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Characterization of an antibody specific for HLA-A36 and not reactive with HLA-A1

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ABSTRACT

Humoral sensitization to HLA often results in antibodies to public determinants shared among two or more antigens. Although monoclonal antibodies to A36 have been produced, there are no reports of polyclonal antibodies that react with A36 but not A1. We report here sera from a heart transplant recipient that reacted with A36 but not A1 in tests with both phenotype and single antigen panels on the Luminex platform. Flow cytometric crossmatch tests yielded positive results with an A36 bearing phenotype but not with a phenotype containing A1. A36 reactivity in solid phase assays was abrogated by absorption with cells bearing A36, but not with A1-positive cells. The frequency of B cells in this patient specific for A1 was comparable to that for individuals not sensitized to A1. These data indicate that reactivity was to an epitope present on A36 but absent from A1.

1. Introduction

HLA–A36 was first identified in 1972 by Festenstein et al. [1], who showed that reactivity with A36 was seen only in sera that also reacted with A1. These data suggested that A36 did not have unique or "private" epitopes but, rather, had only epitopes that were also present on HLA-A1 [2]. In 2002, a murine monoclonal antibody was produced that reacted with A36 but not A1 [3]. This indicated the presence of a xenogeneic epitope. However, the epitope was not necessarily allogeneic. To date there have been no reports of polyclonal antibodies that react with A36 but not A1. We report here a serum reactive with A36 but not A1, occurring in a heart transplant recipient, indicating the presence of an allogeneic epitope on HLA-A36 that is not present on HLA-A1.

2. Subjects and methods

2.1. Patient

The patient is female and 18 years of age, with the HLA phenotype HLA-A29, -A31; -B44, -B60; -Cw10, -Cw16; -DR4, -DR15; -DQ6, -DQ8. She received a heart transplant at 2 weeks of age from a deceased donor with the phenotype HLA-A3, -A24; -B7, -B57; -Cw6; -DR2, -DR7; -DQ1, -DQ2. In 2007, the patient was referred to us as a potential renal transplant recipient, and in April of 2007 she received a kidney transplant from a living related donor who was identical to the patient at the HLA-A, -B, -C, -DRB1, -DR3-4–5, and -DQB1 loci. HLA typing was performed by reverse sequence specific oglionucleotide probe hybridization on the Luminex platform (One Lambda LabType, Canoga Park, CA) according to manufacturer's instructions.

2.2. Antibody testing

Sera were tested by multianalyte bead assays on the Luminex platform according to manufacturers' instructions. The assays used included both phenotype panels (Lifecodes ID, Gen-probe Life Sciences, Stamford, CT) and single antigen panels (Single Antigen Beads, One Lambda, Inc., Conoga Park, CA). Analysis of HLA-specific antibody was performed manually by two or more experienced individuals. Study sera were tested undiluted before and after undergoing hypotonic dialysis to eliminate IgM and other potential interfering factors. Hypotonic dialysis was performed as reported previously [4]. Briefly, the serum was placed in a Slide-A-Lyzer Dialysis Cassette (Thermo Scientific, Rockford, IL) and dialyzed against double distilled, de-ionized water overnight at 4° centigrade. Normal tonicity was restored by dialysis of the supernatant fluid versus phosphate-buffered saline.

2.3. Flow cytometric crossmatch testing

Three-color flow cytometric crossmatch (FCXM) tests were performed on a Becton-Dickinson FACSCalibur as described previously [5,6]. The second antibody used was an IgG heavy chain-specific, murine monoclonal antibody labeled with fluoroscein isothiocyanate (FITC) (BD Biosciences, San Jose, CA). T cells were labeled using monoclonal anti-CD3 antibody, and B cells were labeled using monoclonal anti-CD19 antibody (BD Biosciences, San Jose, CA). Results were expressed as a ratio of the median channel fluores-

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Table 1
Results of flow cytometric crossmatches with serum JN

Target cell source	Subject 1 A30, **A36**; B53; DR1, DR13; DR52; DQ5, DQ6			Subject 2 A1, A3; B35 B57; DR7, DR15; DR51, DR53N; DQ6, DQ9			Subject 3 A2; B41, B37; DR10, DR13; DR52; DQ5, DQ7		
Serum	MESF	Ratio[a]	Interpretation[b]	MESF	Ratio[a]	Interpretation[b]	MESF	Ratio[a]	Interpretation[b]
CD3/ negative control	2194	1.00		2382	1.00		2735	1.00	
CD3/ serum JN-1X	2405	1.09	Negative	2194	0.92	Negative	2588	0.95	Negative
CD19/ negative control	3200	1.00		1589	1.00		2264	1.00	
CD19/ serum JN-1X	11,940	5.23	Positive	1575	0.67	Negative	2811	1.03	Negative

Flow cytometric crossmatches were performed using target cells from individuals expressing A36 but not A1 (Subject 1), A1 but not A36 (Subject 2), and from an autologous surrogate (Subject 3). The HLA phenotypes of each subject are shown below the subject identifier. Sera were tested undiluted (1X).
MESF, molecules of equivalent soluble fluorochromes
[a]Ratio of the median channel fluorescence of the test serum to that of the negative control.
[b]Ratios >3 and MESF values > 6000 are considered positive.

cence of the patient serum to that of the negative control serum and as molecules of soluble fluorochromes (MESF). Ratios greater than or equal to 2.5 and MESF values greater than 6000 were considered positive [7].

2.4. Quantification of HLA-specific B cells

HLA-specific B cells were labeled with HLA-A1 tetramers (Beckman-Coulter, La Brea, CA) as described previously [8]. Briefly, a B-cell enriched lymphocyte preparation was incubated with phycoerithrin (PE)–labeled HLA tetramers and a CD19-specific monoclonal antibody (BD Biosciences, San Jose, CA). Cells were examined by flow cytometry and the percentage of tetramer positive cells among the $CD19^+$ cells was determined.

2.5. Antibody absorption

Absorptions were performed in triplicate using equal volumes of washed packed cells and patient serum. Two absorptions were performed for 1 hour each at 18 –20⁰C and the third at 4⁰C overnight. At the end of each incubation, the cells were sedimented by centrifugation, and the serum was decanted and added to a tube containing fresh cells. Absorption with an autologous surrogate was performed in parallel as a control.

3. Results

3.1. Antibody characterization

Solid phase assays of four sequential sera obtained over a 3 month period yielded positive reactions with only two HLA class I phenotypes: HLA-A36, -A2; -B7, -B72; -Cw2, -Cw7, and HLA-A36, -A66; -B42, -B58; -Cw6, -Cw17 with MFI values of 4675 and 2622, respectively. Seven A1 containing phenotypes yielded negative reactions with MFI values from 63 to 135. One serum was tested on a single antigen panel and had a positive reaction with a bead bearing A*36:01 (MFI = 4429), and negative reactions with all other beads including a normalized value of zero with the A*01:01 bearing bead.

3.2. Flow cytometric crossmatch

Target cells in the crossmatch tests were from three individuals with the following phenotypes: (1) HLA-A30, -A36; -Cw4; -B53; -DR1, -DR13; -DQ5, -DQ6; (2) HLA-A1, -A3; -Cw4, -Cw6; -B35, -B57; -DR7, -DR15; -DQ6, -DQ9; and (3) HLA-A2; -Cw6, -Cw7; -B41, -B37; -DR10, -DR13; -DQ5, -DQ7. The serum yielded a negative reaction with T cells from all subjects and with B cells from subject 2 (ratio = 0.67, 1575 MESF) and subject3 (ratio = 1.03, 2811 MESF). The serum yielded a positive reaction with B cells from subject 1, whose phenotype included the A36 antigen (ratio = 5.23, 11940 MESF) (Table 1).

3.3. Tetramers

We have previously shown that sensitization to an HLA antigen can be detected in the absence of antibody as an increase in the frequency of B cells labeled with a tetramer comprised of that HLA antigen. To test the possibility that the patient producing A36 specific antibody had been sensitized to A1, we tested the B cells of the patient with HLA-A1 tetramers. We found that 0.51% of the $CD19^+$ B cells were positive for HLA-A1 which is comparable to the frequency found in a nonsensitized healthy individual. Unfortunately, A36 tetramers were not available for testing.

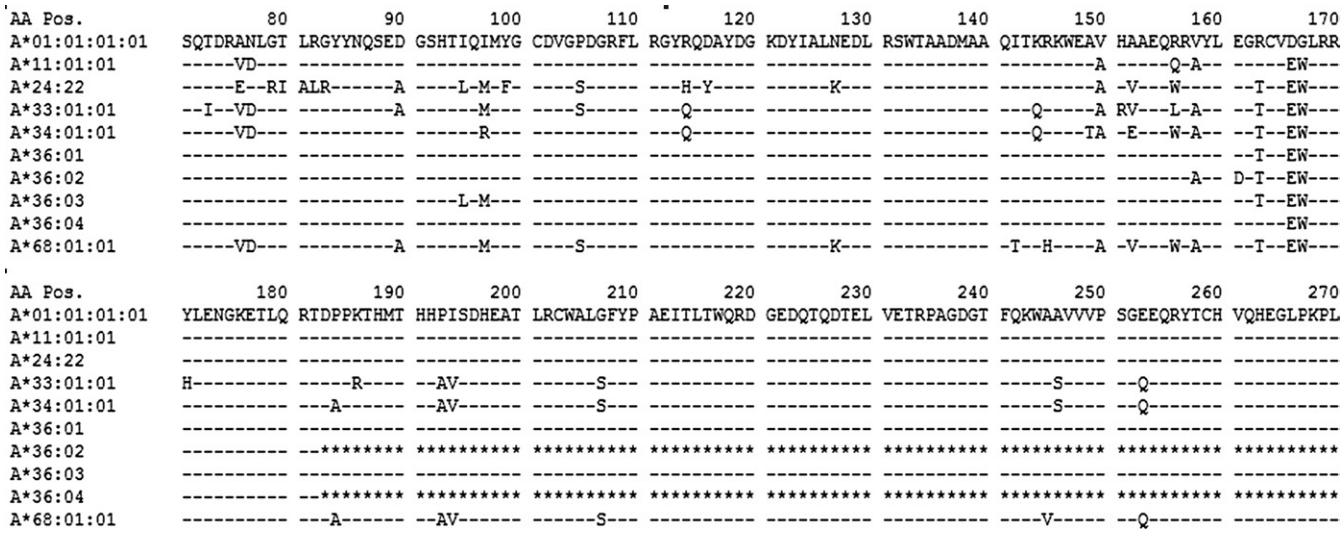
3.4. Antibody absorption

We performed absorptions of the A36-reactive serum to further confirm specificity for A36 and a lack of cross reactivity with A1. Absorptions were performed with cells from EBV-transformed lymphoid cell lines bearing A1 but not A36 (HLA-A1; -C7; -B8; -DR17; -DQ2), A36 but not A1 (HLA-A30, -A36; -C4; -B53; -DR1, -DR13; -DQ5, -DQ6), and neither A1 nor A36 (HLA-A29; -B44; -DR7; -DQ4). The third cell line was a surrogate for the patient and was used to assess any potential nonspecific effect of absorption. As shown in Table 2, absorption with any of the cell lines substantially increased reactivity with the positive control bead as well as with some other beads. This has been observed in other cases and is attributable to removal of nonspecific blocking factors. Reactivity with the A36-bearing bead was abrogated only by absorption with the A36-bearing cells and not with cells bearing A1 or with the autologous surrogate. A24 reactivity is included to show that the sensitization from the patient's heart transplant to A24 did not result in production of A24 antibody.

Table 2
Reactivity of absorbed and untreated serum with selected beads

Bead	Phenotypes of cell lines used for absorption			
	None (untreated serum)	A30, A36; Cw4; B53; DR1, DR13; DQ5, DQ6	A1; Cw7; B8; DR17; DQ2	A29; B44; DR7; DQ4
Positive control	8436	17,715	14,563	19,097
HLA-A*36: 01	4229	0	9876	6373
HLA-A*68: 01	799	1656	2636	1459
HLA-A*24: 02	0	0	0	0
HLA-A*01: 01	0	0	0	0
HLA-A*33: 03	636	1379	2949	1128
HLA-A*34: 02	786	1311	1947	1139

A36-reactive serum was absorbed with A36+A1− and A36-A1+ to confirm specificity for A36 and lack of crossreactivity with A1. Absorption with a third cell line, an autologous surrogate, was performed to assess potential dilution due to the absorption procedure. All absorptions resulted in increased activity with the positive control bead and the A68 bead indicating the removal of nonspecific factors present in untreated serum. Absorptions demonstrate specific removal of antibody reactivity only by A36-bearing cells.



AA Pos.	80	90	100	110	120	130	140	150	160	170
A*01:01:01:01	SQTDRANLGI	LRGYYNQSED	GSHTIQIMYG	CDVGPDGRFL	RGYRQDAYDG	KDYIALNEDL	RSWTAADMAA	QITKRKWEAV	HAAEQRRVYL	EGRCVDGLRR
A*11:01:01	-----VD---	----------	----------	----------	----------	----------	----------	---------A	-----Q-A--	-----EW---
A*24:22	-----E--RI	ALR------A	----L-M-F-	----S-----	---H-Y----	------K---	----------	---------A	-V---W----	--T--EW---
A*33:01:01	--I--VD---	---------A	------M---	----S-----	---Q------	----------	----------	---Q-----A	RV---L-A--	--T--EW---
A*34:01:01	-----VD---	----------	------R---	----------	---Q------	----------	----------	---Q----TA	-E---W-A--	--T--EW---
A*36:01	----------	----------	----------	----------	----------	----------	----------	----------	----------	--T--EW---
A*36:02	----------	----------	----------	----------	----------	----------	----------	----------	-------A--	D-T--EW---
A*36:03	----------	----------	----L-M---	----------	----------	----------	----------	----------	----------	--T--EW---
A*36:04	----------	----------	----------	----------	----------	----------	----------	----------	----------	-----EW---
A*68:01:01	-----VD---	---------A	------M---	----S-----	----------	------K---	----------	-T--H----A	-V---W-A--	--T--EW---

AA Pos.	180	190	200	210	220	230	240	250	260	270
A*01:01:01:01	YLENGKETLQ	RTDPPKTHMT	HHPISDHEAT	LRCWALGFYP	AEITLTWQRD	GEDQTQDTEL	VETRPAGDGT	FQKWAAVVVP	SGEEQRYTCH	VQHEGLPKPL
A*11:01:01	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------
A*24:22	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------
A*33:01:01	H---------	-----R----	--AV------	------S---	----------	----------	----------	-----S----	--Q-------	----------
A*34:01:01	----------	---A------	--AV------	------S---	----------	----------	----------	-----S----	--Q-------	----------
A*36:01	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------
A*36:02	----------	--********	**********	**********	**********	**********	**********	**********	**********	**********
A*36:03	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------
A*36:04	----------	--********	**********	**********	**********	**********	**********	**********	**********	**********
A*68:01:01	----------	---A------	--AV------	------S---	----------	----------	----------	----V-----	--Q-------	----------

Fig. 1. Portion of a HLA sequence alignment from codons 71 to 270 using A*01:01:01:01 as a reference sequence. The alignment compares A*11:01, 24:22, A*33:01, A*34:01, A*36:01, A*36:02, A*36:03, A*36:04, and A*68:01 to illustrate a novel epitope, located between codons 158 and 167, and unique to A36 and not A1.

4. Discussion

We provide here the first report of a polyclonal alloantibody reactive with HLA-A36 but with not with A1. The serum was from a heart transplant recipient and multiple sera from the same patient demonstrated binding to HLA-A36 but not to A1 on solid phase assays using both single antigen assays and phenotype panels on the Luminex platform. It is known that soluble HLA molecules may expose non-native epitopes when bound to solid matrix [9]. Therefore, flow cytometric crossmatch tests were performed to assess reactivity with antigens on cells from healthy individuals whose antigens should be predominantly in their native state. These tests yielded results comparable to those of the solid phase assays with positive reactivity seen with A36-bearing cells but not with A1-bearing cells. In these tests, positive reactions were obtained with B cells but not T cells, which we attribute to the much higher concentration of class I antigens on B cells compared with T cells [10].

The specificity for A36 was defined in both solid phase and cell-based assays. Absorption with A36-bearing cells completely abrogated reactivity, whereas absorption with A1-bearing cells and with an autologous surrogate did not, further confirming specificity for A36. However, it is possible that the reactivity in assays resulted from synergy between antibodies that may have included antibody specific for A1 but was predominantly antibody specific for A36. If so, absorption with A1-bearing cells should have diminished or eliminated reactivity with the A36-bearing bead. Because absorption itself increased reactivity uniformly with the exception of absorption with A36 bearing cells, it could be argued that it is not possible to observe any diminution of reactivity resulting from absorption with A1-bearing cells. However, reactivity with the A36 bead was strongest after absorption with A1, and we believe that this indicates that A1 did not diminish reactivity with A36.

We had also considered that the antibody reactivity might reflect quantitative rather than qualititative differences in epitopes or a difference in binding avidity. Therefore, we determined the frequency of A1-specific B cells as a measurement of sensitization to A1, and we found that the frequency of A1-specific B cells in the patient was comparable to that in a healthy nonsensitized individual, indicating a lack of sensitization to A1. Unfortunately, lack of HLA-A36 tetramers did not permit us to test for A36-specific B cells.

The results presented here are consistent with the presence of an epitope expressed on A36 that is not present on A1. Sequence alignments comparing A*36:01, A*36:03, and A*01:01 indicate a sequence between residues 158-167 that is present on A*36:01, A*36:03, and on A*24:22 but absent from A*01:01 (Fig. 1) [11,12,13]. It is of interest that A24 was a mismatched antigen present in the donor of the heart transplant; however the frequency of the A*24:22 allele is low, and is therefore an unlikely cause of sensitization to this epitope. Moreover, there was no antibody to A24 detected in the solid phase assays. There are additional antigens that possess this same sequence, indicating that the route of sensitization may have been from an alternative source, such as transfusion. Interestingly, removal of interference with the absorption procedure revealed moderately low reactivity with A28, A33, and A34. Sequence alignments show that an epitope shared between A36, A28, A33, and A34, but not found on A1, is formed by substitutions at residues 163 and 166 –167. Identification of this antibody was possible because of the availability of solid phase immunoassays. The strength of the antibody determined by the assays is not sufficient to yield positive reactions in the cytotoxicity assay. It is possible that the widespread use of the solid phase assays will reveal additional sera with similar reactions and may confirm cross-reactivity with A28, A33, and A34.

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