 independent anti-HLA class I and class II antibody specifications and their corresponding DSA-assays, 69 of the virtually defined crossmatch results (32.5%) had to be classified as divergent using the DSA-assay, whereas only 143 results (67.5%) were classified to be in accordance with the assay's outcome. Based on the chosen cohort of recipients (n=106) no less than 62 (58.4%) were characterized by findings that were not supported by the virtual cross-matching. It is noteworthy that this high discrepancy was in all likelihood not due to the well-known possible errors of virtual cross-matching, as all selected donor-recipient combinations were straightforward in terms of the specificities and signal intensities of the assay (Bau et al., 2019). As the set of antibodies involved was the same as that used in the AMS-ELISA, the immunochemical "hardware" could not have been the reason for the diverging results. The problem arose primarily from insufficiencies in the software, which had to classify the recipients' raw data as either positive or negative for DSA. So-called background-adjusted factors (BAF) were subtracted from Mean Fluorescence Intensity (MFI) values measured against the immobilized donor antigens. This BAF generally represented a cut-off value that was calculated using the values of the three control beads Con1 (coated with albumin), Con2 (coated with glycoprotein IV) and Con3 (naked bead) for an equation specific for each lot. The basis of the calculation was unclear, i.e. not revealed in the manual supplied by the company, but resulted in so-called "adjusted MFI-values", which could not be validated. Apparently, a recipient's serum sample was always classified as positive if two of the three adjusted MFI values were positive. Furthermore, serum samples leading to increased raw values of all three control beads were also nearly always classified as positive. Generally, in terms of immunochemical analyses, the integration into the evaluation algorithm of the values for 'naked', untreated Con3-beads must be critically challenged, because any binding of antibodies to the naked carrier material should be prevented through a blocking step. This consideration holds true, of course, for any immunochemical assay. Apart from the fact that the underlying evaluation software is a "black box", including values which are incomprehensible in terms of immunochemistry, apparently no clinical evaluation has ever been performed by the supplier. If this had been done, then the unacceptable deficiencies, which have been similarly commented on by colleagues from three other tissue-typing laboratories, would have been discovered prior to its commercialization.

At the moment only 20 remaining single AbCross assays are still available in our laboratory, which will at the latest be used up by the end of the first quarter of 2020. For this reason, we have tried to evaluate the new ELISA-based crossmatch system named XMatch (Protrans, Ketsch, Germany), which first became available in 2019. Unfortunately, this most recent assay has been designed in strict accordance with the workflow of the original AbCross assay (Fig. 2) and consequently shares all of its disadvantages, described above in detail since again it does not allow the use of donor material lacking detached or intact cells. Thus, the approach has been re-initiated to adapt the system to the workflow of the AMS-ELISA. Initial results, however, do not demonstrate a technically convincing modification, as the sera of many recipients show strong signals even without prior immobilization of donors' HLA-antigens (unpublished data). Using the XMatch system about 25% of recipient serum samples were characterized by antibodies binding unspecifically to the matrix or the blocking material thus leading to false-positive results. Additional experiments using the protein-G column-purified IgG fraction of those sera led to the same outcomes, demonstrating that indeed IgG antibodies lead to the observed artefacts. We must therefore conclude that the XMatch system will likely not be adaptable to the workflow of the AMS-ELISA (Fig. 1), obviating its use for our laboratory's routine work. Our investigations for implementing the Xmatch ELISA are currently ongoing.

Based on the unique features and various fields of

application of solid-phase-based cross-matching successfully carried out in the past, we conclude that remanufacturing of a reliable assay along the lines of the historical AMS-ELISA is one of the most urgent challenges and requirements of current, evidence-based transplantation immunology. In particular, this technical challenge needs to be addressed immediately in order to facilitate the speedy manufacturing of reliable diagnostic solid-phase-based crossmatch systems.

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