2 Preoperative Evaluation of Sensitized Patients

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Q: What kinds of laboratory tests should be performed for characterization of patient sensitization?

- HLA typing
- HLA antibody test for complement dependent cytotoxicity crossmatch, flow cytometry crossmatch and Luminex single antigen bead assay

HLA Typing

Q: Which HLA locus should be typed before renal transplantation?

HLA typing can be divided into serologic typing and molecular typing. Molecular typing can be classified into high-resolution typing and low-resolution typing depending on the reported range. Low-resolution typing can distinguish allele groups to antigenic equivalent and results are reported up to the first field in the DNA-based nomenclature. High-resolution typing can resolve alleles to protein level, which encodes the antigen-binding site of the HLA molecule [1].

According to Eurotransplant guideline, donor and recipient should be typed for HLA-A, -B, -C, -DR, and -DQ. For HLA-A and -B, serological and DNA typing is accepted. For HLA-C, -DR, and -DQ, DNA typing must be performed. The minimum requirement for HLA typing for donors and recipients is at the serological split level [2].

According to Organ Procurement and Transplantation Network (OPTN) policies, deceased donor should be typed for HLA-A, -B, -C, -DRB1, -DRB3/4/5, -DQA1/DQB1, and -DPB1 with molecular typing method and should be reported at the level of serological splits. For transplantation candidates, HLA-A, -B, and -DR should be typed [3].

Recently, Sensitization in Transplantation: Assessment of Risk (STAR) 2017 Working Group recommended that both donor and recipient HLA-A, -B, -C, -DRB1, -DRB3/4/5, -DQA1/DQB1, and DPA1/DPB1 needs to be typed with molecular methods [4]. Anti-HLA antibodies recognize epitopes rather than whole HLA antigen, and there are some opinions that high-resolution typing is needed especially for sensitized patients [5, 6].

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Serologic Typing

Serologic typing method use antisera with well-characterized anti-HLA antibody specificity. Patient's lymphocytes are separated and reacted with various sera with known anti-HLA antibody specificity in a tray well. Complement and vital dye is added, followed by incubation. If the HLA antigens expressed on patient's lymphocytes react with anti-HLA antibodies in the serum, complement cascade will occur and the vital dye is taken up into the cells via membrane attack complex. Tray well is read under the phase contrast microscope [7].

Serologic typing has advantages of getting rapid results, and it can discriminate null alleles, which do not express HLA molecules on cell surface. However, high-quality serum is needed and discrimination between alleles with small amino acid difference can be difficult [7]. These days serologic typing is replaced by molecular typing in clinical laboratories.

Molecular Typing

For molecular typing, sequence-specific primer (SSP), sequence-specific oligonucleotide (SSO), reverse sequence-specific oligonucleotide (rSSO), and sequence-based typing (SBT) methods can be used.

For SSP typing, pre-made primer sets are used to amplify patient DNA. Patient DNA is amplified with each primer sets in each well of PCR tray. Amplification is detected by electrophoresis. By reading which primer set amplified DNA or not, patient's HLA type can be determined [8]. Advantage of SSP is that results can be obtained rapidly, within 4 h. However, it is not suitable for large volume of specimens, and additional step might be needed to resolve ambiguity.

For SSO typing, patient DNA is extracted and amplified according to HLA gene locus. Amplified DNA is blotted on solid support such as nylon membrane. Various sequence-specific oligonucleotide probes are hybridized and detected. SSO is suitable for large number of samples, 88–184 samples can be easily typed. However, ambiguity is often, and it takes almost 2 days to get results [8].

The rSSO is a widely used method with Luminex technology. Patient DNA is amplified using locus-specific primer and denatured. Microbeads attached to allele or group-specific oligonucleotide probe is reacted with patient sample. Patient DNA is bound to complementary DNA sequences on microbead and read on Luminex platform. Low to intermediate resolution is available with rSSO [9].

With SBT, patient DNA is amplified and sequenced by Sanger sequencing. High-resolution typing is available, but ambiguity can still happen and additional allele or group-specific PCR might be needed in that case. Drawbacks of SBT are that running cost is expensive and is time-consuming. With Next Generation Sequencing (NGS) high-resolution typing is available and it is relatively simple and cost-effective. However, since *HLA* is highly polymorphic, generation of consensus sequences is difficult and genotype discordance can occur due to incomplete reference typing, allele imbalance, or software error [10].

HLA Antibody Testing

Q: What kind of HLA antibody test can be done in sensitized patients?

Prevention and early diagnosis of antibody-mediated rejection (ABMR) is critical in renal transplantation. For successful renal transplantation, various tests are performed. In pretransplant phase, complement-dependent cytotoxicity crossmatch (CDC), flow cytometry crossmatch (FCXM), and solid phase immunoassays are done to evaluate the existence of preformed HLA antibody, and to determine acceptable levels of donor-specific antibody (DSA) that allow for successful transplantation [11]. These assays differ in sensitivity and types of antibodies detected. Generally,

combination of these tests are performed to increase sensitivity, and to determine antibody characteristics.

Complement-Dependent Cytotoxicity Crossmatch

Q: What is the condition of false positive CDC?

CDC has been the basic test to perform before transplantation [12]. If there is sufficient amount of antibodies, which can bind to donor antigen and induce complement cascade, cytotoxic response will occur. Various techniques are used to increase the sensitivity of the test such as extended incubation time, addition of washing steps prior to addition of complement, or addition of anti-human globulin (AHG) [13].

Test Principle and Methods

CDC is performed by incubating donor cells expressing HLA antigens and recipient serum with the addition of complement and dyes. If antibody in patient serum can bind to donor lymphocyte and induce complement cascade, the donor cell is killed and the vital dye is taken up so dead cells can be identified by reading with phase contrast microscope [7].

CDC can be performed with unseparated lymphocytes, or T lymphocytes or B lymphocytes with viability more than 80%. Commonly used methods are NIH-CDC, long incubation, AHG-CDC (anti-human globulin augmented), and Amos wash method.

The standard CDC, also known as NIH-CDC, is the least sensitive method. Patient serum is serially diluted and dispersed on Terasaki tray. Donor lymphocytes with 2×10^6 cells/mL is added 1 μL in each well and incubated for 30 min at room temperature. Rabbit complement 5 μL is added and incubated for 60 min at room temperature. After that, vital dyes such as eosin, trypan blue, acridine orange, and ethidium bromide is added and read under phase contrast microscope [13]. OPTN requires to use more sensitive crossmatching assay than basic NIH-CDC.

AHG-CDC is a widely used method to increase sensitivity. Before addition of complement, 1 μL AHG is added and incubated for 2 min at room temperature. AHG allows the antibody Fc portion to be in closer proximity to activate complement [14]. AHG is used to detect low-level anti-HLA antibody and non-complement-fixing antibodies. Long incubation method is basically similar to NIH-CDC, but after addition of complement, incubation is extended to 120 min. For Amos wash method, washing steps are introduced before addition of complement. By this method, probable anti-complementary factors that can prevent complement fixing can be removed [15]. To differentiate IgG and IgM antibodies, pretreatment of patient sera with heat inactivation or treatment with dithiothreitol (DTT) or dithioerythritol (DTE) can be done.

Each HLA laboratory will adopt appropriate method and lymphocyte preparation, incubation time, incubation temperature, AHG treatment, staining, washing, etc. can vary from laboratory to laboratory.

Interpretation

After neglecting the proportion of dead lymphocytes in the negative control well, more than 20% of the dead cells are interpreted as positive. Maximum dilution titer with positive crossmatch is reported.

T cells express HLA class I antigen and B cells express HLA class I and II antigen. Therefore, if both T cell CDC and B cell CDC are positive, it can be concluded that both HLA class I and class II antibodies are present, or that only class I antibodies are present.

In case of negative T cell CDC and positive B cell CDC, HLA class II antibody alone may be considered. In rare cases, non-HLA antibodies or low-level HLA class I antibodies may be considered. Therefore, it is recommended that solid phase immunoassay should be performed for exact identification of anti-HLA antibodies.

Since CDC is the reaction between the patient's serum and donor cell, the reaction does not only occur by anti-HLA antibodies. CDC results may be affected by the drug or patient's underlying disease. Rarely, positive CDC can be due to autoantibodies, such as anti-Lewis antibody. These autoantibodies are usually IgM, and the interference can be reduced by pretreatment of serum with DTT or DTE [16, 17]. In patients who had undergone rituximab (anti-CD20) or basiliximab/daclizumab (anti-CD25) or anti-thymocyte globulin or 6-mercaptopurine treatment, there is a possibility of false positives CDC. In patients who were treated with rituximab, B cell CDC may have a false-positive result over 3 months, since rituximab can be present in the patient's serum for that period [18]. In patients who were treated with these drugs, follow-up with solid phase immunoassay is helpful.

Flow Cytometry Crossmatch

Q: What factors can affect flow cytometry crossmatch?

FCXM is more sensitive than CDC to detect anti-HLA antibodies [19, 20]. Although positive FCXM is associated with poor clinical outcome, it is not an absolute contraindication of transplantation.

Principle

FCXM is similar to CDC in that patient's serum and donor lymphocytes are reacted. Unlike CDC which detects cytotoxic response, fluorochrome-conjugated antibodies are added and the signal is read by flow cytometer.

Methods

In addition to patient serum and donor lymphocyte, negative control serum and positive control serum should be prepared. Negative control serum can be purchased by commercially available human AB serum drawn from blood group AB male donor and should be noncytotoxic. Positive control serum is made by pooling PRA positive sera. Negative control serum and positive control serum is tested in the same way with patient serum.

Donor lymphocytes are isolated and its viability should be more than 80%. Patient's serum and donor lymphocytes are incubated 20–30 min at room temperature. Although the number of lymphocytes and volume of serum varies according to laboratories, ASHI suggest using 500,000 cells and 30 μL of serum to be used [21].

Some laboratories treat lymphocytes with pronase to remove Fc receptor and CD20 on surface of B cells. Since B cells express Fc receptors on cell surface, non-HLA antibodies can bind and cause background noise. In patients desensitized with rituximab, pronase can remove CD20 of lymphocytes and can reduce the rituximab effect on B cell FCXM [22]. There are no standard protocol, but most of laboratories use 0.5–2.0 mg/mL pronase. If higher concentration of pronase is used, cell surface HLA molecules can also be removed, causing reduced HLA antibody reactivity. When pronase is treated in T cell FCXM, sensitivity can be increased, but cryptic epitope of HLA molecule can be exposed, causing nonspecific reactions [23, 24].

After incubating cell and serum, fluorescence-conjugated [$F(ab')_2$] antihuman IgG (Fc specific) is added. Fluorescence-conjugated anti-human CD3 is added to detect T cell reactivity and fluorescence-conjugated anti-human CD19 or anti-human CD20 is added to detect B cell reactivity. Flow cytometry acquisition should be gated at least 5,000–15,000 lymphocytes.

The result of FCXM is reported with median fluorescent intensity (MFI) ratio or median channel shift (MCS). MFI ratio is calculated by patient serum MFI divided by negative control serum MFI, and it is useful when dealing with log scale data. MCS is calculated by subtracting negative control serum value from patient serum value, and is useful with data on linear scale of 256 or 1,024 channels [21].

Interpretation

HLA laboratories should establish their own FCXM cutoff. Since there are many variable factors in FCXM, such as flow cytometers, fluorochromes, reagents, cell-to-serum ratio, and incubation condition, it is difficult to standardize FCXM [25].

FCXM results should be interpreted in context with CDC results (Table 2.1). Low-titer class I DSA can cause negative T cell CDC and positive T cell FCXM. Negative T cell FCXM and positive B cell FCXM can be resulted from HLA class II antibodies, low-titer HLA class I antibodies, non-HLA antibodies such as autoantibodies and MICA antibodies, or nonspecific antibodies [26, 27]. Patients who are treated with rituximab can show false positive B cell FCXM for 3 months after injection [18, 28]. In these patients, monitoring with solid phase immunoassay would be more suitable. Antithymocyte globulin (ATG) can also affect cell based HLA antibody assay. The results of T cell FCXM after ATG treatment can be falsely positive. Moreover, ATG-treated patients can produce false positive result for B cell FCXM in spite of negative B cell CDC [29].

Table 2.1 Interpretations of crossmatch results

CDC		FCXM		
T cell	B cell	T cell	B cell	Interpretation
+	+	+	+	High-titer anti-class I antibodies with or without anti-class II antibodies.
+	–	+	–	Probably not anti-class I antibodies since B cell crossmatch is negative. Further tests needed.
–	+	+	+	Low-titer anti-class I antibodies with or without anti-class II antibodies.
–	–	+	+	Low-titer anti-class I antibodies with or without anti-class II antibodies
–	+	–	+	Anti-class II antibodies and/or low-titer class I antibodies.
– or +	+	–	–	IgM autoantibody possible. DTT treatment would be helpful.
–	–	–	–	HLA antibodies not detectable with CDC, FCXM. SAB assay may detect low-level antibodies.

CDC complement-dependent cytotoxicity; *FCXM* flow cytometry crossmatch

Solid Phase Immunoassay

Q: 1. What are the factors affecting SAB assay?
2. What is the limitation of SAB assay?

Before development of solid phase assay, cell-based panel-reactive antibody (PRA) tests were used. Nowadays, solid-phase assay such as ELISA, flow cytometry, or Luminex platform has been widely used. For luminex platform, pooled antigen panel, phenotype panel, and single antigen beads (SAB) panel can be applied. Pooled antigen panel consists of microbeads coated with HLA class I or class II molecules from multiple donors. Phenotype panel consists of microbeads that are coated with HLA class I or HLA class II molecule derived from a single individual. SAB, each bead is coated with only one kind of HLA allelic antigen. Among various PRA methods, Luminex SAB is the most sensitive and specific method for DSA detection. For HLA sensitized patients, it is recommended that SAB assay be performed at least once before transplantation [25]. Although SAB assay has increased sensitivity and specificity to detect HLA antibodies, it still has limitations. Many factors affect PRA results making inter-laboratory and intra-laboratory MFI results less reproducible. Interference can occur due to serum factors or drugs. Prozone phenomenon and shared epitope phenomenon can also occur. Here, subsequent discussion will be restricted to Luminex SAB assay.

Principle

Luminex SAB assay uses multiplex bead assay technology. It has 5.6 μm diameter microbeads which are impregnated with two fluorescent dyes (classifier signal) [25, 30]. Every beads are uniquely color coded by combination of these two fluorescent dyes. Purified HLA molecules are immobilized to microbeads and up to 100 beads with a unique HLA antigen can be identified. By using phycoerythrin (PE)-conjugated

secondary antibody (reporter signal), specific HLA antibody in patient serum can be detected using dual-laser instrument [25].

Methods

For Luminex SAB assay, patient serum is incubated with purified HLA molecules attached on microbeads and fluorescent-conjugated anti-human IgG. If there are anti-HLA antibodies in patient serum, it will bind to HLA molecules on microbead, and fluorescent-conjugated antihuman IgG will subsequently bind to patient's anti-HLA antibodies. After these steps, sample is analyzed on Luminex platform.

To improve sensitivity and to reduce interference, some laboratories modify the manufacturer methods. With hypotonic dialysis, DTT, ethylenediaminetetraacetic acid (EDTA), or heat inactivation, interference of C1 complex and IgM antibodies can be reduced.

With hypotonic dialysis of serum, IgM antibodies are precipitated, therefore reducing IgM interference. Procedure of hypotonic dialysis is simple and improves PRA specificity, but it is time consuming since it needs overnight incubation [31].

DTT disrupts disulfide bonds of IgM antibodies, therefore reducing IgM interference. 0.05 mol/L DTT 10 μL is added to 90 μL of serum and incubated at 37 °C for 30 min, and then centrifuged for 10 min before testing. DTT pretreatment is simple and fast, but there are some reports that DTT can also increase negative control beads' reactivity [31]. Some laboratories adjust DTT-to-serum ratio to reduce the effect of DTT on control beads.

Complement component C3 can also bind to bead, blocking anti-HLA antibody binding [32]. Heat inactivation of serum at 56 °C for 30 min or pretreatment of serum with EDTA can destroy complement activity [33].

Very high level anti-HLA antibodies can induce prozone effect. Very high level anti-HLA antibodies bind to HLA antigen coated beads, leading to tightly packed antibodies which might interfere binding of the detection antibodies, resulting in falsely low MFI [34]. In this case, serum dilution can be helpful.

Results are presented as antibody specificities and MFI value. One Lambda LABScreen® Single Antigen offers baseline normalized MFI value, which raw MFI of test serum is adjusted with negative control bead and negative control serum.

Interpretation

SAB assay need comprehensive approach, considering patient's disease status, sensitization events, desensitization protocol used, recipient and donor's HLA type, and other related HLA antibody test results [35]. MFI represents degree of saturation, which is degree of antibody binding among total antigens expressed in beads [25]. Although the cutoff MFI 1,000–1,500 is generally used, MFI threshold may be modified on the basis of patient history, different HLA loci, or epitope/antigen groups [4]. De novo DSA after transplantation has clinical implication in relatively low MFI, and HLA-C and -DP antibodies are known to have clinical impact in higher MFI.

It is important to understand that SAB assay is not a quantitative assay, and MFI results are semiquantitative at best [35]. In FDA guideline for the qualification as a quantitative test, acceptable range of coefficient of variance (CV) is 15–25%. However, current CV for MFI variation is mostly more than 25% [36]. Therefore, smaller increases such as 1,000 MFI might not represent a real increase in antibody strength, instead it can be due to day-to-day variability [37]. STAR 2017 group recommended that differences of less than 25% in MFI values should not be interpreted as clinically meaningful [4].

With SAB assay result, calculated PRA (cPRA) can be derived. The cPRA is the percentage of donors with unacceptable HLA to which the patient has been sensitized [38]. The cPRA value is critical when counseling the wait time to highly sensitized patients waiting for deceased donor [39].

By comparing SAB assay and HLA typing results, virtual crossmatch is available. Donors without unacceptable antigens can be identified before having real crossmatch [39, 40].

Limitations

Although SAB assay has revolutionized HLA antibody test, it still has limitations. Various factors can affect PRA result. Vendor, lot-to-lot variability, instrument type, operator, and reagents can all affect MFI value, causing low inter- and intra-laboratory reproducibility [41, 42].

False positive and false negative PRA results are possible. Since HLA molecule is artificially attached to microbead, hidden epitope can be exposed, resulting in false positive results [43, 44]. Or immunologically relevant epitope can be concealed, causing false negative results [35]. SAB assay does not represent every frequent alleles in every ethnicity, it can only include less than 100 HLA alleles. Therefore, antibodies against alleles which are not included in SAB assay cannot be detected. Because of the semi-quantitative nature of SAB assay, careful understanding should be needed when interpreting it. If the concentration of anti-HLA antibody is higher than the range measurable by SAB assay, MFI value does not change even if the concentration of anti-HLA antibody increases. Figure 2.1 shows an example of two HLA antibody titers with similar undiluted initial MFI value. Serum A contains excessive antibodies and remains saturated even with repeated dilution. However, serum B has antibodies enough to saturate bead initially, but not excessive. It means that there may be upper limitation for SAB assay, indicating high MFI value does not quantify how much antibody is present.

Unlike CDC, serum dilution is not routinely performed for FCXM and SAB assay; therefore prozone effect can occur [45]. This phenomenon manifests a strong positive cell based crossmatch in the absence of significant DSA in SAB assay. Figure 2.2 shows how the MFI values change with titration. Generally, antibody titer decreases as the serum is diluted. However, in this figure, some MFI values can increase with repeated dilution. Similarly, some patients with very high anti-HLA antibodies can show similar or increased SAB MFI after plasma exchange, due to prozone phenomenon. It is suggested that low MFI titer does not guarantee low level of anti-HLA antibody and neat MFI does not always reflect antibody strength. Various factors, such as IgM antibody, C1 complex, and IVIG, can also interfere PRA assay. To reduce these interferences, laboratories modify the manufacturer method as mentioned above.

Antibodies against public epitope may show low MFI, because these antibodies can react to multiple beads presenting HLA antigens with shared epitope.

SAB assay is a highly sensitive method to detect anti-HLA antibodies. Not all patients with DSA detected with SAB assay undergo ABMR or have poor graft survival [46]. Therefore, modified SAB assays which can

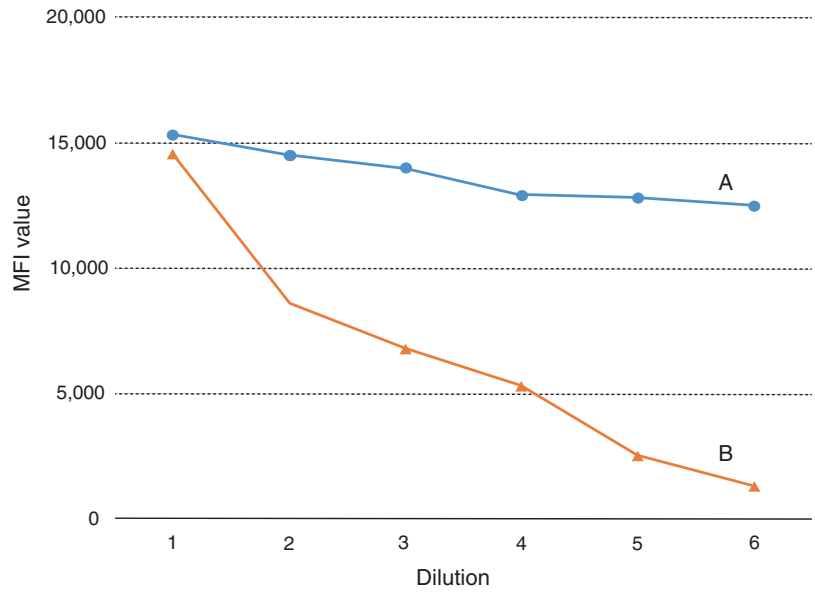

Fig. 2.1 Difference of MFI values with dilution in SAB assay. A: Oversaturated; B: Not excessively saturated

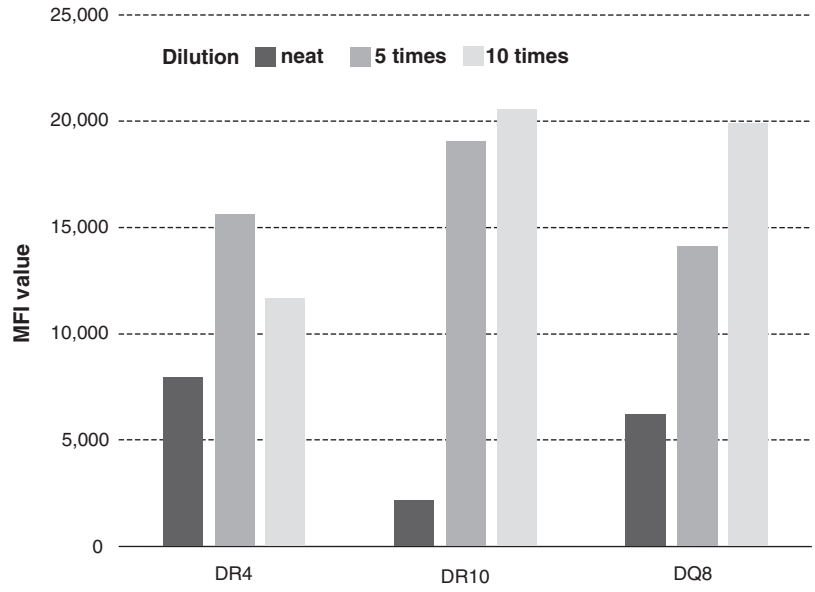

Fig. 2.2 An example of prozone effect

selectively detect anti-HLA antibodies capable of fixing C1q or C3d were introduced.

C1q SAB Assay

Q: 1. What is the rationale of C1q-binding SAB assay?
2. Is this test positively correlated with the clinical outcome?

Since standard IgG SAB assay cannot distinguish cytotoxic antibodies from noncytotoxic antibodies, C1q SAB assay has been developed to detect antibodies which can fix complements. C1q is the first component protein to constitute the complement pathway. Number of studies reported that C1q-binding anti-HLA antibodies are associated with high risk of ABMR and kidney allograft loss [47–49]. C1q SAB assay can be used for antibody screening and desensitization monitoring.

Principle

C1q SAB assay is similar to IgG SAB assay except that it uses PE-conjugated anti-C1q antibody instead of PE-conjugated anti-human IgG antibody. C1q is spiked to patient serum and reacted. If the patient has sufficient anti-HLA antibody and C1q is bound to anti-HLA antibodies, PE-conjugated anti-C1q antibodies are subsequently bound and it is detected by reading the fluorescent.

Methods

Patient sample is heat inactivated (56 °C for 30 min) to remove endogenous C1q. After heat inactivation, the serum is added to well plate and human complement C1q and beads expressing HLA antigens are added. After incubation, PE-conjugated anti-C1q is added and incubated. After washing steps, well plate is read with Luminex instrument.

Like IgG SAB assay, HLA laboratories treat serum with various methods to eliminate possible interference in C1q SAB assay. Some laboratories add AHG to increase sensitivity [50].

Interpretation

Like IgG SAB assay, results are shown as antibody specificity and MFI. With manufacturer provided program, raw MFI and normalized MFI value (normalized with negative control serum and negative control bead) is available. Laboratories set their own cutoffs as MFI 300, 500, 1,000, or some use individual cutoff by adding 1,000 to the lower MFI showing increase of

300 MFI for the first time after sorting MFI from lowest to highest [51].

Limitations

Clq binding to the antigen/antibody complex is the first step in the classical pathway, but anti-HLA antibodies capable of C1q fixing does not necessarily mean that subsequent complement cascade will occur leading to cell lysis [52]. Some argue that the relationship between C1q assay and clinical outcome may be affected by IgG subclass or titer [51]. There are some reports that C1q SAB results are associated with high IgG SAB MFI when interference is reduced through serum pretreatment [50, 53].

Later-developed C3d assay detects the later part of the complement cascade and thus theoretically better reflects the occurrence of the complement cascade.

Epitope Analysis

Q: What is the rationale of epitope analysis in pre-op sensitized renal transplant patients?

Epitope is a part of the antigen that contacts with complementary determining region of an antibody. In the center of the structural epitope lies functional epitope that determines the specific binding of antibody. Duquesnoy et al. introduced concept of eplet which is functional epitope of HLA within radius of 3.0–3.5 Å [54].

The basic concept of HLA epitope analysis is that anti-HLA antibodies are directed against epitopes rather than whole antigen. And it is assumed that patient's anti-HLA antibodies do not react with the self-epitope to cause immune response. With patient and donor's high-resolution HLA type and SAB assay results, epitope matching can be done. It can be analyzed using a free software, HLA-Matchmaker (http://www.epitopes.net). However, the eplets provided by the HLA-Matchmaker are the theoretical epitopes, including those that are not actually confirmed to produce an antigen-antibody reaction. HLA epitope registry (http://www.epregistry.com.br) database offers a list of antibody-verified epitopes.

There may be mismatch at the antigen level, but at the epitope level there may be no mismatch. Epitope matching is especially helpful for highly sensitized patients waiting for a deceased donor to reduce transplantation waiting time and to improve clinical outcome [55]. Eurotransplant conduct Acceptable Mismatch program for highly sensitized (cPRA ≥85%) patients and employs HLA-Matchmaker to define acceptable mismatch [56].

Since there are less than 100 alleles in SAB assay, not every HLA alleles can be analyzed with present SAB assay. Some insist that by using epitope matching, unacceptable epitope can be found and HLA alleles carrying the same epitope could be defined as unacceptable [57].

Correlation of Each Methodology

Q: How can we interpret the various test results?

When interpreting HLA antibody test results, related HLA tests (donor/recipient HLA typing, CDC, FCXM, SAB) should be interpreted together, considering patient's disease status, desensitization protocol used, and sensitizing history such as previous transplantation, pregnancy, and transfusion history.

CDC, FCXM, and SAB assay detect different types of antibodies and have difference in sensitivity. In addition, since these tests have inherent variability and are tested with modifications, cut-offs and correlation of each test may vary between laboratories.

CDC can detect not only HLA antibodies but also non-HLA antibodies. In this case, auto-control or pretreatment of serum to remove IgM antibodies can help. FCXM is more sensitive

Table 2.2 Risk assessment of various test results

CDC	FCXM	SAB	Sensitization history	HLA molecular MM	Risk assessment
+	+	+			Active memory, at risk for hyperacute rejection
–	+	+			Active memory, at risk for ABMR and TCMR
–	–	+			Active memory, at risk for ABMR and TCMR
–	–	–	Pregnancy or prior transplant with repeat MM		At risk for latent memory with a recall B and T cell response
–	–	–	–	High	Increased risk for de novo alloimmunization
–	–	–	–	Low	Baseline risk for de novo alloimmunization
–	–	–	–	0	Low risk for de novo alloimmunization

CDC complement-dependent cytotoxicity; *FCXM* flow cytometry crossmatch; *SAB* single antigen bead assay; *MM* mismatch. Modified and reprinted with permission by Tambur AR, Campbell P, Claas FH et al. from American Journal of Transplantation [4].

than CDC, but it cannot distinguish between HLA and non-HLA antibodies, and cytotoxic and noncytotoxic antibodies. IgG SAB is the most sensitive to detect HLA antibodies, and clinically insignificant low-level HLA antibodies may also be detected. IgG SAB cannot distinguish between complement-fixing and non-complement-fixing antibodies. C1q SAB can detect HLA antibodies which can fix complement, but it does not mean that it is necessarily cytotoxic (Table 2.2) [4].

Cases of Sensitized Patients

A Case with Low-Level Anti-Class I and -Class II HLA Antibodies

56-year-old female patient with previous history of pregnancy was evaluated for renal transplantation from living related donor. CDC was negative, T cell FCXM and B cell FCXM was positive. SAB assay showed she had B54 (MFI 6,228), DQ6 (2,510), and DR15 (1,941) DSAs. She was treated with rituximab 200 mg and went through four sessions of plasmapheresis. After desensitization, T cell FCXM became negative and SAB assay showed DSA B54 (1,792). Since she was treated with Rituximab, CDC and B cell FCXM were not evaluated. After one more session of plasmapheresis, renal transplantation was done. Creatinine was normalized on the second postoperative day (POD). At POD 4, follow-up HLA assays were done and T cell FCXM was negative and SAB assay showed no DSAs. At POD 36, DSA B54 (3,334) was detected but her creatinine level was normal and stable, below 0.7 mg/dL. Her creatinine level was 0.64 mg/dL at POD 8 months and stable up until now.

A Case with High-Level Anti-Class II HLA Antibodies

49-year-old female patient with previous history of pregnancy was evaluated before renal transplantation. AHG T cell CDC and long incubation T cell CDC was negative, but B cell CDC was positive up to 1:2 dilution. SAB IgG assay showed DSA as B61 (5,716), DR8 (13,286), and DQ6 (11,869) with SAB C1q assay DSA DR8 (5,857). She was desensitized with rituximab 200 mg, five sessions of both plasmapheresis and IVIG 200 mg/kg. After desensitization, follow-up SAB IgG and SAB C1q assay revealed IgG DSA B61 (1,911), DR8 (9,089), and DQ6 (5,527) with negative C1q DSA. After one more session of plasmapheresis, renal transplantation was done. Her creatinine level normalized since POD 2. Follow-up SAB assay done at POD 4 revealed DSA DR8 (2,615) and DQB1∗06:01 (1,386). Another follow-up SAB assay done at POD 11

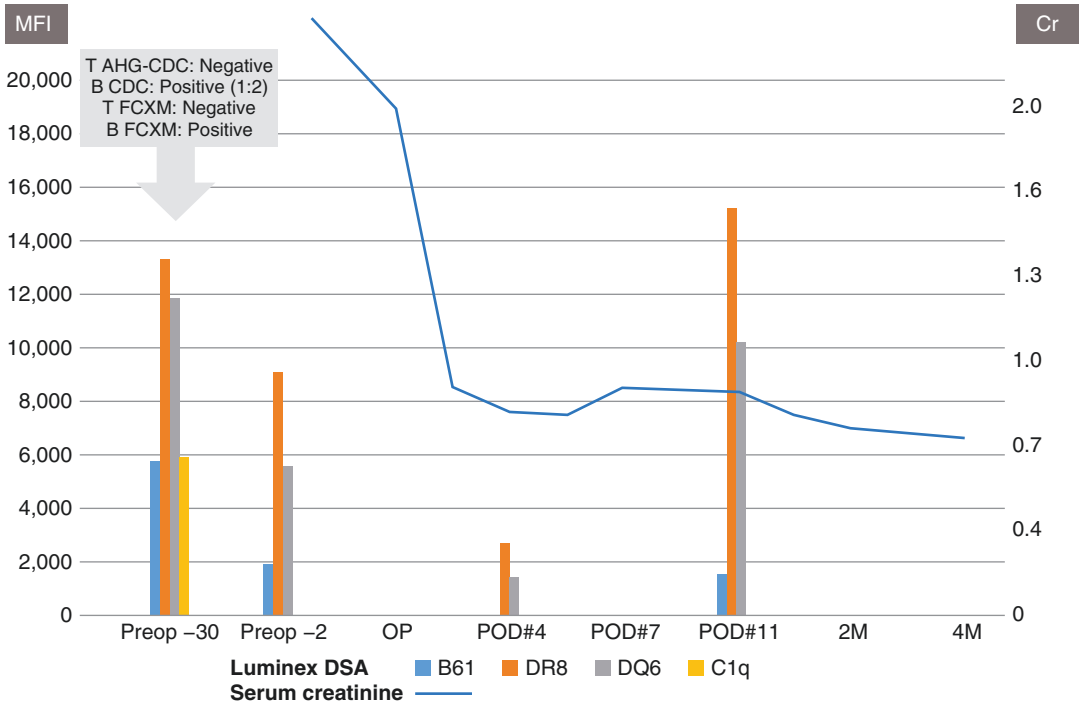

Fig. 2.3 Pre- and post-desensitization in patient two

showed DSA B61 (1,534), DR8 (15,238), and DQ6 (10,196). Although high-level DSA was present, her creatinine level has been stable below 0.8 mg/dL for 4 months post-transplantation up until now (Fig. 2.3).

A Case with CDC(+) and C1q(+) Anti-HLA Antibodies but with Stable Postoperative Course

57-year-old female patient with history of previous pregnancy and renal transplantation was evaluated for second renal allograft from her son. Preoperative AHG T cell CDC was positive (1:1) and B cell CDC was positive (≥1:32). T and B cell FCXM were both positive. SAB IgG showed A24(9,782), DR52(3,328), DQ5(7,666) DSAs, and SAB C1q assay detected DQ5(20,801).

Desensitization with rituximab (500 mg), plasmapheresis (21 sessions), and one cycle of bortezomib was done. Follow-up study showed negative T cell FCXM and DSA as A24 (10,574), DQ5(12,429), and C1q DQ5(22,635). Following renal transplantation, urine output was normal and serum creatinine showed 0.93 mg/dL at POD 3.

Postoperative T cell and B cell FCXM were still positive, and SAB IgG DSA was A24(6,209), DQ5(8,153), and C1q as DQ5(25,290) at POD 4. Even though postoperative DSA persisted, her renal function was good with serum creatinine as 0.67 mg/dL. Renal biopsy at postoperative 2 years and 4 months showed suspicious TCMR with g1, cg0, pct0, c4d0 (0%) and trivial CAN (chronic allograft nephropathy). She was treated with steroid pulse (1.5 g), plasmapheresis (5 sessions), and rituximab (200 mg). PRA IgG and C1q DSAs were persistent even under normal renal function.

At 4 years and 5 months after transplantation, second renal biopsy showed suspicious chronic active ABMR with g1, cg1, ptc1 (focal), c4d0 (0%), and mild CAN. She was treated with steroid pulse (1.5 g), plasmapheresis (4 sessions), IVIG (200 mg/kg ×4), and rituximab (100 g). Follow up PRA IgG and C1q assay were done every two to three month, and DQ DSA MFI remained over MFI 10,000 and

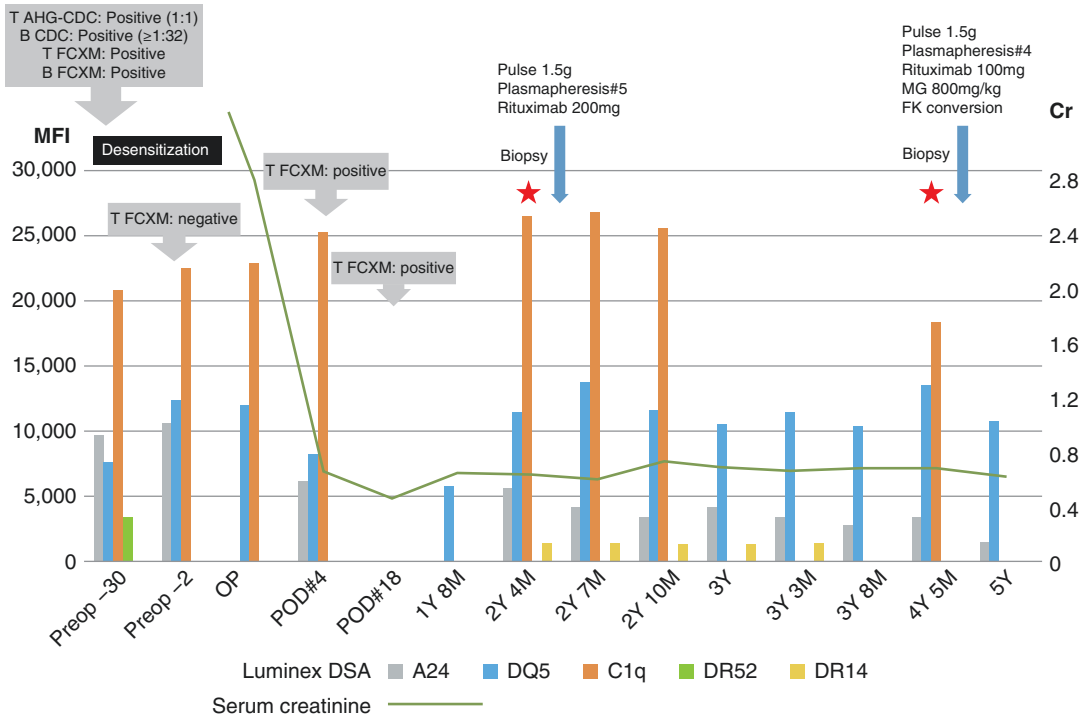

Fig. 2.4 Pre- and post-desensitization in patient three

creatinine level has been stable below 0.8 mg/dL for five years post transplantation up until now (Fig. 2.4). It is known that HLA-DQ is not always expressed on kidney endothelium but its expression is induced by inflammatory cytokines [58]. Therefore, it needs longer time and higher MFI to induce graft dysfunction compared to non-DQ DSA. For de novo DQ DSA, average time to graft dysfunction after its detection was 11 months and MFI over 5,000-10,000 is associated with ABMR [59–62]. ABMR due to preformed DSA occurs earlier compared to de novo DSA ABMR, treated more aggressively and shows better graft survival [63].

A Case with High-Level Anti-Class II HLA Antibodies with Postoperative DSA(−) ABMR

62-year-old female patient with diabetes was evaluated for renal allograft from her daughter. Preoperative CDC and T cell FCXM was negative but B cell FCXM was positive. SAB IgG assay showed B∗13;02(1,242), DR7(13,202), DQ2(2,609) DSA, and SAB C1q assay was negative.

Desensitization with rituximab (500 mg), plasmapheresis (4 sessions), and IVIG (300 mg/kg ×4) were done. Due to persistent DR7(7,662), three more sessions of plasmapheresis and IVIG were treated.

Following renal transplant, immediate postoperative course was not eventful. But at POD 4, creatinine elevated from 1.11 to 2.74 mg/dL. SAB DSA was negative. But under impression of ABMR, steroid pulse (1 g), one cycle of bortezomib, plasmapheresis (9 sessions), and IVIG were treated but without response. At POD 29 under hemodialysis, biopsy showed ATN, suspicious TCMR, and C4d(−) ABMR. DR7(1,316) DSA persisted. Treatment was restarted with steroid pulse (1 g), bortezomib, plasmapheresis (4 sessions), and IVIG. After then renal function recovered with increased urine flow. At POD 45, she was discharged with creatinine 1.30 mg/dL. Her renal function is stable with creatinine 0.81 mg/dL with no DSA for POD eight months up until now.

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