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Detection of Post-Transplant Anti-HLA Donor-Specific Antibodies through the Use of Stored Donors' Cell Lysates and Solid Phase-Based Cross-Matching

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Abstract

Transplant recipients who have sensitizing events such as pregnancies, blood transfusions and previous transplants often develop antibodies directed against human leukocyte antigen (HLA)-molecules of the donors' organs. These pre-formed donor-specific antibodies (DSA) represent a high risk of organ failure as a consequence of antibody-mediated hyper-acute or acute allograft rejection. In order to select recipients without donor-specific anti-HLA antibodies the complement-dependent cytotoxicity crossmatch assay (CDC-CM) was established as standard procedure more than forty years ago. This assay, however, is characterized by several drawbacks such as a high degree of vitality (at least 90%) required for the target lymphocytes of a given donor. This requirement highly limits its applicability for patients treated with therapeutic antibodies, special drugs or patients who suffer from underlying diseases i.e., especially from type III (immune complex) auto-immune diseases. Furthermore, only DSA which exert complement-fixing activity are detected. As a consequence novel crossmatch procedures which act independently of the complement system and which do not represent functional assays have been generated in the format of flow cytometry (FACS-) or solid phase (ELISA-) assays. Especially solid phase-based assays the outcomes of which are not limited by insufficient cell vitalities have in spite of various environmental disruptive factors been shown to lead to valid results and not to false positive outcomes in contrast to CDC-based cross-matching. Our current results show the superiority of ELISA-based cross-matching in a novel context. Data are provided which show the ELISA-based applicability of long term-stored donors' materials to demonstrate or exclude the involvement of DSA in a rejection episode by *de facto* cross-matching and not only by the virtual crossmatch approach i.e., the comparison of the recipients' anti-HLA antibody specificities with the donors' historically identified HLA-pheno- and/or genotypes.

Keywords: Allo-grafting; Complement-dependent cytotoxicity assay; Crossmatch; Donor-specific antibodies; Enzyme-linked immunosorbent assay; Human leukocyte antigens; Major histocompatibility complex; Rejection
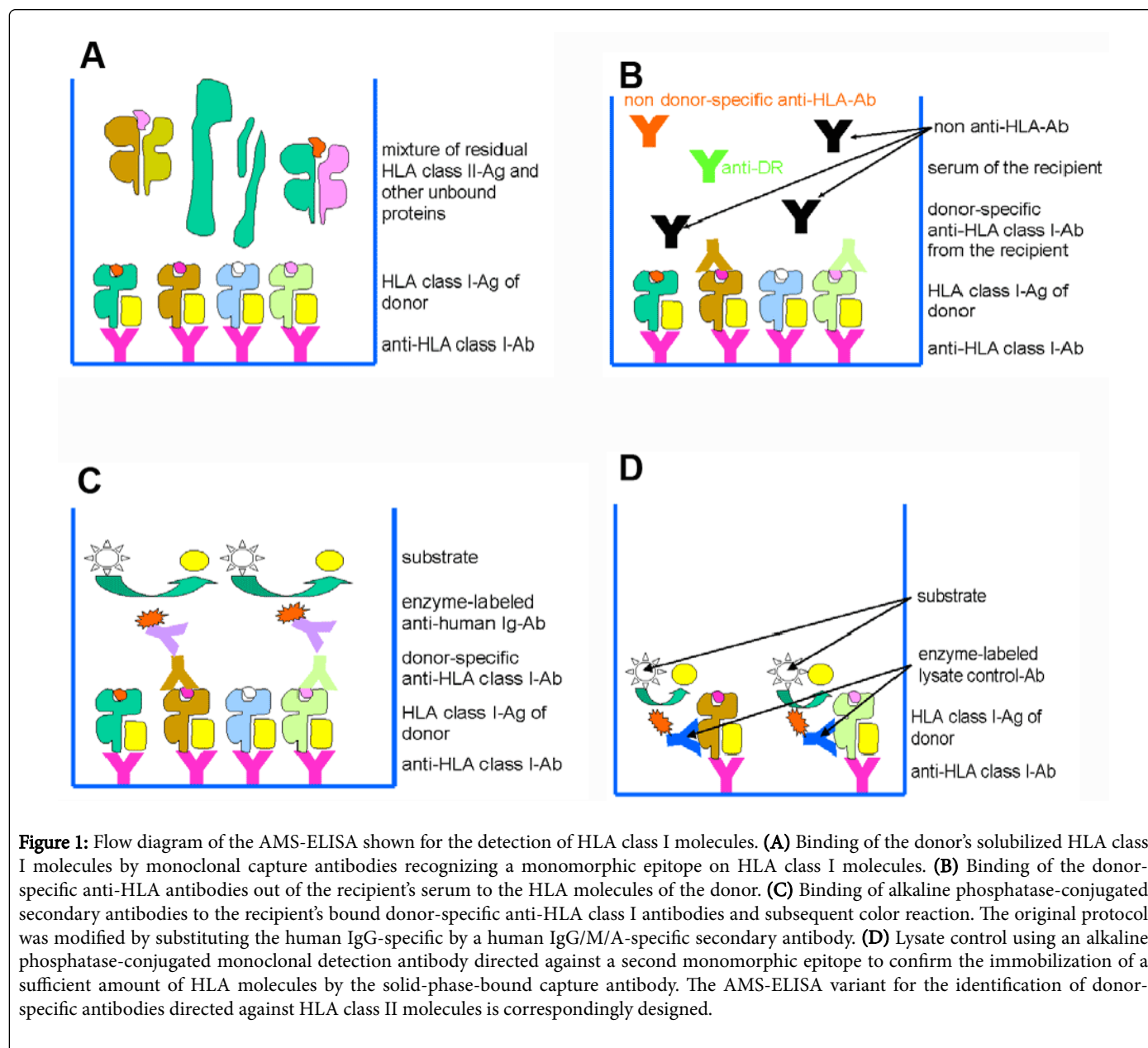
Introduction

It has first been shown for more than forty years that antibodies directed against antigens of donors' tissues represent the dominant reason for hyper-acute or acute rejections of allo-grafted organs [1]. Later studies provided evidence that these donor-specific antibodies (DSA) were in nearly all cases of their detection directed against human major histocompatibility (MHC) antigens, the so-called human leukocyte antigens (HLA) [2,3]. These DSA are thus regarded as a contraindication for allo-grafting according to the transplantation guidelines of most countries and supranational societies (e.g. Eurotransplant Foundation) responsible for the allocation of solid organs. The corresponding technique historically developed in the late sixties of the last century in order to prevent recipients from these antibody-mediated rejections was the complement-dependent cytotoxicity crossmatch (CDC-CM). Until now this technique represents the standard crossmatch (CM) procedure. Lymphocytes isolated from a given donor's blood are incubated with the prospective recipient's serum to lead to a complement-dependent attack after adding rabbit complement. The outcome is analyzed by calculating the number of dead cells (positive reaction) using two-color fluorescence microscopy [4,5]. The procedure, however, is characterized by several drawbacks. Using this functional (vitality) assay leads to the detection only of those DSA which exert their detrimental function by their complement-activating features i.e., the lyses of the donors' cells *via* the classical pathway of complement activation. This technique, however, fails to identify additional DSA without complement-fixing activity although these may as well be detrimental for donor organs [6-8]. An additional drawback is its low sensitivity thus leading to the inability to detect low antibody concentrations. Consequently this assay was modified by introducing secondary anti-human immunoglobulin antibodies, recognizing the primary donor-specific anti-HLA antibodies leading to the design of the anti-human globulin (AHG)-enhanced CDC-CM [9,10]. Regarding the interpretability of the outcomes of the CDC-CM, however, all of its variants depend on a high quality of donor cells and do often not lead to clear results if a given donor's lymphocytes exhibit vitality rates lower than 90%. This holds also true for cell samples contaminated by other leucocytes or precursor cells since the staining procedure leads to adequate results only with lymphocytes. As an alternative approach to circumvent some of these CDC-CM-specific problems the flow cytometric crossmatch (FACS-CM) was first established in 1983, allowing the additional detection of complement-independent DSA [10-13]. The sensitivity of this assay is in the range of the AHG-enhanced CDC. In spite of its increased sensitivity, however, this technique is known to be influenced

by artefacts due to the "irrelevant/unspecific" binding of antibodies *via* their Fc-parts to Fc-receptors especially expressed on B-lymphocytes [4]. This drawback was first reliably overcome by the procedure previously proposed by Hajeer and co-workers to effectively block these receptors [14]. The striking drawback which holds true for CDC-based as well as for FACS-based cross-matching, however, is that both assays depend on the high vitality of the donors' cells, thus, not allowing valid results if only cells of poor quality (i.e., with a vitality rate lower than 80-90%) are available. As this aspect of insufficient cell quality led to many doubtful crossmatch outcomes, novel procedures which work independently of the cell quality in the format of solid phase-based assays (i.e., ELISA- or Luminex microsphere-based) have been established in an increasing number of tissue typing laboratories for the last eight years. Due to its first commercial availability the Antibody Monitoring System (AMS-) ELISA (GTI Diagnostics,

Waukesha, USA) had been established by us already in the year 2005 [4] and, after its discontinuation by the manufacturer for commercial reasons in 2013, was replaced by the AbCross-ELISA (Bio-Rad Medical Diagnostics, Dreieich, Germany). However, to be of any value for us it was established in a completely modified manner (Figure 1). Several publications pointing to the superiority of these solid phase assays over the classical CDC-CM have hitherto been published mainly in the context of disruptive factors falsifying the outcome of the CDC-based technique. These factors include pharmaceutical treatment such as cytostatic agents or therapeutic antibodies [5,15-17] as well as accompanying auto-immune diseases [4,5,16,18-21] of a given recipient. Furthermore, the procedure of ELISA-based cross-matching was successfully implemented for corneal allograft recipients using the outer corneal rim as the only and most acellular tissue available from the donors [4,22].

Figure 1: Flow diagram of the AMS-ELISA shown 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 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 detection antibody directed against a second monomorphic epitope 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 correspondingly designed.

With this contribution we point to the novel aspect of this procedure that by using a given donor's cell lysate including her/his HLA-molecules ELISA-based cross-matching for the first time provides the opportunity to use long term-stored material also of deceased donors for *de facto* cross-matching. Thus, it is possible to substitute or at least to complement the procedure of virtual cross-matching i.e. the comparison of a recipient's antibody specificities with the HLA-phenotypes of the corresponding donor. Furthermore, we show that virtual cross-matching is of very limited value if so-called allele-specific antibodies exist which are not definable at the level of two-digit resolution of HLA-typing since they are directed only against one or several alleles of an (two-digit-defined) HLA-phenotype actually representing a group of alleles.

Patients and Methods

Procedures used for the detection of anti-HLA donor-specific antibodies / Cross-matching

All of the patients presented here were examined for the purpose of routine diagnostics in the tissue typing laboratory (Eurotransplant code GHATT) of the university hospital Halle/Germany between 2009 and 2015. CDC-based cross-matching was generally not required prior to allo-grafting thoracic organs due to the guidelines of Eurotransplant and the German National Medical Association. For the reasons introduced above, it was furthermore not performed due to the lack of the donors' lymphocytic material of adequate vitality. Thus, the procedure of CDC-based cross-matching in detail presented in the references [4,5] which in spite of its drawbacks has been accepted for years and also currently represents the standard procedure for the selection of recipients without DSA has not been performed for any of the lung or heart recipients presented. This unhelpful situation did not change until 2012 when the Eurotransplant guidelines obligatorily dictated a CDC-based pre-transplant crossmatch but only for heart and not for lung recipients.

The alternative procedure of ELISA-based cross-matching was already implemented in the year 2005 using the Antibody Monitoring System (AMS)-class I/II ELISA (GTI, Waukesha, USA; FDA-No. BK060038 given in June 26th, 2006). This assay was successfully used until its discontinuation by the manufacturer in the year 2013. Then it had to be replaced by the AbCross HLA class I/II ELISA (Biotest/BioRad, Dreieich, Germany). However, the ineffective and time-consuming lead through as presented in the manufacturer's manual was completely modified in our laboratory thus resulting in the assay's higher sensitivity, faster results and especially its usability for stored cell lysates of the given donors, an aspect which will be a major point of the discussion. Thus, the AbCross ELISA procedure was not performed as recommended by the manufacturer i.e., by i) the incubation of vital donors' lymphocytes with the recipients' sera in a first step, ii) followed by the detergent-mediated lyses of these immune-complexes out of the cell membranes and iii) not till then binding these lysed aggregates to the monoclonal capture antibodies directed against HLA-class I or HLA-class II molecules, respectively, on the Terasaki-microtest plates in order to iv) detect bound DSA through enzyme-bound secondary anti-human IgG (alternatively anti-human IgG/M/A) antibodies. Quite in contrast, the procedure was modified to be in complete accordance with the AMS-ELISA as formerly used. A workflow scheme is presented in Figure 1. First detergent lysate of a given donor's leukocytes/tissues including HLA class I and II molecules has to be pipetted into the wells of ELISA-strips (GTI) or Terasaki-microtest plates (BioRad), respectively, pre-coated with monoclonal capture antibodies (Figure 1A). These are directed against a monomorphic epitope accessible on all HLA class I or class II molecules, respectively. After this first incubation and several washing steps the recipient's sera (1:3 and 1:6 pre-diluted) are pipetted onto the immobilized HLA-molecules of the given donor and, in case of recognizing them, serve as detection antibodies in this sandwich-assay (Figure 1B). Upon consecutive washing steps the immobilized primary DSA are incubated with enzyme-conjugated secondary anti-human IgG (anti-human IgG/M/A) antibodies to induce the final substrate reaction (Figure 1C). The so-called lysate controls using a second monoclonal anti-HLA class I or class II antibody, respectively, as detection antibody provide evidence that a sufficient amount of donors' HLA-molecules has been immobilized to generally achieve signals of adequate intensity (Figure 1D). The value of a given recipient's serum under investigation has to exceed two-fold the value of the negative control to be classified as positive. The procedure of ELISA-based cross-matching was established in our laboratory more than nine years ago and has been employed for nearly all samples leading to invalid or doubtful results using the conventional CDC-CM due to its susceptibility to various artefacts. In the context of allo-grafting the thoracic organs heart and lung and according to an agreement with the respective transplant center for thoracic organs a retrospective crossmatch-ELISA has generally been performed for nearly all of the recipients one to two days after the transplantation when the respective material reached our tissue typing laboratory. Thus, ELISA-based cross-matching has hitherto been performed in this context more than 250-fold to retrospectively exclude DSA or to adequately adjust the immunosuppressive regimen in order to avoid a rejection episode.

Determination of anti-HLA antibody specificities (antibody monitoring) for virtual cross-matching

The general detection of anti-HLA class I and class II antibodies (antibody screening) was done using the QuikScreen ELISA and the B-Screen ELISA, respectively (both GTI, Waukesha, USA). Serum samples positive in this first step were afterwards investigated in an antibody specification assay named DynaChip. This miniaturized chip technology (Invitrogen/Dynal, Bromborough, UK) which was used until its discontinuation by the manufacturer in the year 2011 was the only completely automated specification system for anti-HLA antibodies. In its second generation design used by us 106 positions on glass microchips were covered with HLA class I molecules and 48 positions with HLA class II molecules of different single donors, respectively. Although this assays did not provide a resolution at the "single antigen level" the combination of the single donors' immobilized HLA class I or class II antigens, respectively, allowed the identification of the patients' antibody specificities in most cases (about 80%) especially when the level of immunization (so-called PRA-level / level of panel-reactive antibodies) was not too high. Generally this PRA-value [%] determined for all patients on the transplant waiting lists for different organs indicates the relative likelihood of a positive *de facto* crossmatch. Furthermore, this statistical value indicates an increased relative risk for allograft rejections mediated by anti-HLA antibodies. Thus, it easily allows the identification of patients who have to be crossmatched and afterwards monitored for rejection episodes very carefully as a consequence of their high anti-HLA pre-immunization status.

The discontinuation of the DynaChip technique led to the implementation of the Luminex-based antibody analysis (Lifecodes/Immucor, Stamford, USA) which currently represents the dominating tool for anti-HLA antibody specification. Its general technical aspects and drawbacks have in detail been reviewed elsewhere [5,23,24]. In brief the assays are composed of a series of polystyrene microspheres, on which single recombinant HLA molecules of only one phenotype (single antigen assay) or a group composed of a single donor's combined HLA class I or II molecules, respectively, (single donor / ID level) are immobilized. The beads binding the respective anti-HLA antibodies are recognizable by a unique signal due to embedded fluorochromes of different intensities. The approach of so-called virtual cross-matching i.e., the identification of anti-HLA antibody specificities directed against the HLA-phenotypes of a given donor was generally used as a plausibility check of the ELISA-based *de facto* crossmatch results. However, a situation of a recipient characterized by so-called allele-specific antibodies is reported where DSA are not distinguishable by virtual cross-matching.

Results

Investigation of a heart recipient's rejection episode using donor's material after its storage for more than four years

A 48-year-old male patient suffering from severe heart insufficiency was phenotyped and genotyped in order to enter the waiting list for heart allo-grafting. His HLA class I antigens were determined as HLA-A1,11; B8,35 (Bw6); Cw4,7 and the HLA class II antigens as DR1,7; DR53; DQ5,3(9). After only two months on the waiting list the patient received a heart allograft from a donor typed HLA-A2; B7,51 (Bw4,6); Cw7 for HLA class I and HLA-DR11,15; DR51,52; DQ6,3(7) for the HLA class II molecules. The resulting HLA mismatch scheme taking only the HLA-A, B and DR phenotypes into consideration was 1-2-2 since the donor was homozygous for the HLA-A2 phenotype offering only one rejection target in this regard. As mentioned above according to the agreement with the heart transplant center also this patient was retrospectively tested for DSA against the organ after being allo-grafted. The consequent analysis using a serum sample taken at the day of the transplantation did not show any DSA using the AMS-ELISA nor anti-HLA antibodies in general as was demonstrable by the Screen ELISA and by Luminex analysis performed in August 2010 (Table 1). Aliquots of the residual detergent lysate of the donor's leukocytes were deep-frozen (-30°C). For a period of more than four years graft function was okay without any immunological complication. This is shown by the annual routine check for the general detectability of anti-HLA antibodies. Every second year (2012 and 2014) a Luminex (Single Donor-based) analysis was performed in addition to the Screen ELISA both of which did not exhibit any existing anti-HLA antibodies (PRA=0%). Thus, over 4.5 years all of the assays performed never led to the conclusion that anti-HLA antibodies may be involved in impairing the graft function and the graft survival (Table 1). However, in March 2015 an unexpected clinically proven rejection episode was diagnosed by the transplant center leading to an immediate analysis of the patient's current serum for a possible involvement of anti-HLA antibodies. As is visible in Table 1 due to the severe indication of a clinically proven rejection additional antibody specification assays, the One Lambda Single Antigen Class I ELISA (LAT 1HD) and the Lambda Antigen Single Donor Class I and II ELISA (LAT12/88) (both *via* BMT, Meerbusch, Germany) were performed in addition to the standard Screen-ELISA and the Luminex Single Donor specification analyses either not leading to identifiable anti-HLA antibodies in

general and clearly allowing the conclusion of a negative virtual crossmatch. Additionally, a *de facto* crossmatch using the deep frozen splenic leukocyte-derived material was performed with the procedure of the modified Ab Cross ELISA. In best accordance with virtual cross-matching the modified AbCross assay did not detect donor-specific anti-HLA class I or II antibodies with the serum taken in March 2015 (Table 1). The validity of these negative data of the patient's serum was confirmed by clearly positive reactions of the lysate controls (Figure 1D). Thus, evidence was provided that sufficient numbers of HLA class I and class II molecules were still accessible in the donor's leukocyte-derived detergent sample to perform this assay showing that the proteins had not been degraded during their storage for more than 4.5 years. A sufficient amount of HLA-molecules was thus immobilized to result in an adequate signal of these positive LCR-controls. This report may lead to novel diagnostic conceptions through the use of a given donor's long term stored spleen or blood-derived leukocytes in order to monitor a humoral rejection episode characterized by an involvement of anti-HLA DSA which in this special case was excluded with very high probability as DSA were not demonstrable both by virtual and *de facto* cross-matching.

Serum sample	GTI-Screening Class I / II ELISA	Luminex (SD) Class I / Class II	ELISA-Crossm. Class I / Class II
06/2010 &	neg./neg.	n.d./n.d.	n.d./n.d.
08/2010 $	neg./neg.	neg./neg. (PRA=0%)	neg./neg. (AMS)
07/2011 *	neg./neg.	n.d./n.d.	n.d./n.d.
08/2012 *	neg./neg.	neg./neg. (PRA=0%)	n.d./n.d.
08/2013 *	neg./neg.	n.d./n.d.	n.d./n.d.
11/2014 *	neg./neg.	neg./neg. (PRA=0%)	n.d./n.d.
03/2015 #	neg./neg.	neg./neg. (PRA=0%)	neg./neg. (AbCr.)

for 03/2015 additionally:
LAT 1HD (anti-HLA class I Single Antigen ELISA): negative
LAT 1288 (anti-HLA class I/II Single Donor ELISA): negative

n.d.: not done; neg.: negative; pos.: positive; PRA%: Panel Reactive Antibodies %; SD: Single Donor Resolution; &: analyses for entering the waiting list; $: analyses at the date of the transplantation; *: routinely performed post-transplantation analyses; #: analyses at the date of the clinically proven rejection episode; (AMS), (AbCr.): after its discontinuation in 2013 the AMS-crossmatch ELISA was replaced by the highly modified AbCross-crossmatch ELISA

Table 1: Results of different antibody detection and specification analyses in comparison with crossmatch-ELISA outcomes most probably excluding an involvement of anti-HLA DSA in a rejection episode of the 48-year old heart recipient.

Confirmation of an anti-HLA B7 donor-specific antibody by ELISA-based cross-matching as cause for a rejection episode of a lung allograft recipient

A 42-year-old prospective female lung allograft recipient suffering from cystic fibrosis was phenotyped and genotyped HLA-A11,26;

B7,62 (Bw4,6); Cw 2,3 for class I and genotyped HLA-DR13,15; DR51,52; DQ6 for class II antigens in order to be registered on the waiting list for this organ. Due to prior immunizations the origin of which remained undefinable for our laboratory (most probably due to pregnancies), this patient had already been immunized for several HLA-antigens when entering the waiting list in 08/2012. As an initial routine procedure anti-HLA class I and class II antibody screening was initially performed using the GTI QuikScreen and the B-Screen assays, respectively. As the QuikScreen ELISA was positive antibody identification was performed for both HLA classes using the Luminex single donor (single ID) assay. This Luminex-based specification exhibited a PRA of 54% against HLA class I and of 0% against HLA class II molecules. The antibodies' specificities were definable as anti-HLA A9 (23,24), -B7, -B27, -B40 (60,61), -B47 and -B81 (Table 2). Thus, the immunological parameters which were possibly relevant for the prospective transplantation outcome including the patient's antibody status were sufficiently investigated when entering the waiting list in August 2012. After about six months on the waiting list (02/2013) a lung allograft was offered. It was typed HLA-A3; B7,38 (Bw4,6); Cw7,12 for class I and DR13; DR52; DQ6 for class II antigens resulting in the HLA A-B-DR mismatch scheme of 1-2-0. Due to the incompatibility of the Cw antigens two additional theoretical HLA targets existed whereas the HLA class II antigen DQ6 was present on the patient's as well as on the allograft's cells thus representing no additional antigen. Consequently the positivity of the patient's virtual crossmatch was due to identifiable anti-HLA B7 antibodies directed against the corresponding antigens expressed on the allograft's cells. As mentioned above it is again noteworthy in this context that due to the guidelines of the Eurotransplant Foundation and of the German Federal Medical Association a pre-transplant CDC-CM which is strictly mandatory prior to any kidney allo-grafting is generally not required as a pre-requisite for lung transplantations. Unfortunately, the donor's antigen HLA B7 leading to the positive virtual crossmatch was overlooked for representing a so-called "unacceptable antigen". Furthermore, this donation was one of the few for which no crossmatch ELISA was retrospectively performed to adequately adjust the immunosuppressive regimen if DSA were detectable. In spite of its probability it can only be speculated whether the positive virtual crossmatch would have been supported by positive ELISA-based cross-matching indicating the serious situation already at that date of the transplantation. Anyway, allo-grafting was performed in spite of these unfavorable circumstances leading to a clinically apparent rejection episode (07/2013). Consequently our laboratory was asked for analyzing anti-HLA antibodies as possible cause. In best accordance with the antibody specifications performed for the waiting list entry all of those antibodies mentioned above were identifiable again using Luminex analysis (single donor level of resolution) for a second time resulting in a very similar PRA of 60% (Table 2). Accidentally a deep-frozen splenic leukocyte cell pellet of the lung donor was available since that given donors material had been HLA-typed in our laboratory about five months ago and afterwards been stored for the purpose of DNA preparation. Thus, the idea arose to perform ELISA-based cross-matching using this material's detergent lysate as donor material. Of course no vital cells existed after thawing the pellet and any vitality assay such as CDC-based cross-matching was a priori impossible. The serum taken at the date of the rejection episode (07/2013) was investigated for DSA against HLA molecules of the donor and clearly exhibited positive values for anti-HLA class I antibodies at dilutions of 1:3 and 1:6 whereas no antibodies directed against HLA class II molecules were demonstrable (Table 2). The donor's spleen-derived leukocyte pellet, stored for five months

however, was successfully used to demonstrate the existence of DSA not only on the basis of virtual cross-matching. Unfortunately the donor's cell pellet had been too small to get a sufficient pellet for a continuing series of monitoring DSA. Thus, only one follow up analysis using the AMS-ELISA was performed after three apheresis cycles (08/2013) which clearly showed that this therapeutic approach did not lead to the removal of DSA or at least to a significant decrease in their number still detectable at dilutions of 1:3 and 1:6 (Table 2). These ELISA-based *de facto* crossmatch data were in best accordance with the accompanying Luminex-based specification data still exhibiting a PRA-value of 58% and exactly the same spectrum of anti-HLA class I antibodies clearly including those directed against the donor's HLA B7 antigen as in the two previous analyses (Table 2). Successive antibody-reducing therapeutic steps were monitored only virtually through the use of Luminex specification analyses (data not shown).

Serum sample	Luminex- (Single Donor/ ID) Ab specificities	PRA (%)	AMS-ELISA-CM	
			Class I	Class II
08/2012 &	Class I: anti-A9 (23,24), **-B7** -B27, -B40 (60,61), -B47, -B81	54%	n.d.	n.d.
	Class II: neg.	0%		
07/2013 #	Class I: anti-A9 (23,24), **-B7** -B27, -B40 (60,61), -B47, -B81	60%	**pos. (1:6)**	neg.
	Class II: neg.	0%		
08/2013 §	Class I: anti-A9 (23,24) , **-B7** -B27, -B40 (60,61), -B47, -B81	58%	**pos. (1:6)**	neg.
	Class II: neg.	0%		

n.d.: not done; neg.: negative; pos.: positive; &: antibody specification as prerequisite for entering the waiting list; #: antibody analyses at the date of the rejection episode; §: antibody analyses after three apheresis cycles; bold lettering: donor-specific antibodies as detected by virtual (Luminex) or *de facto* (AMS-ELISA) cross-matching at the highest dilution (parentheses).

Table 2: Results of Luminex-based anti-HLA antibody specification analyses and corresponding outcomes of the AMS-crossmatch ELISA for the 42-year old lung recipient highlighting an involvement of anti-HLA DSA (anti-HLA-B7).

Rapid upcoming of anti-HLA DSA in a lung recipient after four weeks as shown by virtual and ELISA-based *de facto* cross-matching

A 47-year-old female prospective lung allograft recipient was pheno- and genotyped HLA-A3,31; B27,35 (Bw4/6); Cw1,4 for HLA-class I and genotyped DR1; DQ5 for HLA-class II antigens in order to enter the waiting list for a lung allograft (02/2014). As a routine approach both the QuikScreen and the B-Screen ELISA analyses were performed not exhibiting any anti-HLA antibodies of the patient in general. Consequently no additional Luminex-based specification analysis was performed. Only four months later (06/2014) she received an organ typed HLA-A2; B7,40(60) (Bw6); Cw3,7 for HLA-class I and DR4,8; DR53; DQ4,3(8) for HLA-class II molecules leading to the HLA A-B-DR mismatch scheme 1-2-2. As is visible both the HLA-class I Cw antigens and the class II DQ antigens of the donor represent possible additional transplantation antigens, which are, however, not represented by this scheme. As a routine approach antibody screening (GTI QuikScreen and B-Screen) and specification analyses (Luminex class I and II single donor analyses) were performed using a patient's

serum sample taken at the day of grafting. As a result no anti-class I and class II antibodies were generally detectable by the screening assays or identifiable by the specification analyses (Table 3) leading to a negative outcome of virtual cross-matching. Due to an agreement with the transplant center the modified AbCross crossmatch ELISA was used to exclude DSA additionally by *de facto* cross-matching using the donor's blood leukocyte pellet and the patient's serum taken at the day of grafting. Also this crossmatch assay did not exhibit any DSA leading to the conclusion of low risk transplantation in terms of expectable immunological complications. For this reason it was completely unforeseeable that about four weeks later the patient suffered from a clinically apparent rejection episode. A new serum sample taken at that date (07/2014) exhibited a completely different outcome in comparison with the sera taken before. Both the QuikScreen and the B-Screen ELISA showed an unequivocally positive outcome. The consequent Luminex (Single Donor)-based specification resulted in PRA-values of 44% for anti-HLA class I and 80% for anti-HLA-class II antibodies. Anti-HLA A2 and anti-B60 antibodies against the donor's class I molecules were distinctly identifiable as well as anti-DR4, anti-DR8, anti-DQ3 (7,8,9) and anti-DQ4 antibodies against the donor's class II molecules, thus clearly indicating a positive virtual crossmatch (Table 3). Additional identifiable antibody specificities are listed in Table 3. Furthermore, a remaining aliquot of the donor's blood-derived leukocyte lysate used for ELISA-based cross-matching one day after the transplantation and afterwards stored as deep-frozen sample was thawed after four weeks and used for the same purpose in combination with the current serum sample (07/2014). In best accordance with the virtual crossmatch outcome this second AbCross clearly exhibited anti-HLA class I as well as anti-HLA class II DSA (Table 3). In analogy to the previous case there was no sufficient amount of donor material to monitor the resulting treatment performed to reduce the patient's antibody load also by ELISA-based cross-matching. Right from the first cross-matching no opportunity of storing donor material sufficient for several possible future ELISA-crossmatch runs was provided as no spleen but only a limited volume of blood was allocated to our laboratory. Thus, the outcomes of several runs of antibody reducing treatment were monitored only virtually (data not shown).

Serum sample	GTI-Screening Class I/II ELISA	Lum.- (ID) Ab- specificities	PRA (%)	AbCr.-CM ELISA Class I/ Class II
02/2014 *	neg./neg.	n.d.	n.d.	n.d./n.d.
06/2014 $	neg./neg.	class I: neg	0 %	neg/neg.
		class II: neg	0 %	
07/2014 #	pos./pos.	class I: **anti-A2**, **-B60**, -B13	44%	**pos./pos. (1:3)/(1:6)**
		class II: **anti-DR4**, **-DR8**, **-DQ3(7,8,9)**, **-DQ4**, -DR3(17,18), -DR5(11,12)	80%	

n.d.: not done; neg.: negative; pos.: positive; *antibody specification as prerequisite for entering the waiting list; $: antibody analyses at the date of transplantation; #: antibody analyses at the date of the rejection episode; bold lettering: donor-specific antibodies as detected by virtual (Luminex) or *de facto* (AbCross-ELISA) cross-matching at the highest dilution (parentheses).

Table 3: Rapid upcoming of anti-HLA DSA in a lung recipient after four weeks as shown by virtual and ELISA-based de facto cross-matching.

Identification of allele-specific DSA not definable by virtual cross-matching based on the level of low resolution (two digit) typing

Some years ago a case was investigated in our laboratory dealing with the very special situation that unforeseeable allele-specific antibodies led to the loss of a kidney allograft. These DSA had arisen although the patient had received a fully matched (so-called full house) post mortem kidney as defined at the level of two-digit resolution. The 10-year-old male patient with end stage renal insufficiency was HLA-typed A3,25; B8, 18 (Bw6); Cw7,12; DR15,17; DR51,52; DQ6 (Table 4a). In 1998 he received a graft with no HLA-mismatch. Due to the donor's homozygosities in DR and DQ phenotypes no rejection targets were presented leading to the most favorable HLA A-B-DR -mismatch scheme of 0-0-0 (Table 4a). As required by the guidelines for kidney allografting in those days and as expected on the basis of perfect HLA-matching the pre-transplant CDC-based crossmatch performed in 1998 was negative for PBL, T-cells and B-cells. As a matter of course the transplantation was realized. Unexpectedly this allograft lost its function after eight years leading to the re-entry of the patient onto the waiting list in the year 2006. Using the antibody screening ELISA the variant for anti-HLA class I antibody detection was clearly positive whereas no anti-HLA class II antibodies were detectable using the B-Screen variant. Afterwards performed assays for antibody specification (Luminex single donor class I and II assays and LAT 1HD anti-class I single antigen ELISA) both clearly identified anti-HLA A25, -A26, -A34 and -A66 antibodies whereas the Luminex single donor anti-class II assay did not exhibit any antibodies (Table 4a). All of these antigens against which antibodies were detectable belong to the so-called broad antigen HLA-A10 strongly suggesting that antibodies against this common antigen (shared by all of the mentioned split antigens) had been generated by the young patient. Consequently the situation was not at all clear through the use of virtual cross-matching based on the two digit-level of typing as anti-HLA A25 antibodies theoretically represented auto-antibodies. However, as a matter of fact this must be regarded as an ultra-rare phenomenon. It should be mentioned in this context that in most cases the pseudo-identification of auto-antibodies directed against self-HLA molecules is a consequence of artificially positive outcomes of the CDC-CM (here the CDC-based auto-crossmatch). This assay is highly susceptible to various artefacts and disruptive factors which in many cases remain unknown for the individual patient due to insufficient consecutive diagnostic follow up analyses [5,16-20]. Real anti-HLA class II antibodies have in contrast recently been detected in cases of auto-immune hepatitis [25]. When re-entering the waiting list the meanwhile 18-year-old patient, however, did not exhibit a positive CDC-based auto-crossmatch nor did he suffer from any auto-immune disease. Thus, auto-reactive anti-HLA antibodies were not very probable right from the beginning of the anti-HLA A10 specification. Nevertheless, a transplant kidney offered in the year 2009 had to be refused due to a clearly positive pre-transplant CDC-crossmatch although virtually no antibodies were detectable against this potential graft (Table 4b). The conclusion that indeed DSA had been the reason both for the allograft loss and the refusal of that kidney offer in 2009 was finally based on high resolution genotyping of the patient who exhibited the very rare allele HLA-A*25:14 in contrast to the most frequent and expected one HLA-A*25:01. Of course no material from the primary donor from 1998 was available after 11 years. However, in view of the antibody specification analyses which exhibited only anti-HLA A10 (anti-A25) antibodies it is

highly probable that the immune response resulted in DSA directed against the phenotype of the donor's frequent allele HLA-A*25:01. Furthermore, the AMS-ELISA used as HLA-specific auto-crossmatch assay in the year 2009 was as well negative. To retrospectively support the hypothesis of allele-specific DSA the AMS-ELISA was used to detect the respective patient's antibodies directed against three lysates of selected and stored donor lysates exhibiting the HLA-A25 phenotype derived from the frequent HLA-A*25:01 allele (Table 4b).

Patient's typing	Donor's typing (1998)§	Typing of the refused kidney offer (2009)#
A3, 25 (10)	A3, 25 (10)	A3, 25 (10)
A*03:01	(high res. not done)	A*03:01
A*25:14		A*25:01
B8, 18 (Bw6)	B8, 18 (Bw6)	B18, 49 (Bw4,6)
Cw7, 12	Cw7, 12	Cw7, 12
DR15, 17	DR15, 15	DR4, 14
DR51, 52	DR51, 51	DR52, 53
DQ2, 6	DQ6, 6	DQ3 (8), 5

Patient's antibody screening results:

anti-HLA class I: positive; anti-HLA class II: negative

Patient's identified anti-HLA antibodies:

anti-A25 (10);

other HLA-A10 specifications: anti-A26 (10), anti-A34 (10), anti-A66 (10)

§,#, Patient's CDC-based crossmatch results:

§: Crossmatch results using the patient's pre-transplant serum from 1998:

Negative CDC-based pre-TX crossmatch in 1998 using PBL, T-cells and B-cells

#: Crossmatch results using the patient's pre-transplant serum from 06/2009:

positive CDC-based pre-TX crossmatch in 2009 using PBL, T-cells and B-cells

Table 4a: HLA typing results of the patient, his donor from 1998, and a refused kidney offer from 2009. Identifiable HLA-specific antibodies and crossmatch-ELISA results of the patient are presented.

Patient's serum sample	AbCross-ELISA-CM results against selected virtual donors		
	B.H. class I / II	K.P. class I / II	T.H. class I / II
06/2009	**pos.** / neg.	**pos.** / neg.	**pos.** / neg.
07/2011	**pos.** / neg.	**pos.** / neg.	**pos.** / neg.
12/2012	**pos.** / neg.	**pos.** / neg.	**pos.** / neg.
Virtual donors' HLA-A High Res. typing results:	B.H.: HLA-A*24:02, *25:01		
	K.P.: HLA-A*25:01, *26:01		
	T.H.: HLA-A*02:01, *25:01		

Table 4b: AbCross-crossmatch-ELISA results using three sera of the patient (taken in 06/2009, 07/2011 and 12/2012) which were tested for DSA against three selected stored leukocyte pellet lysates of virtual donors bearing the allele HLA-A*25:01.

All runs of the AMS-crossmatch ELISA using three serum samples of the patient (06/2009, 07/2011 and 12/2012) resulted in unequivocally positive signals by demonstrating anti-HLA class I DSA directed against these "donors" whereas anti-HLA class II antibodies were never detectable (Table 4b). It is easy to conclude that the availability of stored material from the primary donor in the year 1998 would have been resulted in adequate material for a crossmatch-ELISA-based analysis of DSA against this historical donor. Thus, allele-specific DSA as the reason for the rejection of the virtually HLA-identical graft would most probably have been detected earlier and with a higher validity/plausibility by ELISA-based *de facto* cross-matching than by unmeaning two-digit virtual cross-matching alone. This led only to the right conclusions with a long way round gone by high resolution (four-digit) typing.

Discussion

Based on the four exemplary cases it was the aim of the reports presented to provide data on the possibility to use deep frozen donors' material for cross-matching. Thus, the cases represent the initial approach to reliably and routinely use deceased donor's material in order to monitor possible emerging donor-specific anti-HLA antibodies accompanying a rejection episode. This approach presented here first provides a reliable opportunity to monitor a humoral allo-reactive anti-HLA immune response using a deceased donor's stored material for *de facto* cross-matching. Thus, an opportunity is provided to get data highly relevant for rejection episodes which are not solely based on the theoretical approach of virtual cross-matching.

Although the CDC-based crossmatch procedure, which was established as the prototype of cross-matching in the late sixties of the last century, still represents the current standard procedure an increasing number of this technique's drawbacks has been reported in the last 10 to 15 years. These reports have been authored mainly in the context of this assay's insufficiency in leading to valid results under certain prerequisites [4,5,15-21]. All these publications clearly demonstrate that the CDC-CM under certain circumstances has hardly the capacity to result in reliable and in the whole context of immunological diagnostics plausible identification of DSA. The discrepancies shown are due to the fact that the CDC-CM is a vitality assay the outcome of which depends on the activation of complement components added to the bound DSA. However, as discussed in the above mentioned publications, artificial factors which do not represent DSA also lead to an activation of the complement system, consequently falsifying this assays outcome by simulating positive reactions as mediated by DSA. Pharmaceutical treatment, especially the use of therapeutic antibodies such as Rituximab and Basiliximab both of which belong to the complement-activating IgG1-isotype, as well as cytostatic agents are noteworthy in this context [5,15-17]. Additionally, autoimmune diseases especially those of the complement-activating type III (immune complex type) such as lupus erythematosus and rheumatoid arthritis lead to false positive results of the CDC-CM [5,16,18-20].

Regardless of these increasingly discussed artefacts leading to false positive results of the CDC-based crossmatch and severely hampering the allocation of kidney allografts to certain groups of patients on the waiting list for kidney allografts it was the aim of the current report to point to the methodic aspect of using stored donor's material in order to perform a *de facto* crossmatch also against post mortem donors possibly deceased for years and consequently no more available in order to provide vital donor material. Generally DSA are in these cases identified solely by virtual cross-matching i.e., the comparison of antibody specificities of a given recipient with the HLA-antigens of the

corresponding donor. Limitations of virtual cross-matching based on HLA-typing at the low resolution (two-digit) level are shown using example 4. ELISA-based cross-matching first provides a diagnostic tool to overcome this limitation through the use of a given donor's retain sample which consists of non-vital but deep-frozen and in the long term storable material. To our best knowledge only one historical approach was performed about 18 years ago in order to use stored material. This approach was performed in the context of corneal grafting and used retinal pigment epithelial cells isolated from explanted eyes which had to be stored in liquid nitrogen. After their thawing the cells had to be re-cultured and stimulated with IFN-γ to upregulate the surface expression of HLA-molecules for the subsequent flow cytometry-based analysis [26,27]. This historical approach mandatorily using vital cells, however, must be regarded as time-consuming, expensive and technically very challenging. Thus, it is inappropriate for the routine task of any tissue typing laboratory in complete contrast to the ELISA-based technique presented here. Its most prominent advantage is that it does not require vital lymphocytes or other vital cells in general. In terms of technical and terminable practicability it is easily implementable in any laboratory without expensive and complex technical equipment.

There is an additional aspect which strongly supports the idea to perform *de facto* instead of virtual cross-matching or at least to supplement the virtual procedure by ELISA-based cross-matching which is based on so-called allele-specific antibodies increasingly described in literature [28-30]. These antibodies are directed only against one allele or a limited number of alleles of an HLA-phenotype defined by the two-digit level of resolution which as a matter of fact represents a whole group of alleles and may thus be characterized by various epitopes on different alleles. Therefore, the typing results performed at the level of two-digit typing of a given donor do not always plausibly allow the virtual identification of DSA. These antibodies may virtually appear as auto-antibodies [28-30] although directed against another allele of the same HLA-antigen if defined at the level of two-digit resolution. Furthermore, DSA defined by virtual cross-matching are generally not detectable if they are directed against rare alleles which are not immobilized as antigens in the various specification/identification assays commercially available. However, these antibodies are necessarily detectable if material of the real donor (i.e., material unavoidably including also a given donor's rare HLA-antigens) is used.

For a more systematic approach of these initial attempts a sufficient amount of donor-derived material is generally required for storing in order to possibly perform several future attempts of ELISA-based cross-matching including all donors. This has not been done for the initial exemplary cases presented here since using the samples for the detection of DSA was not the initial aim of their storage. However, in the meantime the conclusion has been drawn that the availability of splenic tissue, as is possible for nearly all post mortem donors, provides sufficient material. Thus, the opportunity is provided to establish something like a tissue bank comprising all donors for this special application. This systematic approach of providing sufficient donor material in order to enable at least five crossmatch-ELISA-based analyses using double preparations of the respective recipient's serum at two dilution steps (1:3 and 1:6) has been implemented in our laboratory since November 2014 for all lung and heart recipients.

Taken together the cases presented here clearly demonstrate the benefit by implementing ELISA-based cross-matching as an alternative technique first allowing the usage of stored donor's material as a routine application of any laboratory in order to monitor a donor-specific anti-HLA immune response.

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