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Detection of Anti-HLA Antibodies by Flow Cytometer

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Additional information is available at the end of the chapter

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Abstract

Lives of patients with solid organ failure depend physically, emotionally, and economically on others. Improvement in organ transplantation is one of the most important medical breakthroughs of the twenty-first century. Being healthy upon organ transplantation is the second chance to live the life. This is frequently observed in heart-, lung-, and liver-transplanted patients. For instance, upon kidney transplantation, dialysis dependence terminates and life quality of the patients increases. The major difficulty in organ transplantation is the low number of organ donation. Thus, the number of patients in the waiting list for the cadaveric transplantation increases day by day. Under these limited circumstances, required conditions should be further provided for the long survival rates of recipients with allogeneic graft without any problem. Human leukocyte antigen (HLA) tissue typing and anti-HLA antibodies produced before and after the transplantation adversely affect the graft survival and thus the survival of an individual. Investigation of pretransplantation immune status of recipients is significant. Particularly, donor-specific anti-HLA antibodies determine early and long-term graft survival. Flow cytometer is one of the most important devices used in anti-HLA antibody detection and also for other clinical and scientific purposes. Compared to conventional methods, it supports transplantation clinics due to its high sensitivity and specificity. The use of flow cytometer dependent methods in transplantation field increases progressively.

Keywords: solid organ transplantation, HLA, flow cytometer, cross match, panel reactive antibody

1. Introduction

Transplantation can be defined as the transfer of cells, tissue, or organ from one body to another. Solid organ transplantation is the replacement of the ablated organ or a part of it from a donor to the recipient instead of his/her damaged or absent organ [1]. There are two important organ

resources in transplantation: related donors and deceased donors. Although there have been many studies aiming to increase the number of deceased donors in various countries, the number of patients in the waiting list for the transplantation increases every passing year due to low ratio of organ donation. Currently, there are 122,403 patients waiting for organ transplantation in the United States. Of these patients, 101,189 of them are waiting for kidney transplantation (as of 8 October 2015). The average waiting time for the first kidney transplantation is 3.6 years, and it depends on the health, compatibility, and availability of organs. Of 17,105 kidney transplants in the United States, it was reported that 11,570 of them were cadaver transplants and 5535 of them were related transplants in 2014. However, 3000 new recipients are currently included in the waiting list every month and 12 patients die each day while waiting for kidney transplantation [2]. It was reported that totally 70,000, 8500, 3850, 2200, 1600, and 70 patients were, respectively, waiting for kidney, liver, heart, lung, pancreas, and small bowel transplants in European Union countries such as Iceland, Norway, as well as Turkey, on 31 December 2013. It was also estimated that 6000 recipients died in 2013 while they were waiting to be transplanted in Iceland, Norway, and Turkey [3].

During eighteenth century, researchers tried to achieve organ transplantation in animals and humans. Despite failures, the first successful kidney transplantation was performed between monozygotic twins by Murray in 1954. Solid organ transplantation that is performed between genetically identical individuals, such as monozygotic twins, is called “syngeneic transplantation.” In syngeneic transplants, graft would not undergo rejection. Consecutive syngeneic transplantation of more than 30 kidneys led to the consideration of patient treatment with end-stage kidney disease by transplantation. It was understood that tissue and organ transplantations can be performed between genetically non-identical individuals of the same species by the developments in immunosuppressive therapy. This type of transplantation is called allogeneic transplantation [4–6]. Currently, liver, heart, pancreas, lung, and intestine, as well as kidney, transplantations are commonly performed in this way. The most transplanted organ is kidney because there are double kidneys in an individual and one of them is sufficient for a healthy life. In recent years, increasing number of successful solid organ transplantations has been performed between genetically non-identical individuals [7].

Human leukocyte antigen (HLA) and ABO blood group compatibilities and detection of donor-specific anti-HLA antibodies (DSA) in recipients are important for the success of the organ transplantations as well as for the immunosuppressive therapy [8]. As it is well known, acceptance or rejection of a tissue or an organ is under the control of proteins encoded by HLA genes. The immunizations of recipients against HLAs due to pregnancy and blood transfusions are important. Also, previous transplantations are also important for the sake of the success of the organ transplantation. Hyper acute rejections can be observed in this kind of transplants [9, 10].

2. Human leukocyte antigen (HLA)

HLA genes reside on the short arm of sixth chromosome. It contains more than 200 genes. The region is in four centimorgan in length and is about 4 Mb in size [11]. HLA contains loci of

genes that encode proteins present on various cells. These proteins play critical roles in the graft rejection. Thus, they are also termed as “transplant antigens” [12].

In 1958, Dausset, Snell, and Rappaport found HLA antigens in human leukocytes for the first time. In the same year, van Rood et al. reported the production of antibodies against leukocytes in sera of multitransfused individuals and multiparous women [13]. These antigenic structures were defined as HLAs because they were first identified on leukocytes. It was observed that these antigens were present not only on leukocytes but also on tissue cells [11, 14, 15].

In the mid-1930s, Gorer and Snell determined that HLA antigens played an important role in the rejection of the allograft. HLA molecules are expressed co-dominantly in each individual and in three groups of gene regions (class I HLA, class II HLA, and class III HLA) were identified in major histocompatibility complex (MHC) (**Figure 1**) [16, 17].

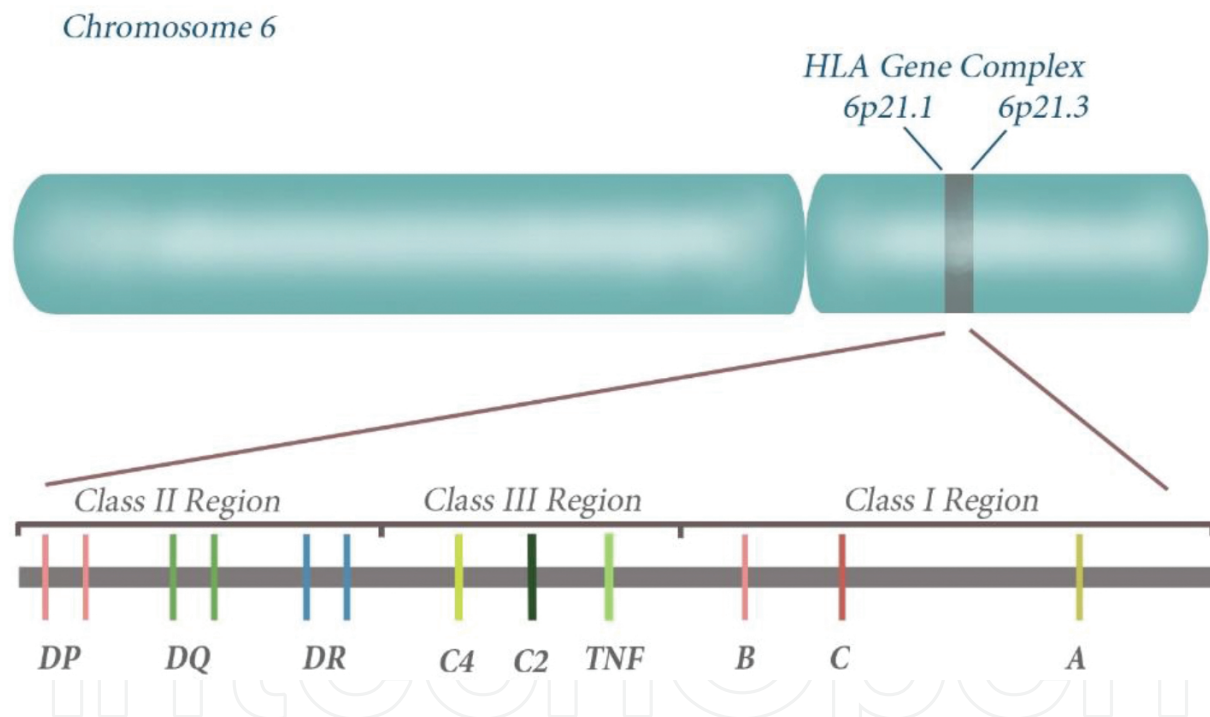


Figure 1. Gene map of HLA region on chromosome 6.

2.1. HLA class I molecules

HLA class I molecules are present in all nucleated cells in human. This region includes HLA-A, -B, -C, -E, -F, -G, -H, -J, and HLA-X loci. HLA-A, HLA-B, and HLA-C loci are polymorphic and functional classical class I loci. The rest of HLA loci are known as non-classical loci. These loci are pseudogenes and they do not encode proteins. HLA class I molecules contain two different polypeptide chains (α -chain and β_2 -microglobulin) that are bound non-cova-

lently. The α -polypeptide chain is 44 kDa and consists of three loops that are 338 amino acids (aa), and they are bound to each other by disulfide bonds. This molecule is divided into three parts: extracellular hydrophilic region (1–281 residue), transmembrane hydrophobic region (282–306 residue), and intracellular hydrophilic region (307–338 residue). Extracellular parts of heavy chain (α_1 and α_2) are located on distal membrane and they constitute peptide binding region of the molecule. These regions are encoded by second and third exons and the variability is determined by them. The other part (α_3) on proximal membrane carries interaction region for CD8 molecule on T cells [16–18]. β_2 -microglobulin, which is encoded by a gene on fifteenth chromosome, is a 12-kDa subunit. Non-polymorphic β_2 -microglobulin is associated with HLA class I heavy chains without the membrane binding. It was shown that peptides that bound to HLA class I molecules were not longer than 8–9 amino acids, and they were exogenous peptides. β_2 -microglobulin is required for heavy chain and antigenic peptide association. This molecule stabilizes heavy chain connection (**Figure 2A**) [17–19].

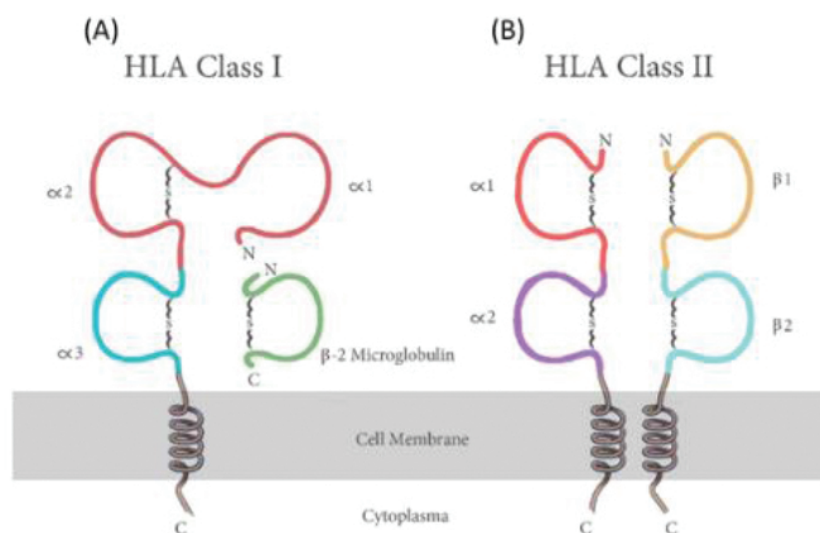


Figure 2. (A) The structure of class I HLA molecule. (B) The structure of class II HLA molecule.

2.2. HLA class II molecules

HLA class II molecules are located on B lymphocytes, macrophages, dendritic cells, endothelial cells, and active T cells. They consist of six different loci (HLA-DM, DN, DO, DP, DQ, and DR). DM, DN, and DO loci are pseudogenes and they do not encode proteins. They are type I integral membrane proteins with heterodimer structure. HLA class II molecules have two polypeptide chains (α , β), which bound non-covalently to each other. The α -chain is 24–32 kDa and β chain is 29–32 kDa. Second exons of α and β chains (α_1 and β_1) encode the variable peptide-binding region. This variable region is formed by two distinct chains. It can bind to peptides that are more than 11 amino acids in length in order to present them to $CD4^+$ T_H cells (**Figure 2B**) [17–19].

Foreign HLA molecules can be presented to immune cells by two ways (**Figure 3**):

- A. **Direct presentation:** When unprocessed foreign HLA molecules and recipient HLA molecules are structurally similar, direct presentation occurs. Antigen presenting cells (APCs) of donors on the graft and migrant leukocytes are transported to lymphoid system of the patient. This activates T lymphocytes of the patient (**Figure 3A**)
- B. **Indirect presentation:** Donor HLA molecules are processed, and peptides are presented by HLA molecules of the recipient (**Figure 3B**) [20, 21].

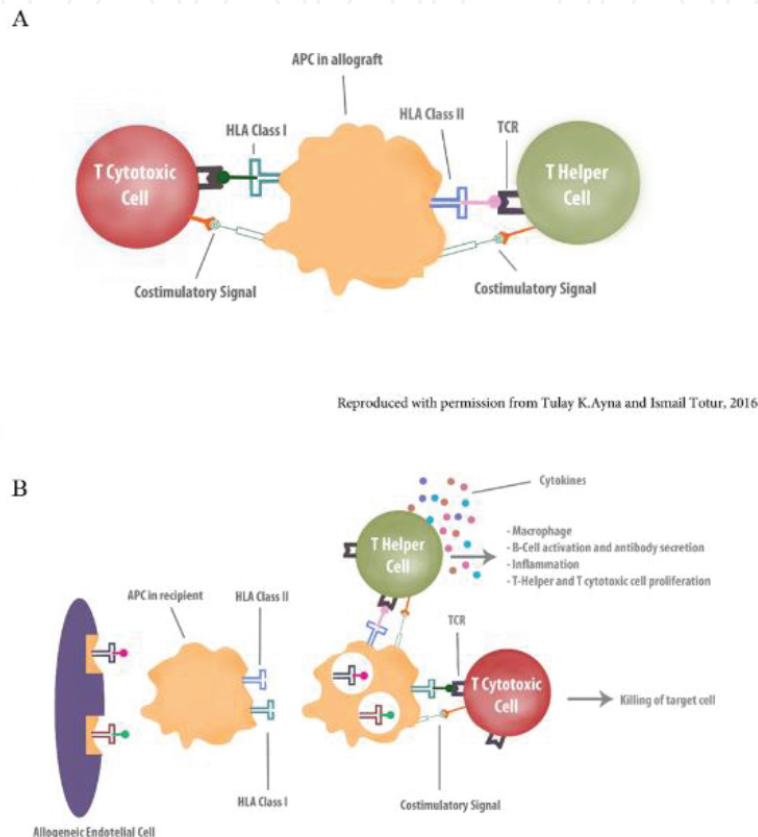


Figure 3. (A) Direct and (B) indirect recognition in allogeneic transplantation.

CD4⁺ T cells usually develop by indirect presentation. Foreign antigens are processed and obtained by APCs through endosomal vesicular pathway, and HLA class II molecules present these antigens at the end of pathway. In addition, some antigens derived from graft cells are presented by HLA class I molecules and they are recognized indirectly by CD8⁺ T cells. HLA similarity is not sufficient alone for the acceptance of the graft. When a graft is transplanted between genetically non-identical individuals, there is always a rejection risk; even if they have similar HLA antigens. The reason is the variability in minor histocompatibility loci. HLA antigens can be directly recognized by helper and cytotoxic T cells. This is called alloreactivity. However, minor histocompatibility antigens can be recognized only if they are presented by their own HLA molecules. Graft rejections due to major histocompatibility differences are stronger than rejections that are developed by minor histocompatibility differences. Graft rejection phenomena may be divided into phases: sensitization and effector [22, 23].

2.2.1. Sensitization phase

Foreign major and minor histocompatibility antigens that are expressed on graft cells can be recognized. Helper and cytotoxic T cells proliferate when they recognize these molecules. Even though a response against minor histocompatibility antigens is generally weak, combined response against numerous minor histocompatibility antigens may be stronger. Both HLA molecules and the peptide ligand in HLA cleft are recognized during the response. Peptides in the peptide-binding groove of HLA class I molecule are derived from proteins synthesized by foreign cells. On the other hand, peptides in the peptide-binding groove of HLA class II molecule are processed by foreign APCs via endocytic pathway [22, 23].

Under some conditions, fragments of foreign HLA class I molecule can be presented by class II HLA molecule. Costimulation signal by APC is required for the T-cell activation in recipients. Patient's APCs may migrate to the graft and take foreign antigens by endocytosis. They also present foreign peptides and minor/major histocompatibility molecules, which are processed by their HLA molecules [22, 23].

It was reported that donor APC populations (called as passenger leukocytes) migrated to lymph nodes. These cells are dendritic cells that express class II HLA molecules and they are common in majority of mammalian tissues except brain. Passenger leukocytes activate T cells in lymph nodes since they express foreign HLA antigens and thus they are recognized as foreign [22, 24].

Another type of APCs is endothelial cells. They are present in Langerhans islets and blood vessels. They can express HLA class I and II molecules [24].

Lymphocytes are transported to spleen and localized in lymph nodes to generate effector cells. When effector cells are generated, these cells are transferred back to the graft. The recognition of foreign antigens activates T-helper cells. Upon activation, T cells proliferate. It is considered that activated T cells have an important role in the initiation of various rejection mechanisms [22, 24].

2.2.2. Effector phase

The most common effector mechanisms are graft failure by delayed type hypersensitivity (DTH) and cytotoxicity developed by CD8⁺ T cells (CTL). T cells and macrophages located in the graft lead to the graft rejection. Foreign class I HLA molecules are recognized by cytotoxic T cells of the patient, and this may lead to cell death by CTL. In some situations, when helper T cells bind to class II HLA molecule, they may function as cytotoxic T cells and lead to graft rejection [25].

Graft is rarely injured by antibody-dependent cell cytotoxicity (ADCC). These antibodies are immunoglobulin (IgG) isotypes. IgGs can be divided into four groups (IgG1, IgG2, IgG3, and IgG4) according to their hinge size, place, and the number of disulfide bonds between two heavy chains. There are 2, 4, 11, and 2 disulfide bonds in IgG1, IgG2, IgG3, and IgG4, respectively. Even though IgG2 and IgG4 have the same number of disulfide bonds, IgG heavy chain components are different. The relationship between IgG1 and IgG3 activates the comple-

ment system. This activation is initiated by C1 protein binding to CH2 or CH3 region on Fc area of IgG. This induces another complement protein cascade. As a result, pores are formed on the cell membrane, and thus cell lysis occurs [26, 27].

IgG2 and IgG4 are complement-independent antibodies, which play roles in ADCC. Some cells with cytotoxic activity express receptors specific for Fc region of the antibody on their membranes. Thus, these cells (natural killer cells, macrophage, monocyte, and eosinophil) can bind to target cell in case the antibody binds to the target cell. This leads to lysis of the target cell due to the release of lytic substances (such as tumor necrosis factor (TNF) and perforin) by cytotoxic cells from their lysosomes and granules [26, 27].

3. Anti-HLA antibodies

3.1. Panel reactive antibodies

Anti-HLA antibodies are generally defined as panel reactive antibody (PRA). They are one of the immunological factors affecting the graft survival. In recent studies, researchers have assessed the pretransplant incidence of anti-HLA antibodies and their clinical significance. Numerous studies reported that post-transplant anti-HLA antibody detection was associated with high rejection ratio [28–31]. Anti-HLA antibodies can directly recognize undamaged foreign HLA molecules on the cell surface. Thus, these antibodies play a critical role in solid organ transplantation and also in hematopoietic stem cell transplantation (HSCT). The humoral response directed against foreign HLA molecules can be because of encountering them during pregnancies, blood transfusions, and/or previous transplantations. Transplant candidates are tested for these anti-HLA antibodies by different techniques, such as PRA test. HLA laboratories perform PRA tests routinely (once in 3 months) in patients waiting for solid organ transplantation. Physicians can measure probability of the negative cross-matched donor [32, 33]. Earliest studies determined class I and II anti-HLA antibodies with complement-dependent lymphocytotoxic techniques (CDC). However, antibodies at low levels cannot be detected using this technique. In addition, only complement-dependent antibodies (IgG1, IgG3, and partly IgG2) can be detected by this method. Over the years, other analytical methods have become available, such as flow cytometer and solid-phase flow methods. It was observed that flow cytometer methods were more sensitive compared to CDC methods. All of IgG subtypes (IgG1, IgG2, IgG3, and IgG4) can also be detected by flow cytometry. The first flow cytometric PRA test experiment was reported by Cicciarelli et al. in 1992 [34, 35].

Complement-dependent cytotoxic crossmatch (CDCXM) and flow cytometric crossmatch (FCXM) are the two main crossmatch methods used nowadays, and they are used for the detection of donor-specific antibodies in most of transplant centers. It has been accepted that crossmatch (CM) and antibody screening methods should have similar sensitivity. Thus, a flow cytometric PRA would be suitable for the laboratories that use FCXM method for cross-matching [36–38].

There are two different flow cytometer PRA methods:

1. **Cell-based method:** It is used to estimate the percentage of PRA by incubating the patient serum with a panel of HLA-typed lymphocytes. In other words, a cell pool is generated from 10 healthy donors whose HLA class I and II tissue types are identified by serological or molecular methods. This cell pool should include HLA antigen variability in population. The cells from the pool are added onto negative, positive, and patient sera samples. If there is antibody specific to HLA antigens expressed on cells in the pool, they can be detected by fluorescence-labeled secondary antibody (FITC- or PE-labeled anti-human IgG). Anti-HLA class I antibodies can be detected by this cell-based method, because most of the cells in peripheral blood are T cells and they express HLA class I molecules. Lymphocytes are gated on FSC/SSC dot plot during the analysis by flow cytometer instrument. Anti-human IgG fluorescence density is detected depending on these cells [29]. The percentage of positive cells is identified by median channel shift considering the negative control serum [39]. This method is not very applicable because of the additional test requirements for antibody specificity determination. In addition, anti-HLA class I and II antibodies should be analyzed simultaneously (**Figure 4**) [32, 33, 40].
2. **Based on bead method:** These kits (PRA kits) were developed including beads coated with specific HLA antigens (HLA-A, HLA-B, Cw, DR, DQ, and DP) (**Figure 5A** and **Figure 5B**). Then, single antigen beads (SABs) were coated with only one HLA antigen (**Figure 5C**). This method allows identification of PRA specificity and definition of acceptable mismatches in hypersensitive patients. It is possible to differentiate anti-HLA antibodies from

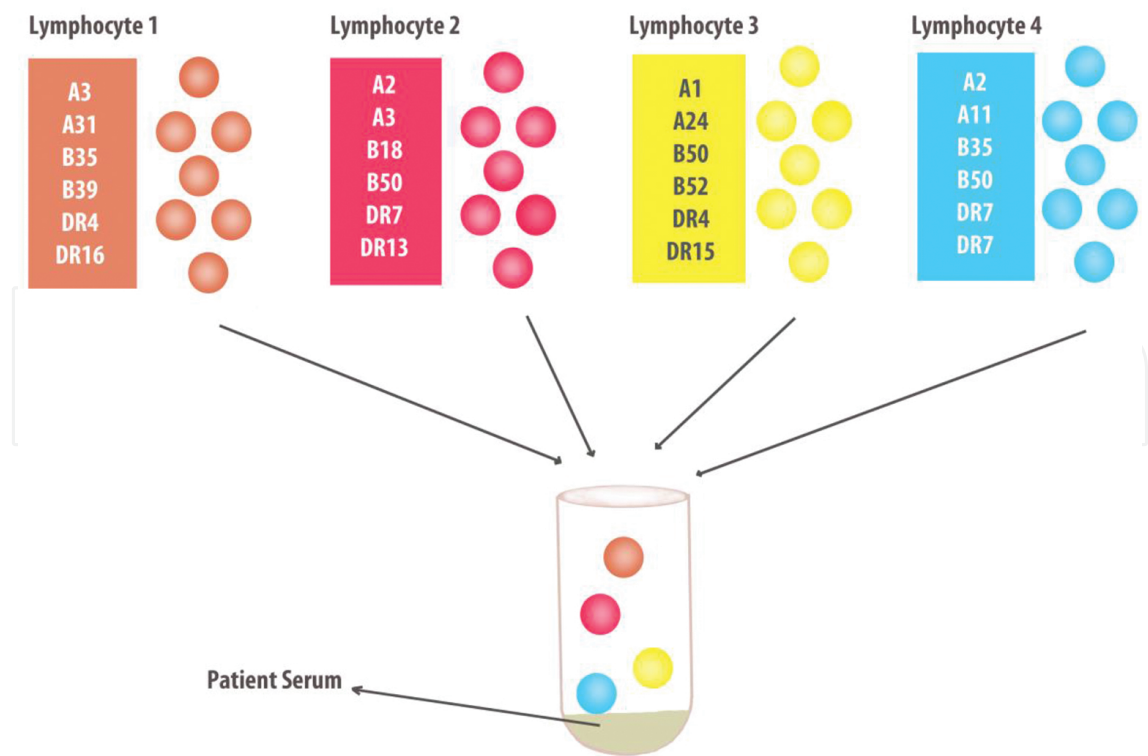


Figure 4. Cell-based PRA method by flow cytometer.

HLA antibodies because only HLA antigens are available on the commercially prepared beads.

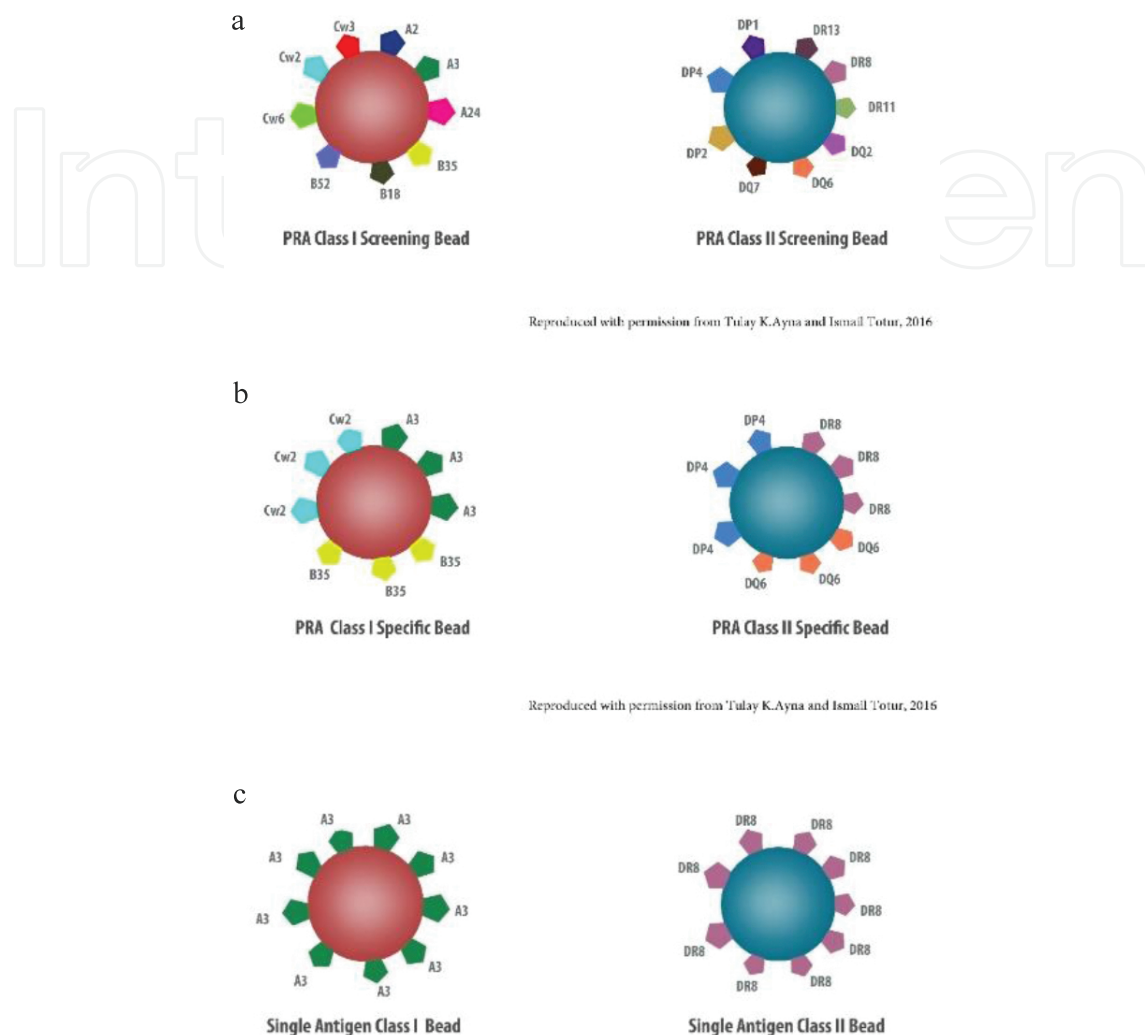


Figure 5. Bead-based PRA method by flow cytometer. (A) PRA screening beads. (B) PRA identification beads. (C) Single antigen beads.

Class I and II HLA antigens were isolated from B cell lines, which were transformed by an Epstein-Barr virus, and used for coating PRA beads. When the number of cells is increased in a culture medium, HLA molecules are released from these cells. Then, HLA antigens are kept on affinity columns. Class I and/or II HLA antigens are isolated from these columns. These antigens are accepted as “natural proteins” because of their expression on the cell surface. However, SABs are coated with only one HLA antigen. These HLA antigens are obtained by recombinant DNA technology. Thus, these HLA antigens can be defined as “recombinant proteins” instead of “natural HLA antigens”.

PRA screening, identification, and SAB assays have similar test principle consisting of two main incubation steps. Primarily, patient and control sera are incubated with appropriate beads, while fluorescence-conjugated anti-human secondary antibodies are added during the

second incubation step. At the end of the assay, a fluorescence peak is generated, which indicates a positive result (binding of antibody to HLA antigens on the beads). We will now examine each of these tests [19].

3.1.1. PRA screening

PRA screening tests can be used for the first evaluation of anti-HLA antibodies and PRA percentage in recipient serum. Two pools of micro beads are coated with class I or II HLA antigens, each of them containing 30 different bead preparations, which represent all common antigens as well as rare HLA alleles (**Figure 5A**) [31, 33]. HLA class I antigen frequencies in PRA class I screening beads are shown in **Table 1**, and HLA class II antigen frequencies in PRA class II screening beads are shown in **Table 2**.

Specif icity	Number in panel	Speci ficity	Number in panel	Specif icity	Number in panel	Specif icity	Number in panel
A1	4	A80	2	B51	2	B75	1
A2	5	B7	2	B52	1	B78	1
A3	5	B8	2	B53	1	B81	2
A11	5	B13	2	B54	1	Bw4	18
A23	3	B18	2	B55	2	Bw6	26
A24	4	B27	2	B56	1	Cw1	6
A25	2	B35	2	B57	2	Cw2	6
A26	2	B37	2	B58	2	Cw4	4
A29	2	B38	1	B59	1	Cw5	1
A30	3	B39	1	B60	2	Cw6	7
A31	2	B41	2	B61	2	Cw7	9
A32	3	B42	1	B62	2	Cw8	5
A33	3	B44	2	B63	1	Cw9	2
A34	2	B45	1	B64	1	Cw10	4
A36	2	B46	1	B65	1	Cw12	1
A66	3	B47	2	B67	1	Cw14	2
A68	2	B48	2	B71	1	Cw15	3
A69	1	B49	1	B72	2	Cw16	2
A74	2	B50	1	B73	1	Cw17	3
PRA I screening Lot 017-One Lambda						Cw18	3

Table 1. Frequencies of HLA class I antigens in PRA class I screening beads.

Speci ficity	Number in panel	Speci ficity	Number in panel	Specif icity	Number in panel	Specif icity	Number in panel	Speci ficity	Number in panel
DRB1*01	4	DRB5*(51)	8	DQB1*02	8	DPB1*01	8	DPB1*20	0
DRB1*0103	2	DRB3*(52)	20	DQB1*04	8	DPB1*02	9	DPB1*21	0
DRB1*04	3	DRB4*(53)	9	DQB1*05	16	DPB1*03	4	DPB1*40	0
DRB1*07	5			DQB1*06	6	DPB1*04	15		
DRB1*08	4			DQB1*0301 (7)	8	DPB1*05	5		
DRB1*09	3			DQB1*0302 (8)	1	DPB1*08	0		
DRB1*10	3			DQB1* (9)	6	DPB1*09	0		
DRB1*11	3					DPB1*10	0		
DRB1*12	4					DPB1*11	1		
DRB1*13	5					DPB1*13	1		
DRB1*14	4					DPB1*14	3		
DRB1*15	4					DPB1*17	1		
DRB1*16	5					DPB1*18	2		
DRB1*0301 (17)	4					DPB1*19	1		
DRB1*0302 (18)	3					DPB1*105	1		

PRA II screening Lot 020-One Lambda.

Table 2. Frequencies of HLA class II antigen in PRA class II screening beads.

PRA screening test has two beneficial applications. First, the percentage of PRA may give an idea about the CM results. Second, analysis of PRA reactivity in detail can lead us to predict HLA antigens that are targets for antibodies. PRA screening test results give us the clue about which tests should be performed next. Targeted HLA antigens are determined in sera samples with low levels of PRA detected by PRA identification test, whereas these antigens are detected by SAB method in hypersensitized patients (PRA screening result >80%) [41, 42]. Therefore, unnecessary cost, time, and labor losses are prevented.

PRA screening test kit includes micro particle beads with diameter of approximately 2–4 µm. Concurrent analysis of HLA class I and II beads can be performed because class I beads are non-fluorescent, whereas class II beads are stained with a fluorochrome similar to phycoerythrin (PE) [43].

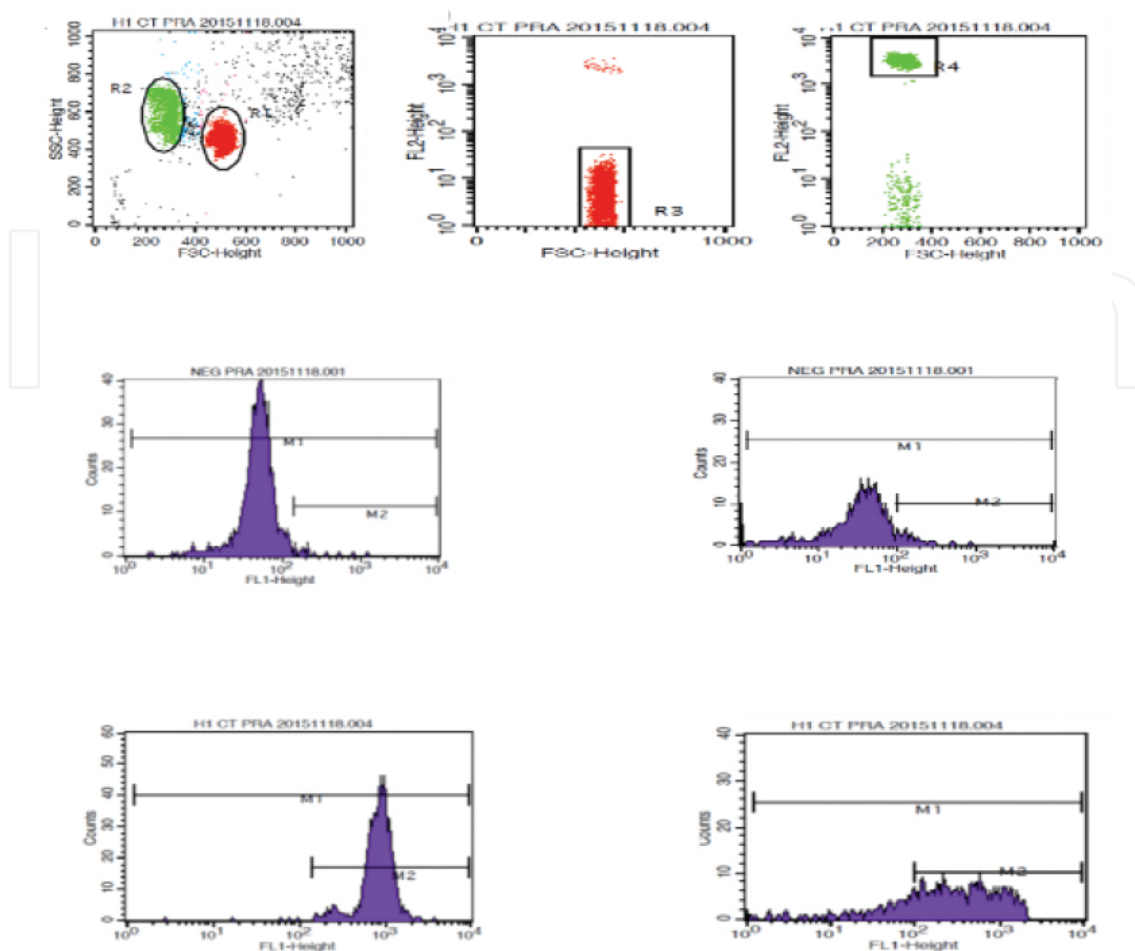


Figure 6. Class I and II PRA screening beads on SSC/FSC dot plot (A). Class I beads on PE/FSC dot plot (B). Class II beads on PE/FSC dot plot (C). Anti-human IgG-FITC staining with a negative control serum (D: class I, E: class II). Anti-human IgG-FITC staining with a patient serum (F: class I, G: class II). The results are the original results from our laboratory.

Class I and II beads are added into the control and patient tubes. Negative control serum does not have any anti-HLA antibody, and it is commercially available. It is important for the evaluation of positive results. Positive control sera are important for the evaluation of secondary antibody activity and observation of test performance. Sera are added onto the beads and incubated at room temperature. After incubation, the samples are washed and fluorescent-labeled anti-human IgG secondary antibody is added on the samples. After the samples are washed, beads are gated on a forward scatter (FSC)/side scatter (SSC) dot blot during the analysis by flow cytometer (**Figure 6**). Class I and II beads are gated on a PE/FSC dot blot. HLA class II beads stained with fluorochrome dye are indicated in **Figure 6C**, whereas the beads represent non-fluorescent HLA class I coated beads are shown in **Figure 6B**. The PRA ratio (%) can be estimated by determining positively reacting bead ratio (%). It is considered that if there is an antibody against a single antigen, fluorescence percentage will increase more than 3% because there are 30 different beads in the pool ($100/30=3.3$). Sera without antibody have single homogenous peak and its positivity ratio is under 3%. Sera antibodies have multiple peaks which represent heterogeneous population [41–43].

3.1.2. PRA specific assay

PRA identification test kit is composed of micro particle bead pools with diameter of 2–4 μm . Each of class I and II PRA test kit consists of a 32-bead panel coated with purified different class I and II antigens. Class I beads are coated with HLA-A, HLA-B, and HLA-C antigens, whereas class II beads are coated with HLA-DR, HLA-DQ, and HLA-DP antigens. This 32-

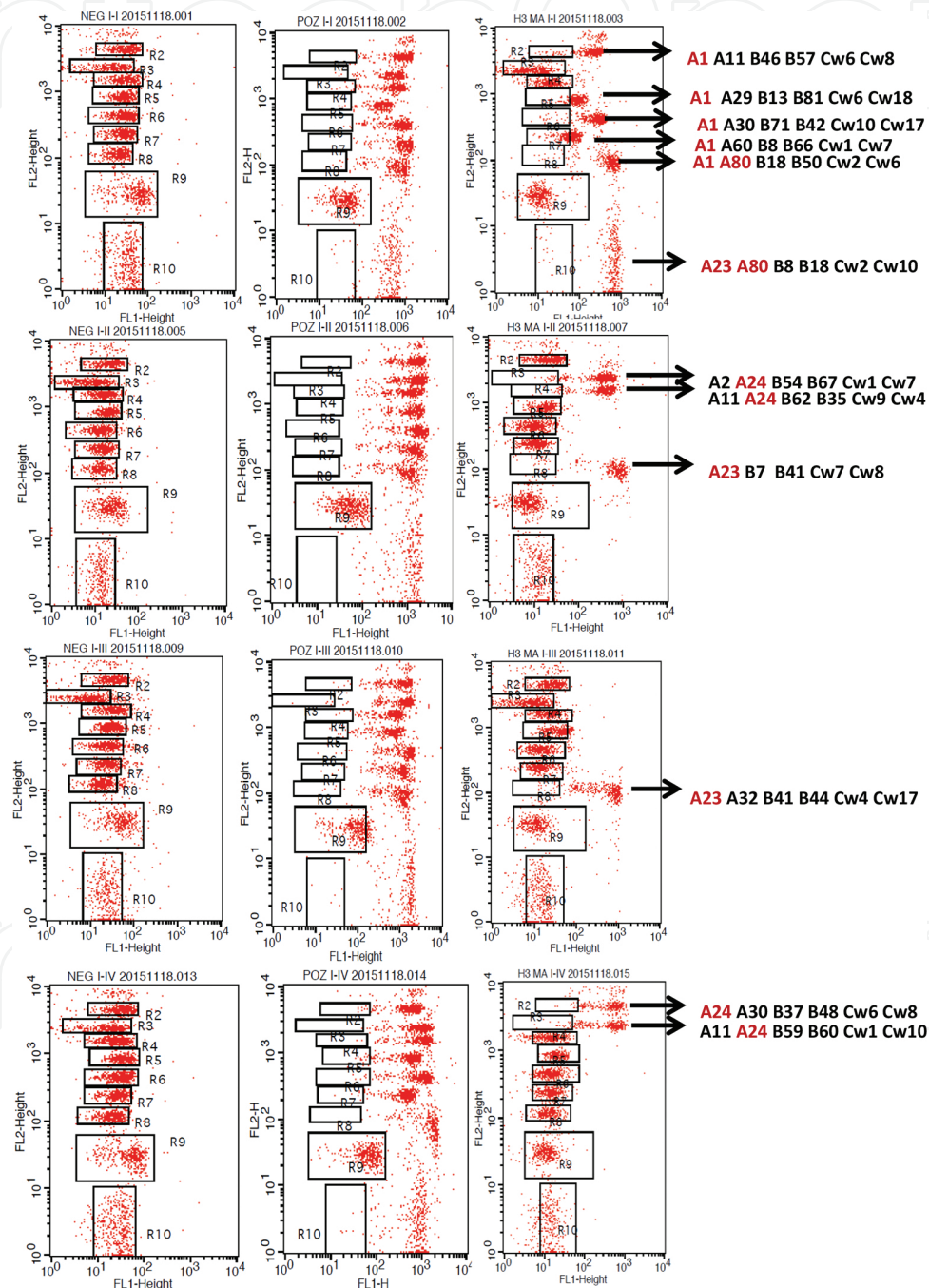


Figure 7. Evaluation of class I PRA identification tests (including negative, positive, and patient sera). PRA screening result is 31% positive. The results are the original results from our laboratory.

bead panel is divided into four HLA groups with eight antigens in each group. There is also a control bead group without HLA antigen in four groups. A serum sample should be tested with each group of beads in order to complete 32-bead panel analysis of HLA class I and II antibodies. During the procedure, each group of beads is incubated with the patient and control sera. Upon incubation, FITC-conjugated anti-human IgG secondary antibody is added [41–43]. During analysis, each group of beads is gated on FSC/SSC dot blot. A positive reaction has a FL1 channel shift when compared to negative control serum. Each of the eight beads has different FL2 channel shift. HLA specificity can be determined by analyzing the samples on FL1 vs. FL2 dot blot. The channel shifts over 50% are accepted as positive (Anti-HLA A1, A23, A24, A80 positive serum is shown in **Figure 7**). Results can be assessed by scoring the channel shifts on software program.

3.1.3. SAB assay

As mentioned before, PRA screening test gives an idea about the following analysis which is required for the fate of the transplantation. Targeted HLA antigens in sera samples with low level of PRA are determined by performing PRA identification test. However, anti-HLA antibody specificity cannot be detected in >80% positive sera samples by PRA identification test. These patients are termed as hypersensitive patients who have very low chance to find a CM negative donor. Testing the patient serum by using SABs is one of the best methods used for the detection of acceptable antigens (**Figure 5C**). When we compare studies in which virtual CM results are estimated by SAB technique and studies in which two important crossmatching methods are used before transplantations, the efficiency between virtual CM and CDCXM was 70–75%, whereas it was 85–95% between virtual CM and FCXM. Detection of clinical compatibility by the detection of anti-HLA antibodies and detection of donor-specific antibody compatibility increase the significance of SAB [44, 45]. Occasionally, there may be some incompatibilities between PRA screening and SAB results. For instance, if the copy number of the targeted antigen is low, a weak signal occurs even if there is a sufficient antibody titer in the serum and a positive fluorescence cannot be obtained. However, identical antibody level can be detected by SABs. If antibody level is limited in the serum, results may be negative due to the high antigen/epitope ratio. In this example, antibodies are dispersed over varied beads carrying the same epitope. In addition, new epitopes of HLA antigens may also occur as a result of the denaturation during SAB production. These new epitopes which are not present on cell surface *in vivo*, may lead to false positive results. Thus, HLA antibodies determined by SAB technique should be confirmed by cellular techniques [19, 33].

The test procedure of flow SAB test is similar to the procedure of PRA identification test. In this test kit, each of the bead group is coated with a single HLA allele. Class I SAB beads consist of 10 groups of beads. Each group is composed of eight different bead sets, and each bead has a single HLA-A, HLA-B, and HLA-C allele. The first four groups contain HLA-A, HLA-B, and HLA-C alleles, which are common in the population, whereas six groups have rare HLA alleles. Class II beads consist of five groups. The first four groups contain common HLA-DR, HLA-DQ, and HLA-DP alleles, whereas fifth group has rare alleles [43]. SAB results of a PRA class I screening (%100) positive patient is shown in **Figure 8**.

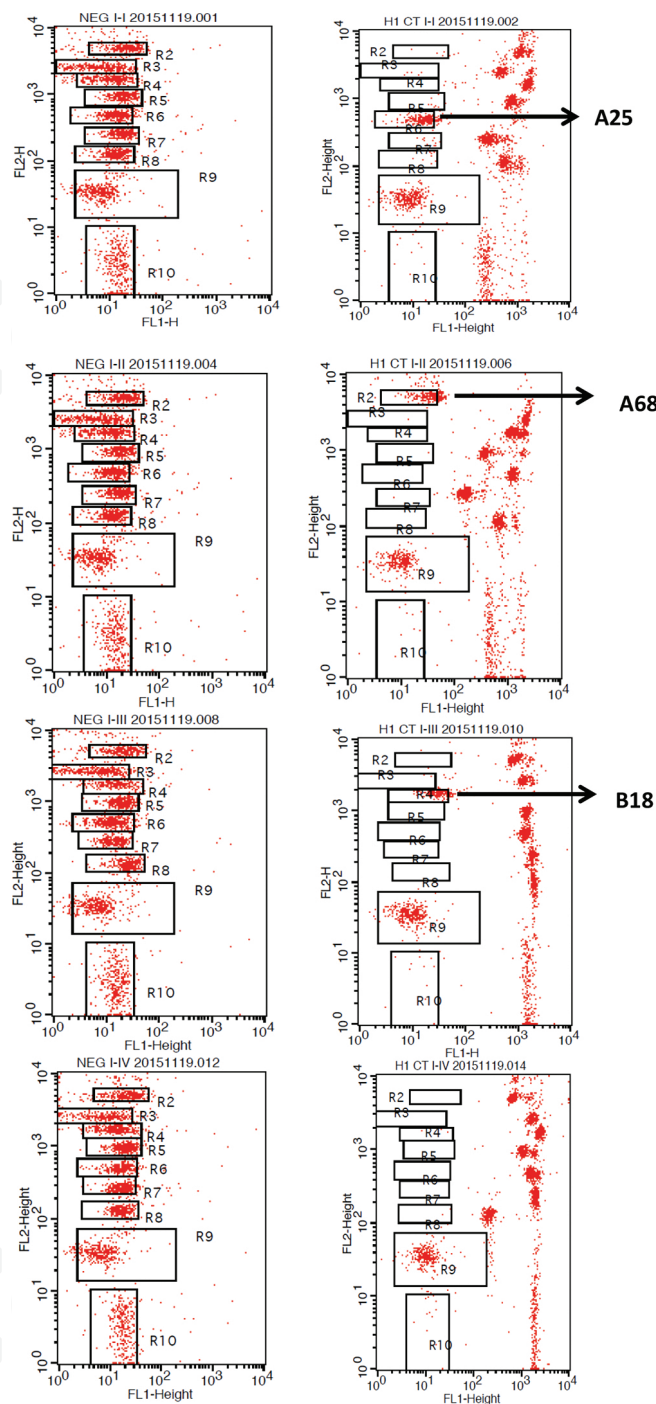


Figure 8. Class I SAB test result of the patient with 100% PRA class I screening. HLA type of patient is A*25, A*68, B*15, B*18, DRB1*11, and DRB1*16. Testing of negative control serum and patient serum with four bead groups. The results are the original results from our laboratory.

3.2. Donor specific antibody detection by flow cytometry

CM test is also performed in addition to tests mentioned above. It is a very important and common test in organ transplantation field, particularly in kidney, lung, heart, and intestine

transplantations. CM is a test system that shows the presence of DSA in the patient serum. The presence of anti-HLA antibodies in recipient is tested in PRA tests, whereas only the presence of DSA is tested in CM test. Detection of pretransplant antibodies provides an insight into the identification of transplants with high rejection risk [46, 47]. Lymphocytes are selected as target cells for the detection of DSAs because they express high levels of HLA antigens. If pre-existing anti-HLA antibodies in the recipient serum are specific to donor graft antigens, the graft is rejected very quickly [48]. These hyperacute rejection reactions can occur within the first 24 hours after transplantation. Acute rejection reactions generally begin in the first few weeks of transplantation, whereas chronic rejection reactions occur months to years after transplantation; as well as pre-existing DSAs, de novo DSAs produced after transplantation, are important for the graft survival. Thus, CM tests can be performed after transplantation in order to follow-up the immunological status of recipients [49, 50].

CDCXM test is the gold standard technique, which is frequently used for the detection of these antibodies. The significance of this test was shown in 1960s. This test is based on the detection of donor-specific anti-HLA antibodies in the patient serum. In 1980s, the ratio of early graft failure risk was 35–50% upon cadaveric kidney transplants. Since 1990, the risk has been decreased progressively. Introduction of more sensitive CM techniques, such as anti-human globulin (AHG) and FCXM plays an important role in this success. The very first experiments on FCXM method for transplants were initiated in 1983 by Goravay. Bray [19] had significant contributions to the development of multi-colored FCXM method [50–52].

Lymphocytes, which are used in FCXM test, can be isolated from peripheral blood, spleen, or lymph nodes. Cell viability should be >80%. For the test, totally $1\text{--}5 \times 10^5$ cells are sufficient. During the analysis in flow cytometer, it is sufficient to have 1000–2000 B cells and 9000–10,000 T cells. Monoclonal antibodies are used to determine the target cell population. To do this, anti-human-CD3 and anti-human-CD19/CD20 monoclonal antibodies are used for T and B cells, respectively. Indirect immunofluorescence is used for the determination of anti-HLA antibodies. Primarily, unlabeled antibody binds to the cells. Then, a second labeled antibody (anti-human IgG) is used for the analysis of unlabeled antibody-cell complexes. CD3-PE, CD19-PE/CD20-PE, and IgG-FITC are usually used for two colored analyses performed by flow cytometer. If three colored analysis is used, CD3-PerCP, CD19-PE, and IgG-FITC should be preferred (**Figure 9**). Secondary antibody should be specific to Fc region of the antibody, F(ab')₂ fragment, and should not react with mouse and horse IgGs [19, 35].

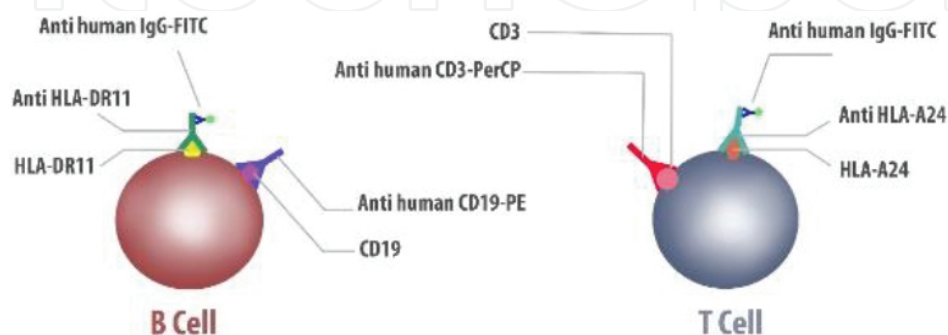


Figure 9. The principle of flow cytometric CM test.

Negative control should not have any alloimmunizations (pregnancy, blood transfusion, previous transplantations), should not be collected from an individual with autoimmune disease, and it should contain healthy sera with AB blood group.

In the FCXM protocol, the use of PBS control (wash buffer control), auto control, and diluted patient serum samples are important in addition to having negative and positive controls. Pretreatment of the serum may be useful before its use. Pretreatment includes high-speed or ultracentrifugation in order to remove large immune aggregates. Inactivation of complement proteins is also recommended [19, 26, 33].

A cut-off value should be determined for the evaluation of CM results. Negative serum and 20 different sera samples without anti-HLA antibodies, and at least 20 cell samples from healthy individuals should be tested. Once the cutoff value is determined, the FCXM test can then be performed. During FCXM protocol, donor cells and patient serum samples are incubated. Then, samples are again incubated with monoclonal and secondary antibodies. Results are analyzed and expressed as positive or negative according to the shift in the fluorescence intensities of samples compared to the results of the test serum (**Figure 10**). Laboratories can determine their own cutoff value by evaluating mean values, median values, and channel shift or fluorescence percentage values [19, 35].

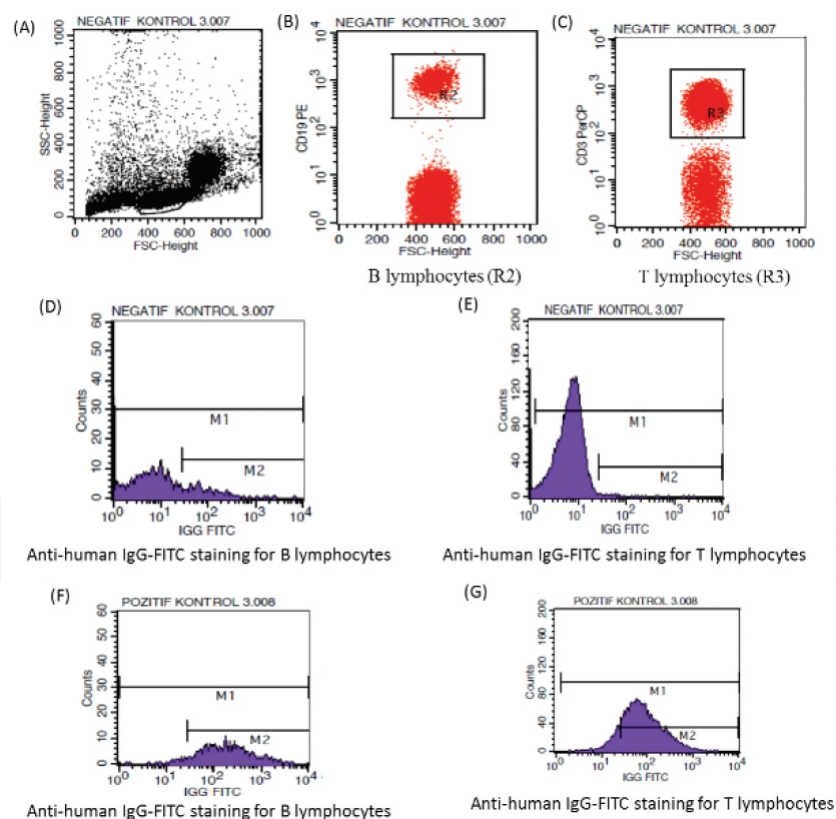


Figure 10. Flow cytometer CM test. Lymphocyte population gated on FSC/SSC dot plot (A). B and T lymphocytes on PE/FSC dot plot and PerCp-/FITC dot plot, respectively (B and C). Anti-human IgG-FITC staining with a negative control serum (D and E). Anti-human IgG-FITC staining with a positive control serum (F and G). The results are the original results from our laboratory.

3.2.1. *T FCXM*

FCXM is a more sensitive method compared to CDCXM test. Thus, DSAs that cannot be detected by CDCXM can be detected by FCXM technique. In this situation, the antibody concentration may be insufficient to generate hyperacute rejection. However, positive T FCXM and negative T CDCXM results indicate the high risk of graft failure. Positive FCXM may not affect the graft survival in non-sensitive patients who transplanted for the first time. Hypersensitive patients with previous transplantations and patients with positive FCXM would have shorter graft failure compared to patients with negative FCXM. Positive FCXM results obtained after previous transplantations can be due to the production of memory cells, their differentiation, and antibody production. If these antibodies recognize new HLA antigens during the new transplantation, they will lead to the rejection. Low levels of antibodies may indicate the memory cell activation [51, 53–55].

It has been also known that sensitive patients with positive FCXM are at high risk for the graft failure, even though they are going to be transplanted for the first time. This may occur because of IgG subgroups. If a patient has complement-independent antibodies, it cannot be detected by CDCXM and PRA tests [46, 54, 55].

3.2.2. *B FCXM*

Roles of class II anti-HLA antibodies in transplantation are contradictory. There are some reports indicating that when there is no class I HLA antibody and cytotoxicity, low levels of anti-HLA-DR and HLA-DQ antibodies are not harmful for the graft. However, high levels of antibodies may lead to hyperacute rejection.

Positive B FCXM is used for the class I and II antibody assessment. It is known that class I antigens are highly expressed on B cells compared to T cells. Thus, low level of class I antibody can be detected by B cell FCXM. However, false positive CM results can be obtained because B lymphocytes express immunoglobulin Fc receptors and anti-IgG antibodies can bind to these receptors non-specifically. In recent years, the proteolytic enzyme pronase has been used before FCXM test in order to remove Fc receptors from B lymphocytes [47, 52].

Although it is known that mostly DSAs lead to rejections, it has been reported that non-HLA antibodies can also be related to rejection. It was observed that antibodies produced against non-classical HLA molecules, such as MHC class I polypeptide-related sequence A (MICA) and B (MICB), also led to acute kidney rejection. It was shown that other antibodies such as anti-vimentin and anti-myosin were associated with long allograft survival in heart transplants. Some researchers reported that antibodies produced against vascular endothelial cells led to chronic rejection in heart and kidney transplantations. Therefore, endothelial precursor cells were isolated, and the antibody detection method was performed which was similar to FCXM [22, 53–55]. FCXM is important for clinical work due to its effect on graft and patient survival. If T/B FCXM is negative, it is generally considered that there is no DSA or DSA is at very low level. If T FCXM is negative and B FCXM is positive, then it is due to the presence of

strong class I antibody or class II antibody. If T FCXM is positive and B FCXM is negative; antibodies are most probably non-HLA antibodies.

In conclusion, transplantation can be performed if FCXM and CDCXM are negative. If CDCXM is positive and FCXM is negative, the patient cannot be transplanted due to the high risk of hyperacute rejection. Patient can be transplanted if FCXM result of a patient is positive without alloimmunizations and CDCXM is negative. Nevertheless, this patient should be considered as if he/she is at high risk for graft failure in case he/she has an alloimmunization.

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