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Anti-HLA-A, -B, -DR, -DQB1 and -DQA1 antibodies reactive epitope determination with HLAMatchmaker in multipare awaiting list for heart transplant

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ABSTRACT

Human leukocyte antigen (HLA) antibodies represent a significant risk factor for transplant failure.

It is very important to characterize anti-HLA antibodies as epitopes rather than antigens so that this knowledge can be applied clinically.

The aim of the study was to investigate the extra reactivity patterns in sensitized multipare. Here, we have used the HLAMatchmaker program, a theoretical algorithm, to explain these unexpected antibody reactivity patterns in multipare awaiting for heart transplant.

The patient was sensitized during pregnancy by alleles HLA-A*24:02, HLA-DRB1*07:01, HLA-DRB4*01:01, DQB1*02:02 and DQA1*02:01 mismatches with development of respective antibodies. However, the patient' sera were shown an unexpected reactivity not directed toward HLA mismatches of daughters: A*23:01, A*24:03 and B*15:12 for class I and DRB4*01:03, DRB1*09:02, DRB1*09:01, DQB1*03:01, DQB1*03:03, DQB1*03:02, DQB1*04:02, DQB1*04:01 and DQB1*02:01 for class II. By HLAMatchmaker analysis we found that these antibodies reacted with eplet shared by antigens in single allele Luminex panels. These eplets were: 62EE, 66GKH, 70KAH, 71HS, 127K, 113YH, 144KR, 150AAH, 151AHV, 163TG and 167DG for class I and 4Q, 74RRAE, 71RRA, 98KN, 120N, and 135G, 25FT, 34HE, 41ER, 47EK2, 48LF for class II. Thus, HLAMatchmaker software together with to solid phase techniques could open new horizons for a more precise characterization of the HLA-antibodies.

1. Introduction

HLA antibodies, usually produced by alloimmunization resulting from transfusions, pregnancies or transplants, increase the risk of transplant failure because they play a crucial role in the incidence of acute and chronic rejection [1]. Thus, identify specificities of antibodies to HLA accurately, could help organ allocation without disadvantaging sensitized patients. Currently, the importance of antibody-mediated rejection in cardiac and kidney transplant is widely described and accepted as one of the leading causes of graft dysfunction [2–4].

For many years, the complement-dependent cytotoxic (CDC) assay was the only available test to detect anti-HLA antibodies and it allowed preventing disastrous hyperacute rejections [2]. In the last decade, the identification anti-HLA antibodies has benefited of the introduction of more sensitive techniques as enzyme-linked immunosorbent assay (ELISA) and bead-based technology (including Flow Cytometry and Luminex) which detects both cytotoxic and non-cytotoxic antibodies. In particular, the detection of specific anti-HLA antibodies by single antigens is more specific, accurate and reproducible [5,6]. They have made it possible to characterize not only antibodies directed against antigens HLA-A, -B, DR but also C, DQA1, DQB1 and DP [7], in sera from sensitized patients. In addition, the improvement of the knowledge of the

Abbreviations: HLA, human leukocyte antigen; CDC, complement-dependent cytotoxicity crossmatch; ELISA, enzyme-linked immunosorbent assay; PRA, panel reactive antibody; SA, single antigens; MFI, mean fluorescence intensity units; PCR-SSP, polymerase chain reaction-sequence-specific primer; CDR, complementary determining region.

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crystallographic structure of HLA antigens and amino acid sequencing of HLA alleles have allowed advances the predicting immunogenicity [8].

Humoral sensitization is generally associated with anti-HLA antibodies specific for epitopes rather than antigens [9]. Epitopes are important not only for identifying acceptable mismatch for sensitized patients but also for a better understanding of the sensitization process induced by HLA mismatch [10].

The part of the antigen that makes contact with the antibody is defined as 'structural epitope'[11]. A structural epitope has about 15–25 contact residues in a surface area of 700–900 $Å^2$. Inside this area it lies the so-called 'functional epitope' consisting of amino acid residues that play a major role in the specific binding with antibody [12–16]. To be immunogenic, a structural epitope must have a functional epitope with at least one antibody-accessible nonself residue [15,16].

These concepts have been applied for antibody analysis to HLA-Matchmaker, which was developed by Duquesnoy [17]; this theoretical algorithm considers that each HLA antigen represents a collection of aminoacid patches (eplets) in antibody-accessible positions. These represent potential functional epitopes that can elicit HLA-specific alloantibody responses [18,19].

The eplets are characterized by a patch aminoacid residue within a radius of about 3 Å from a polymorphic residue on the HLA molecular surface [20]. The HLAMatchmaker program determines the eplets of HLA typed patients using high resolution (4-digit); successively, when it is used for antibody analysis, it defines the eplets that are different from those on the patient's HLA antigens for each allele in the Luminex panel. The eplets on patient alleles with positive reactions are compared to mismatched donor eplets that may explain the antibody reactivity pattern [20]. Certain antibodies are specific for single eplets, but many others recognize epitopes represented by pairs of eplets in different sequence locations.

Pregnancy represents a physiological condition in which the woman is confronted with an antigenically foreign body. Although during pregnancy the woman acquires a state of tolerance, however, a lot of multiparous showed anti-HLA antibodies against the paternal antigens [21–23]. Frequently, the study of patients sensitized for anti-HLA antibody, showed, also, an unexpected antibodies reactivity pattern.

In this study, our aim was to investigate the extra reactivity patterns by HLAMatchmaker antibody analysis in a sensitized multipare with anti-HLA-A, -B, -DR, -DQA and -DQB antibodies directed not only to paternal HLA mismatch(es).

2. Case description

A 54-year-old Caucasian woman patient, affected by heart failure secondary to ischemic heart disease, was listed for heart transplant; she was sensitized for anti-HLA antibodies.

The immunological tests for hepatitis B and C were negative. There was no history of transfusion or previous transplants or abortion. The patient had two daughters, with her last pregnancy 22 years before. Her husband was died.

The patient and daughters were HLA-A, -B, -C, -DR, -DQB1 and typed in low and high resolution using kits commercial (PCR-SSP-GenoVision-Olerup). Since the high-resolution typing was not available for all HLA loci, HLA typing was obtained as the most likely 4-digit alleles using the converter in the HLAMatchmaker program (http://www.HLAMatchmaker.net). HLA class I and II typings are summarized in Table 1. The daughters (not twins), have the same HLA phenotype. The mismatches between mother and daughters were: A*24:02, B*49:01, C*06:02, DRB1*07:01, DRB4*01:01, DQB1*02:02 and DQA1*02:01.

The patient's sera were obtained on different dates and collected by separation of clotted whole blood aliquots and frozen at −80 °C. They were screened for anti-HLA antibody by CDC and Luminex assay [5].

The CDC was performed by long incubation in order to increase the sensitivity of the cross-match test. This panel population consisted of 40 cells, from blood donors with known and well represented in our population HLA phenotypes. The B and T lymphocytes (2000 cells/μl) were separated by beads (Dynabeads Invitrogen Oslo, Norway). These panels were based on the availability of surrogate daughter cells with HLA specificities corresponding to patient antibodies. Briefly, to 1 μl of cells was dispensed serum undiluted and diluted 1:2 (2 μl) and positive and negative controls included for the purpose of quality control. After 1 h the complement and 2 h later the colorant, composed of acridine orange, ethidium bromide and quenching ink (Fluoroquench, One Lambda), were added to each well of the tray. If antibodies were present, the classical complement pathway was activated resulting in the lysis of cells by the membrane attach complex. Positive reactions were scored when the number of lysed cells in each well was 20% or more, negative reactions were scored for wells with 0–20% cell death. The number of lysed cells in the negative and positive control wells was monitored for each tray. The patient's sera were negative with all tested cells for both B and T cells. In addition, the CDC performed with both B and T cells of daughters resulted also negative; this was probably from the low titres of alloantibodies.

For the Luminex method, the study of antibodies were carried out using the LABScreen Panel Reactive Antibody (PRA) beads (LABScreen PRA: LS1PRA e LS2PRA; One Lambda) coated with purified class I and class II HLA antigens. Differently from the CDC, the patient's sera were always positive by Luminex assay. Particularly, the patient's sera were characterized by PRA = 30% for class I and PRA = 40% for class II. Subsequently, in order to characterize the antibody specificity, sera were tested with HLA class I and II coated beads recombinant rHLA single antigens (SA) (LS1A04 for class I and LS2A01 for class II; One Lambda). Tests were carried out according to the manufacturer's instructions as previously reported [5]. Briefly, 5 μl of serum samples were incubated with HLA class I- and class II-beads for 30 min in the dark under gentle agitation. The specimen was then washed three times before being incubated with 100 μl anti-human IgG-conjugated phyco-erythrine in the same conditions as in the first incubation. When the sample is passed through the detector, one laser excites the fluorochrome in the bead, which exhibits a unique signal and the other laser excites phyco-erythrine bound to the second antibody. The combination of these signals defines the specificity of the antibody in the test serum. The Labscan 200 flow analyzer (Luminex) was used for data acquisition. Data were then exported to HLA software (Fusion 2.0 One Lambda) for analysis.

Fluorescence intensity units (MFI) values of patient's sera were normalized with the appropriate negative control. MFI values greater than 1000 were accepted as "true" antibodies and 700–999 MFI as "possible" antibodies.

Fig. 1(a and b) shows how use of SA has revealed antibodies specificities directed toward the mismatches A*24:02, except for B49 and C*06:02 for class I and DRB1*07:01, DRB4*01:01, DQB1*02:02-DQA1*02:01, for class II; also, it has detected extra reactivity patterns: A*23:01, A*24:03 and B*15:12 for class I and DRB4*01:03, DRB1*09:02, DRB1*09:01, DQB1*03:01(DQ7), DQB1*03:03 (DQ9), DQB1*03:02 (DQ8), DQB1*04:02 (DQ4), DQB1*04:01(DQ4) and DQB1*02:01(DQ2) for class II.

For better interpretation of this reactivity pattern, we have used HLAMatchmaker. This program is posted on the website http://www.HLAMatchmaker.net.

Table 1
HLA class I and II typing of the patient and daughters.

HLA	Locus A	Locus B	Locus C	Locus DRB1	Locus DRB3/4/5	Locus DQB1	Locus DQA1
Patient	A*29:02; A*30:01	B*08:01; B*58:01	C*07:01	DRB1*08:01; DRB1*03:01	DRB3*01:01 (DR52)	DQB1*02:01; DQB1*04:02	DQA1*04:01; DQA1*05:01
Daughters	A*24:02; A*30:01	B*08:01; B*49:01	C*06:02; C*07:01	DRB1*07:01; DRB1*03:01	DRB3*01:01 (DR52) DRB4*01:01 (DR53)	DQB1*02:01; DQB1*02:02	DQA1*02:01; DQA1*05:01

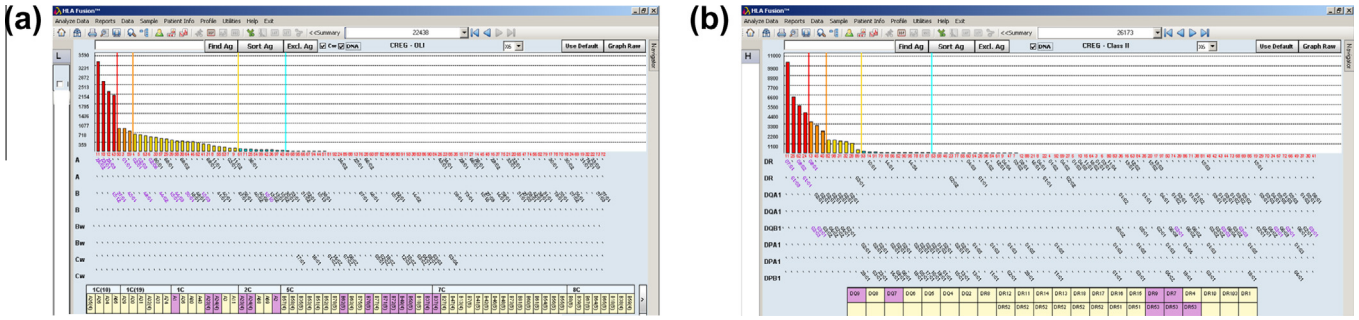

Fig. 1. (a) LabScreen single-antigen assay with an R-PE-conjugated anti-human HLA-A, -B, -C, monoclonal antibody, tested with regular beads. (b) LabScreen single-antigen assay with an R-PE-conjugated anti-human HLA-DR, -DQ monoclonal antibody, tested with regular beads. The *y* axis indicates the mean fluorescence intensity values and the *x* axis denotes single-antigen beads.

The HLAMatchmaker program determines HLA antibody reactivity using an algorithm to assign an eplet to each HLA antigen. Therefore, with this program, we searched one or more eplets present on the same group of antigens and/or alleles of the Luminex panel and with similar aminoacid residue compositions.

In Table 2a, we showed antibody reactive patterns class I of serum samples determined in the SA assays and informative eplet on reactive alleles by HLAMatchmaker analyses program. The Luminex results are presented as the normalized trimmed mean of MFI values. In particularly, are listed allele specificity of class

Table 2
(a) Antibody reactive patterns class I of serum samples determined in the SA assays and informative eplet on reactive alleles by – HLAMatchmaker analyses program: A*24:02, A*23:01, A*24:03 and B*15:12; (b) antibody reactive patterns class II of serum samples determined in the SA assays and informative eplet on reactive alleles by HLAMatchmaker analyses program: DRB1*07:01, DRB4*01:03, DRB1*09:02, DRB4*01:01, DRB1*09:01, DQA1*02:01–DQB1*03:01, DQA1*02:01–DQB1*03:03, DQA1*02:01–DQB1*03:02, DQA1*02:01–DQB1*02:02, DQA1*02:01–DQB1*04:02, DQA1*02:01–DQB1*04:01, DQA1*02:01–DQB1*02:01.

Bead	MFI	Specificity	Allele specificity		Informative eplet on reactive alleles
(a)					
	6205		Positive control		
	19		Negative control		
011	2655	A24	A*24:02 immunizer	11	,,,,,,62EE,,66GKH,,70KAH,71HS,,,,,,127K,,,,113YH,,,144KR,150AAH,151AHV,,163TG,167DG,,,,,,,,
010	2394	A23	A*23:01	8	,,,,,,62EE,,66GKH,,70KAH,71HS,,,,,,127K,,,,113YH,,,,,,163TG,167DG,,,,,,,,
012	1721	A24	A*24:03	9	,,,,,,62EE,,66GKH,,70KAH,71HS,,,,,,127K,,,,113YH,,,144KR,150AAH,151AHV,,,,,,,,,,
043	1317	B76, Bw6	B*15:12	5	,,,,,,,,,,,,,,,,,,,,,,145ERA,150AAH,151AHA,152HA,163RW,,,,,,,,
(b)					
	4522		Positive control		
	14		Negative control		
011	7018	DR7	DRB1*07:01 **immunizer**	9	4Q,,,,,,26QF3,26KFE,,,,40EFD,47EYR,,,,67IR,,,,,76GDT,180VM,,,98ES,,,,,
025	3595	DR53	DRB4*01:03	10	4Q,,,,,,,,32IYN,,,,48YQ6,,,,67LR,70LRRA,71RRA,73AEDT,74RRAE,,,,,98KN,,120N,,,
062	3354	DR9	DRB1*09:02	1	4Q,,,14FEH,,,,26KYH,31QGIY,32IYN,,40HFD,,,,,,,71RRA,73AEDT,74RRAE,,180VM,,,98ES,,,,,
				1	
024	3200	DR53	DRB4*01:01, **immunizer**	1	4Q,,,,,,,,32IYN,,,135S,48YQ6,,,,67LR,70LRRA,71RRA,73AEDT,74RRAE,,,,,98KN,,120N,,,
				1	
013	2010	DR9	DRB1*09:01	11	4Q,,,14FEH,,,,26KYH,31QGIY,32IYN,,40HFD,,,,,,,71RRA,73AEDT,74RRAE,,180VM,,,98ES,,,,,
040	1827	DQ7	**DQA1*02:01, immunizer** DQB1*03:01	5	,,25FT,34HE,41ER,,47EK2,48LF,,,,,,,,,,
045	1779	DQ9	**DQA1*02:01, immunizer** DQB1*03:03	5	,,25FT,34HE,41ER,,47EK2,48LF,,,,,,,,,,
082	1001	DQ8	**DQA1*02:01, immunizer** DQB1*03:02	5	,,25FT,34HE,41ER,,47EK2,48LF,,,,,,,,,,
031	1055	DQ2	**DQA1*02:01, immunizer**	5	,,25FT,34HE,41ER,,47EK2,48LF,,,,,,,,,,
			DQB1*02:02 immunizer	1	,,,,,,,,,,,,,,,,,,,,,,135G,,,
033	903	DQ4	**DQA1*02:01, immunizer** DQB1*04:02	5	,,25FT,34HE,41ER,,47EK2,48LF,,,,,,,,,,
032	785	DQ4	**DQA1*02:01, immunizer** DQB1*04:01	5	,,25FT,34HE,41ER,,47EK2,48LF,,,,,,,,,,
028	815	DQ2	**DQA1*02:01, immunizer** DQB1*02:01	5	,,25FT,34HE,41ER,,47EK2,48LF,,,,,,,,,,

I (A*24:02; A*23:01; A*24:03 and B*15:12) gave positive reactions (MFI > 1000) and the informative eplets that are mismatched for the patient alleles.

These positive reactions can be explained by antibody reactivity toward 11 eplets on daughters mismatches: eplets 62EE, 66GKH, 70KAH, 71HS, 127K, 113YH, 144KR, 150AAH, 151AHV, 163TG and 167DG (shared by HLA-A*24:02).

In Table 2b are listed allele antibodies specificity of class II (DRB1*07:01; DRB4*01:03; DRB1*09:02; DRB4*01:01; DRB1*09:01; DQA1*02:01–DQB1*03:01; DQA1*02:01–DQB1*03:03; DQA1*02:01–DQB1*03:02; DQA1*02:01–DQB1*02:02; DQA1*02:01–DQB1*04:02; DQA1*02:01–DQB1*04:01; DQA1*02:01–DQB1*02:01) gave positive reactions with their MFI values and the informative eplets.

These positive reactions can be explained by antibody reactivity toward 10 eplets on daughters mismatches: eplet 4Q (shared by HLA-DRB1:*07:01, DRB4*01:01), eplets 71RRA, 74RRAE and 120N (shared by HLA-DRB4*01:01), eplets 25FT, 34HE, 41ER, 47EK2, 48LF (shared by HLA-DQA1*02:01) and eplet 135G (shared by DQB1*02:02).

Indeed, this would explain unexpected reactivity not directed to daughters HLA mismatches: anti-A*23:01, A*24:03 due to eplets 62EE, 66GKH, 70KAH, 71HS, 127K, 113YH, and partially 144KR, 150AAH, 151AHV, 163TG and 167DG; anti-B*15:12 due to eplet 150AAH; anti-DRB4*01:03 due to eplets: 4Q, 74RRAE, 71RRA, 98KN, 120N; anti-DRB1*09:02 and DRB1*09:01 due to eplets: 4Q, 74RRAE, 71RRA; anti-DQB1*03:01, DQB1*03:03, DQB1*03:02 due to eplets: 25FT, 34HE, 41ER, 47EK2, 48LF. In the present case, the patient has a positive reaction against all beads coated with DQA1*02:01 in combination with others beads coated with non-self-β (31, 32, 40, 45, 82, 98) and self-β (28, 89) chains. Beads coated with molecules different from DQA1*02:01 are negative, indicating that the epitopes were confined to the DQα (DQA1*02:01) (Fig 1b).

3. Conclusion

In our study, the analysis antibodies pattern with the help of HLAMatchmaker program, suggested potential additional reactions based on identification of shared eplets between the immunizing HLA and the extra antibody reactivity observed in the single antigens bead Luminex analysis.

We hypothesized that the sensitization of the patient was caused by her exposure to paternal HLA alloantigens expressed by fetal cells during pregnancies. Previous studies on human monoclonal antibodies have demonstrated that certain HLA epitopes require a pair of eplets on the same allele [24]. These eplets must be close enough on the molecular surface so they can make contact with the antibody-specificity-mediating complementary determining region (CDR) loop, and the second CDR loop referred to as a critical contact site necessary for sufficient antibody binding, allowing high affinity and avidity. Antigens that have only the specific eplet will give negative or weak reactions with antibody. This concept may also explain why antibodies react with certain antigens in binding assays but not in complement-dependent lymphocytotoxicity [25]. In addition, many studies have been done for a correlation between mean fluorescence intensity by Luminex with positive CDC [26–28]. More recently, Yanagida et al. [29] reported that MFI > 10,000 correlates with positive CDC in a cohort of patients waiting time to heart transplant.

HLAMatchmaker software together with to solid phase techniques could open new horizons for a more precise characterization of the HLA-antibodies. In conclusion, the key points illustrated by the case are the followings: (1) HLAMatcmarker has expanded our knowledge on the interpretation of positive MFI values with alleles in Luminex panels. Indeed, the ignoring of such shared epitopes may lead to erroneous interpretations about the identification of antibody specificities; (2) often sera so-called 'polyspecific' reflect an overvaluation of the sensitization status and this could be avoided by a specific epitope analysis; (3) a large debate still remains on which ones are clinically relevant; (4) extention of HLA typing 4-digit alleles (HLA-A, -B, -C, -DR, -DQA, -DQB, and -DP), for both immunizer and recipient, facilitates the interpretation of results.

In the future, more cases are needed to validate application of the HLAMatchmaker in clinical practice.

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