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ORIGINAL ARTICLE

Complement-dependent cytotoxicity and Luminex technology for human leucocyte antigen antibody detection in kidney transplant candidates exposed to different sensitizing events

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Abstract

Background: The aim of this study was to determine the frequency of exposure to different sensitizing events (SEs) and to assess their effects on human leucocyte antigen (HLA) alloimmunization in transplant candidates using two different HLA antibody screening techniques: complement-dependent cytotoxicity (CDC) and Luminex.

Methods: This retrospective study included HLA antibody screening results for 163 patients on the kidney transplant waiting list (WL) tested from March 2012 until the end of December 2015 at the Tissue Typing Laboratory, Rijeka, Croatia. All sera samples were tested using the CDC and Luminex techniques in parallel.

Results: Two-thirds of the patients [114 (70%)] on the WL were exposed to transfusions, pregnancies and/or kidney transplant. The pre-transplant sera of 104 (63.80%) patients were negative for antibodies. In the sera of 23 (14.11%) patients, HLA antibodies were detected by CDC and Luminex and in the sera of 36 (22.09%) patients by Luminex only.

Conclusion: In patients on kidney WL, previous organ transplantation represents the strongest immunogenic stimulus, followed by blood transfusions (the most frequent SE) and pregnancies. Although Luminex is more sensitive than CDC in HLA antibody detection, the decision on unacceptable HLA antigens in WL patients has to be based on the results of both assays and the patient's immunization history.

Key words: HLA antibodies, HLA antibodies techniques, kidney transplantation, sensitization, waiting list

Introduction

Different sensitizing events (SEs), such as blood transfusions, pregnancies and previous transplants, may induce the development of alloantibodies against human leukocyte antigen (HLA) [1, 2]. In the organ recipient, preformed antibodies against graft antigens [donor-specific antibodies (DSAs)] may cause acute (hyper-acute) or chronic transplant rejection. The patients wait longer for transplantation and extended dialysis treatment results in lower graft survival than in non-sensitized patients [3–5]. Post-transplant development of *de novo* donor-specific antibodies causes a higher incidence of graft rejections and increased risk of graft loss [6].

The HLA system is the most polymorphic system in humans. The complexity of antigenic epitopes represents a significant challenge for tissue typing laboratories (TTLs) in developing appropriate methods to detect and characterize the repertoire of HLA antibodies present in sensitized transplant candidates.

The conventional and most widely used method for determining HLA antibodies is the cell-based complement-dependent cytotoxicity (CDC) assay that was introduced by Terasaki and McClelland in the early 1960s [7]. The CDC technique has been in use at TTL Rijeka since 1971, when the laboratory was founded as the first one in Croatia. The technique is based on HLA molecules displayed in their natural configuration [2]. In HLA antibody screening, the patient's serum is incubated with a panel of HLA-typed T and/or B lymphocytes, whose HLA alleles provide a representative sample of the studied population. Results are expressed as the percentage of panel lymphocytes that react with a patient's serum [panel reactive antibodies (PRA)]. As the same technique is used for crossmatching between the recipient serum and the potential donor's lymphocytes, PRA is useful in assessing the probability of a negative crossmatch result [8]. However, the CDC assay has a number of shortcomings related to the possibility of false negative (due to low antibody titres, non-cytotoxic antibodies or antibodies to HLA class II) or a false-positive result (the presence of autoantibodies, non-HLA antibodies or immune complexes) [9].

One of the main consequences of the CDC method's lack of sensitivity is the considerable rate of graft failure in transplantation after a negative CDC crossmatch [10]. In order to improve graft survival, new antibody detection methods were introduced, including flow cytometry (FC) and solid phase assay (SPA)–enzyme-linked immunosorbent assay (ELISA) and Luminex technology. While FC is cell based, SPA methods use purified HLA molecules attached to plates (ELISA) or microspheres (Luminex). These methods can detect lower levels of HLA antibodies, allowing more precise determination of the HLA antibody specificity and differentiation of antibodies that activate complement from those that are non-complement fixing [11]. Currently the Luminex technology is the most sensitive and has been in use at TTL Rijeka since 2012 [12]. It is a semi-quantitative bead-based immunoassay for detection of immunoglobulin G (IgG) and IgM antibodies to class I and class II HLA molecules that combines FC (xMAP technology) and fluorescent microparticles coated with HLA antigens at a high concentration [9]. This technique enables identification of very low titres of class I and II HLA antibodies, accurate definition of acceptable and unacceptable HLA antigens in highly sensitized patients, and determination of epitope specificity, which is important for graft outcome. It is very useful in DSA monitoring after transplantation. This technology is also highly sensitive, leading to the detection of clinically irrelevant antibodies. While some specificities detected by SPA are considered to be relevant, they are not an absolute contraindication to transplantation [1, 8].

The purpose of this study is to determine the frequency of exposure to SEs such as organ transplantation, blood transfusion and pregnancy and to assess their effects on HLA alloimmunization in patients on the kidney transplant waiting list (WL) using two different HLA antibody screening techniques in parallel: CDC and Luminex.

Materials and methods

We performed retrospective analysis of the HLA antibody screening results for 163 patients on the kidney transplant WL in Rijeka. A total of 664 sera samples were tested from March 2012 until the end of December 2015. Sera from all patients were tested by CDC, with or without di-thiotheitrol (DTT), and Luminex techniques in parallel. In order to compare the two techniques in terms of their detection of HLA IgG antibodies only, nine patients were excluded from the study, as their sera revealed the presence of non-HLA and/or IgM antibodies. Patients with a positive result in at least one serum sample were considered to be sensitized.

Information on SEs was obtained from potential recipients, their nephrologists and transfusion protocols for patients who underwent haemodialysis in Rijeka and from the questionnaire that accompanied each sample.

In our centre, patients on the WL are screened for HLA antibody presence every 3 months, four times per year, using CDC and Luminex techniques in parallel.

In the CDC assay the patient's serum is incubated with a panel of 50 HLA-typed unseparated T and B lymphocytes following standard procedures. HLA antibody screening was performed with and without DTT addition. A PRA > 5% was considered positive.

Serum screening by Luminex was performed in two stages according to the manufacturer's instructions. The first stage was tested by LIFECODES LifeScreen Deluxe Beads, LMX (Immucor Transplant Diagnostics, Stamford, CT, USA). Serum with a positive result in the LMX test was subjected to a second stage of testing by Luminex Single Antigen (LSA) beads (LIFECODES LSA Class I and/or LSA Class II Single Antigens, Immucor Transplant Diagnostics). Analysis was performed using a fluorocytometer (LABScan 200 Flow Analyser, Luminex, Austin, TX, USA). The results were analysed using MatchIt software (Immucor Transplant Diagnostics). Currently, no standard median fluorescence intensity (MFI) cut-off values exist, therefore the raw values of patient's serum >1000 were considered positive, as has been previously indicated in the literature.

Continuous data were expressed as the arithmetic mean or median and categorical data as n (%). To determine statistically significant differences between the two techniques used in HLA antibody detection, data were compared with the use of the chi quadrat test and the Mann–Whitney U test. A two-sided P-value <0.05 was considered statistically significant. Statistical analysis was performed using MedCalc version 12.13. (MedCalc Software, Ostend, Belgium).

Results

Of the 163 patients included in the analysis, 65 (39.88%) patients were female and 98 (60.12%) were male. The average age was 55.85 ± 11.86 years.

One-third of the patients [49 (30%)] on the WL were not exposed to any SEs. Two-thirds of patients [114 (70%)] were exposed to one of the SEs (63 subjects) or to combinations of two (43 subjects) or three SEs (8 subjects) (Figure 1).
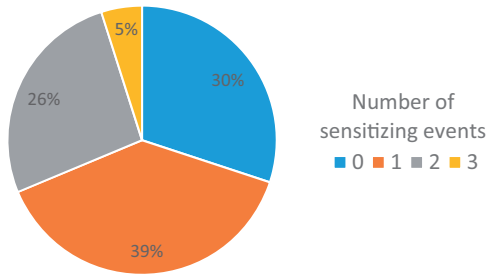
Fig. 1. Distribution of patients on the kidney WL exposed to SEs (previous transplantation, pregnancy and/or blood transfusion).

The number of sensitized patients grew proportionately with the number of sensitizing factors. HLA antibodies were detected (by Luminex assay) in 10 (20.41%) patients who did not have any SEs, in 19 (30.16%) patients after one SE, and in 22 (51.16%) patients after two SEs. Exposure to three SEs caused HLA antibody generation in all patients. The relationship between HLA sensitization and the number of SEs is shown in Figure 2.

Regardless of the combination, the most frequent SEs were blood transfusions, in 92 (56.44%) patients, while previous pregnancies were reported in 57 (34.97%) patients and previous transplants were performed in 24 (14.72%) patients.
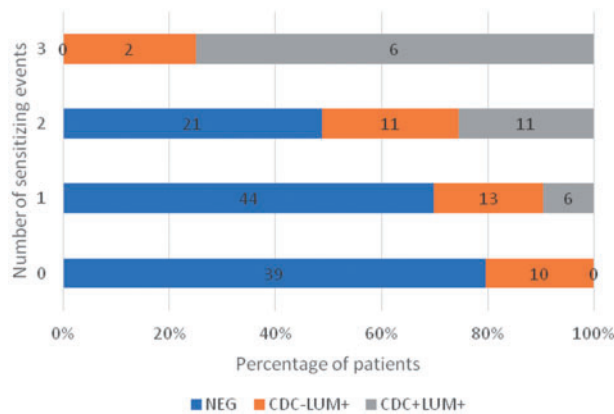

Fig. 2. Distribution of patients with and without detected HLA antibodies according to the number of SEs.

Considering the type of SE, 42 (25.77%) patients received a transfusion only, 20 (12.27%) women reported a history of previous pregnancies only and 1 (0.61%) patient had a previous transplant. In terms of combinations of SEs, 28 (17.18%) women had a history of both transfusions and pregnancies, 14 (8.59%) recipients had previous transfusions and transplants, only 1 (0.61%) patient had both a previous transfusion and pregnancy, while all three SEs were reported in 8 (4.91%) patients.

Figure 3 shows the proportion of the patients with detected HLA antibodies in pre-transplant sera according to the type of SE. Previous transplantation represents the strongest immunogenic stimulus regardless of the combinations with other SEs, while pregnancy as an isolated event was the weakest sensitizing factor.

HLA antibodies were not detected by either the CDC or Luminex techniques in the pre-transplant sera of 104 (63.80%) patients, while the sera of 59 (36.20%) patients were found to be positive for IgG HLA antibodies by the CDC and/or Luminex method.

In terms of the technique, HLA antibodies were detected in 23 (14.11%) patients by CDC and Luminex (CDC+LUM+) and in an additional 36 (22.09%) by Luminex only (CDC−LUM+). The difference between the proportion of patients with HLA antibodies detected by CDC and/or Luminex was not statistically significant ($\chi^2 = 0.18$, P = 0.673).

Table 1 shows HLA sensitization detected by different techniques according to exposure to the SEs in patients on the kidney transplant WL. Considering different SEs, all patients with a history of previous transplantation only, or in combination with another SE, were sensitized.

SEs induced the HLA sensitization in 49 patients; however, HLA antibodies were also detected in 10 patients without a history of SEs. These antibodies were detected by Luminex only. Figure 4A–C shows the prevalence of sensitized patients according to exposure to SEs and the class I and/or class II HLA antibodies detected by the two different techniques. Antibodies against mixed HLA antigens class I/II were mostly detected by the CDC and Luminex in parallel. Luminex was more sensitive in the detection of HLA class I, and especially of class II, compared with the CDC assay. Thus the CDC technique finds it difficult to distinguish class II antibodies using a panel of T and B lymphocytes.

The strength of the HLA antibody expressed as MFI was significantly higher in patients with antibodies detected by both techniques (CDC+LUM+) compared with the group of patients with antibodies detected by Luminex only (CDC−LUM+) for HLA class I antibodies (MFI 4789 versus 3086; P < 0.001) and class II
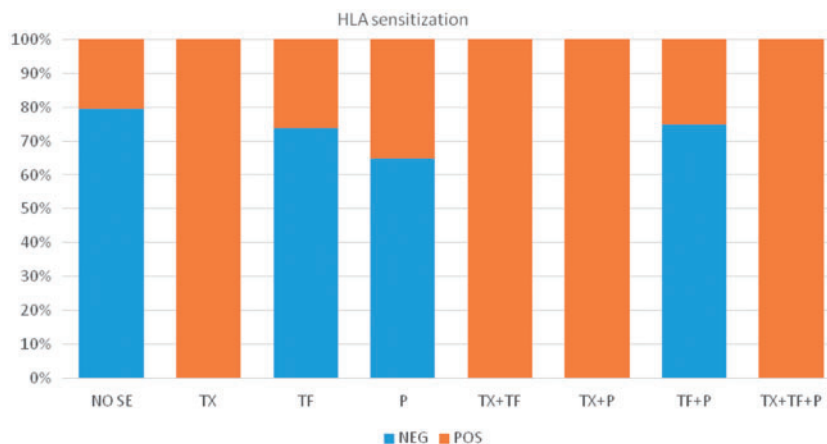

Fig. 3. Proportion of patients with and without HLA antibodies according to exposure to SEs. TX, transplantation; TF, blood transfusion; P, pregnancy.

Table 1. Total number and proportion of patients exposed to different SEs according to the HLA antibody screening results by the CDC and Luminex techniques

Type of SE	HLA antibodies screening results		
	Neg, n (%)	CDC−LUM+,n (%)	CDC+LUM+,n (%)
No SE (n = 49)	39 (79.59)	10 (20.41)	0
TX (n = 1)	0	1 (100.00)	0
TF (n = 42)	31 (73.81)	7 (16.67)	4 (9.52)
P (n = 20)	13 (65.00)	5 (25.00)	2 (10.00)
TX + TF (n = 14)	0	6 (42.86)	8 (57.14)
TX + P (n = 1)	0	0	1 (100.00)
TF + P (n = 28)	21 (75.00)	5 (17.86)	2 (7.14)
TX + TF + P (n = 8)	0	2 (25.00)	6 (75.00)
Total (n = 163)	104 (63.80)	36 (22.09)	23 (14.11)

Neg, negative; TX, transplantation; TF, blood transfusion; P, pregnancy.

antibodies (MFI 5715 versus 4606; P < 0.001). The results are shown in Figure 5A and B.

Ten patients who were not exposed to any senstizing event were HLA antibodies positive using Luminex technique and negative using CDC technique. Using Luminex technique Class I and Class II antibodies were detected. Among Class I antibodies 32 specificities were detected, and among Class II 21 specificities. Median values of peak MFI of HLA antibodies class I were significantly higher from median values of peak MFI of Class II specificities. (MFI 3324 versus 1779; P = 0.010) (Figure 6).

Discussion

Previous transplants, blood transfusions and pregnancies are major SEs that can cause HLA alloimmunization [13]. Two-thirds of the patients (69.94%) on the WL at our centre were exposed to one or more sensitizing factor, which is consistent with the literature [14, 15].

The risk of developing HLA antibodies is proportional to the number of sensitizing factors. The combination of SEs represents a stronger stimulus for HLA antibody generation than exposure to a single SE. In our study, most patients (38.65%) with a history of immunization had only one SE and HLA antibodies occurred in one-third (30.16%) of them, based on Luminex results. Fewer patients (26.38%) had been exposed to combinations of two SEs, and more than a half (51.16%) of these became sensitized. After exposure to three SEs (4.91%), HLA antibodies were detected in all patients (100%). Concerning the techniques used in antibody

detection, the difference in sensitivity between CDC and Luminex are interesting; the sensitivity mirrors the number of SEs. The higher sensitivity of Luminex is pronounced after exposure to a single SE (more patients had antibodies detected by Luminex only, while the CDC technique gave negative results). The difference between Luminex and CDC results disappears after two SEs, while exposure to three SEs caused sensitization detected by both techniques in almost all patients. The increased number of SEs causes antibody generation in higher concentrations that can be detected by less sensitive methods. The cumulative immunizing effect of different SEs has been recognized by several studies [13, 16].

The most frequent SE was blood transfusion. More than half (56.44%) of the patients received one or more red blood concentrates (RBCs), which is less than in previously published studies [14]. Most studies do not specify whether patients received transfusions after being placed on the WL or during the pre-transplant period. In this study, almost all transfused patients received RBCs (with or without leucocyte reduction) before registration on the WL. While being on the WL, only 11 (6.75%) patients were transfused. The US Renal Data System reports that 30% of transplant candidates on WLs received at least one blood transfusion, while Yabu et al. [13] reported only 8.87% [13, 17]. Other SEs were less frequent; about one-third of transplant candidates had a history of pregnancy (34.97%) and the SE with least prevalence was previous transplants (14.72%).

Evaluation of the impact of SEs on HLA sensitization varies according to the techniques used. Transfusions caused HLA antibody generation in only 9.52% of transplant candidates when the serum screening was performed by CDC, but when tested by Luminex, the rate of sensitized patients increased to 25.77%. In our study the positivity rate detected by CDC is lower than in published data. Opelz et al. [18] reported that 28% of transfused patients developed HLA antibodies in the CDC assay. However, Luminex results in our study are in accordance with other reports. Hyun et al. [19] reported a 33% positive rate in transfused patients, while Leffell et al. [20] reported 20%. The reports on the positivity rate detected by CDC are mostly old, and lower results in our study can be explained by the differences in lymphocyte panels among centres and the various approaches to anaemia treatment by transfusions of different types of blood units.

HLA sensitization was detected in 10% (CDC) versus 12.27% (Luminex) of women with a history of pregnancies. The reported incidence is 18–30% tested by the CDC method and >50% when tested by Luminex [21–23]. The age of women in the tested groups may be the cause of the different results, as the titre of HLA antibodies declines over time [24]. In our study, only one patient had previous transplant as the sole SE.
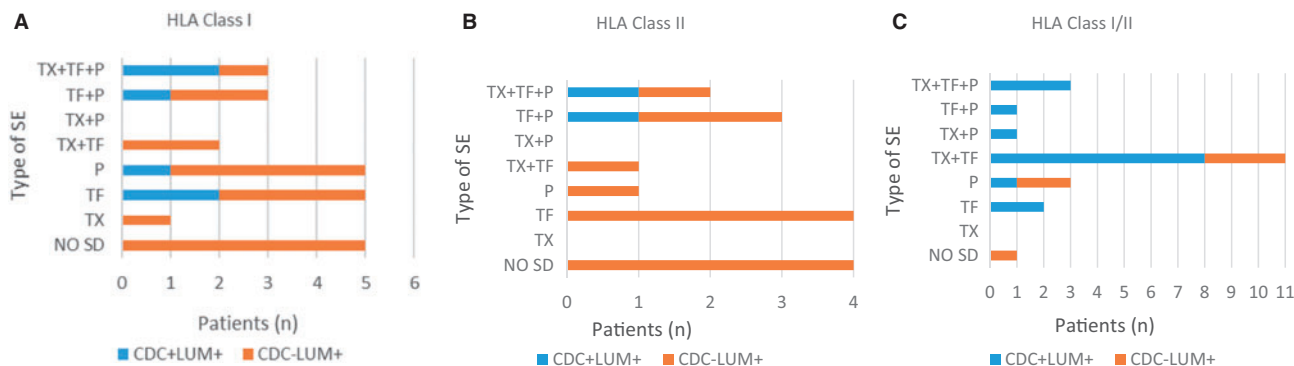

Fig. 4. Total number of patients with HLA antibodies (**A**) class I, (**B**) class II and (**C**) class I/II detected by CDC and/or Luminex. TX, transplantation; TF, blood transfusion; P, pregnancy.
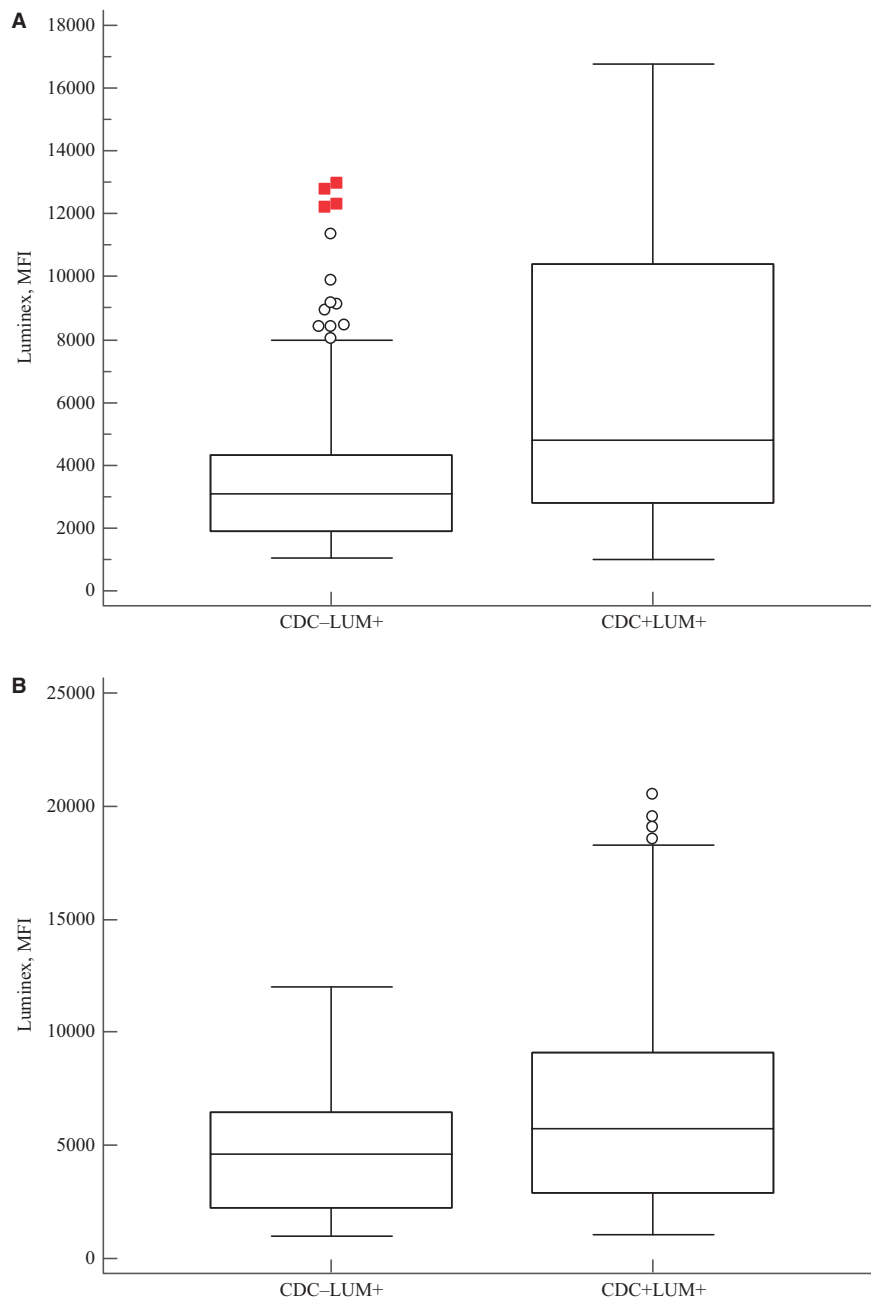
Fig. 5. Comparison of MFI values between HLA antibodies (**A**) class I and (**B**) class II detected by CDC and Luminex (CDC+LUM+) and antibodies detected by Luminex only (CDC−LUM+) in sensitized patients on the kidney transplant WL exposed to SEs. The box-and-whisker plot shows median values of peak MFI for every specificity of HLA antibody that is considered positive (MFI ≥ 1000) in each patient.

Considering the difference between HLA class I and II antibodies detected by the two techniques, the higher sensitivity of Luminex is evident when a single class is present in the tested serum, especially class II antibodies. In our laboratory, the CDC technique is performed using a panel of unseparated (T and B) lymphocytes. HLA class I antigens are expressed on T lymphocytes and class II antigens on B lymphocytes. In the whole lymphocyte population used in the CDC assay, the proportion of B lymphocytes is low, therefore the titre of HLA class II antibodies is below the sensitivity threshold of the CDC test. In patients with the presence of both class I and II antibodies, Luminex sensitivity is not so dominant when compared with the CDC technique. A likely explanation may be that most patients positive for both antibody classes had a history of previous kidney transplantation, confirming that solid organ transplantation represents the strongest immunizing event [19].

In our study, 10 male patients without reported SEs had antibodies that were detected by Luminex only. More specificities were found for class I antibodies. The MFI was low for both classes, but class I antibodies showed significantly higher MFIs compared with class II antibodies. The presence of HLA antibodies in non-immunized patients may be explained by the response to cross-reactive epitopes found in microorganisms, ingested proteins and allergens (natural alloantibodies), or as reactivity
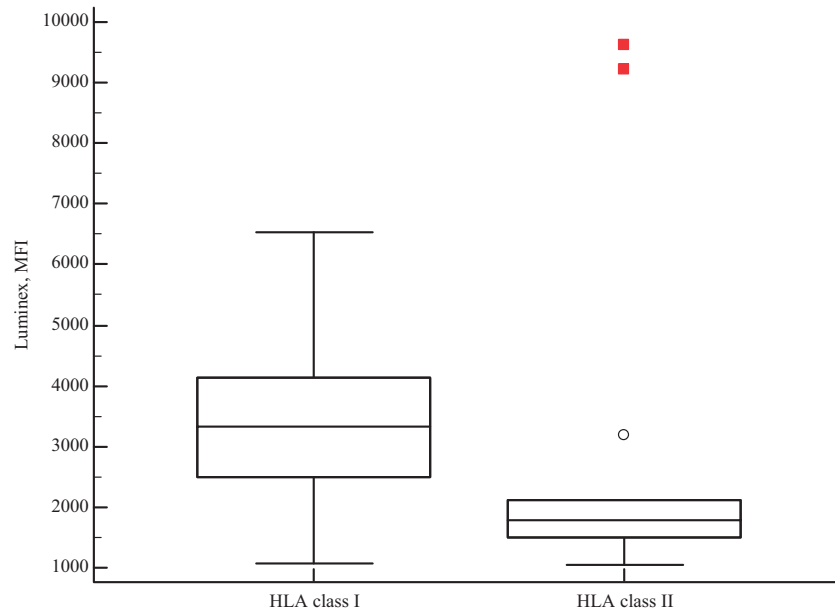
Fig. 6. Comparison of MFI values between HLA antibodies class I and II detected by CDC and Luminex in patients not exposed to SEs. The box-and-whisker plot shows median values of peak MFI for every specificity of HLA antibody class I and II that is considered positive (MFI ≥ 1000) in each patient.

directed against denatured molecules (neo-epitopes) and exposed cryptic epitopes resulting from possible conformational changes of the HLA protein that may occur during the manufacturing process [25–28].

Implementation of more sensitive techniques in HLA antibody detection has enabled new insights into their occurrence, activity and clinical relevance in organ transplantation. The association between DSAs detected by CDC with hyperacute or acute graft rejection has been known for decades [10]. Reports on the clinical importance of DSAs detected in Luminex-based assays only are conflicting. Several studies have shown that the presence of DSAs detected exclusively by Luminex has no clinical relevance to graft survival [29, 30]. Others, however, reported an association between DSAs and significantly increased graft loss even with a negative CDC and/or FC crossmatch [3, 31, 32]. The threshold of clinically relevant MFI values is also a matter of debate. Low levels of HLA DSAs detected by a Luminex assay before transplantation (MFI <1000 or <2000) are unlikely to have a deleterious effect on the graft [33, 34]. Several authors have demonstrated significantly lower graft survival in patients with higher MFIs [3, 35]. To assess the clinical impact of complement binding antibody on graft survival, several modifications of Luminex-based assays have been made. However, while some studies have shown that complement-fixing DSAs are more relevant in graft survival than non-complement-fixing DSAs [13, 36, 37], some authors were unable to demonstrate such an association [38, 39]. Further investigations and methodology advances are needed to bring us closer to these answers.

Conclusion

Exposure of patients on WLs to blood transfusions, pregnancies or previous transplants represents a risk factor of developing HLA antibodies that are considered a major immunologic barrier to successful transplantation. Blood transfusions are the most frequent and the most susceptible to our influence compared with other SEs. They should be minimized or avoided in transplant candidates whenever possible. HLA antibody testing

is critical in transplantation risk assessment, and CDC is widely used as a conventional cell-based method. It has low sensitivity and low resolution in determining antibody specificity, but high positive predictive value for antibody-mediated graft rejection. Luminex is a semi-quantitative SPA that is more sensitive than CDC, enabling better characterization of antibodies, but due to increased sensitivity, it may reveal the presence of antibodies with dubious clinical relevance. Based on the characteristics of both methods, the results have to be evaluated in combination with the clinical background and history of the patient's exposure to SEs in order to determine unacceptable HLA antigen mismatches in the pre-transplant period.

Conflict of interest statement

None declared.

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