Reviews

Flow Cytometry Application in Organ Transplantation

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The use of flow cytometry in solid organ transplantation has evolved significantly since the initial description of the flow cytometric crossmatch in 1983. Many transplant laboratories currently use crossmatch by flow cytometry as their standard method since it represents the most sensitive test available for the detection of anti-donor antibody. The logistics of a flow cytometry assay also facilitate the distinction between T cell and B cell reactivity. Data from numerous laboratories have demonstrated the clinical utility of the flow crossmatch in predicting early graft failure and increased episodes of rejection. On the other hand, opponents to this approach claim that its major advantage — increased sensitivity — is also its drawback. Hence, the clinical significance and need for additional sensitivity in assigning the most appropriate donor is an area of debate.

In this article we review current applications of flow cytometry in the field of organ transplantation, namely donor-specific crossmatch and assignment of alloimmunization, introduce new developments in the field such as a solid phase assay that differentiates between anti-class I and class II antibodies, and discuss our experience with a look to the future.

In recent years the methods to verify donor/recipient compatibility for bone marrow and organ transplantation have become more sophisticated and more sensitive. Among others, fluorescence-activated cell sorter analysis became a valuable tool with increasing applications. Transplant candidates may become sensitized to alloantigens following previous pregnancies, blood transfusions or a prior transplant. Consequently, an essential role for tissue typing laboratories in solid organ transplantation is to analyze patient's serum for antibodies that may react with transplant donor tissues. Hence, contrary to classical FC-based immunophenotyping, the foremost application of cytometry in organ transplantation is to detect circulating alloantibodies in the recipient's circulation.

In a landmark study about 30 years ago, Patel and Terasaki [1] reported that 80% of renal allograft recipients with detectable donor-specific antibodies rejected the kidney within 24–48 hours of transplantation. In contrast, less then 5% of recipients who did not display anti-donor antibodies lost their grafts in the same time period. It was therefore recognized that the single best predictor of short-term allograft survival was a negative crossmatch between donor lymphocytes and recipient serum. The original assay relied upon complement-dependent cell lysis. In combinations where the recipient exhibits anti-donor antibodies in his or her sera, the antibodies bind to the donor lymphocytes in the *in vitro* assay. The addition of complement would then induce cell death, which can be assessed in the presence of a vital dye (e.g., trypan blue, eosin).

Crossmatch

Following the publication by Patel and Terasaki [1], it became evident that a prospective crossmatch is warranted in order to prevent "hyperacute" rejection, and that an allograft should be transplanted only in the presence of a negative crossmatch. Alas, it was soon apparent that a significant number of grafts still failed due to early episodes of rejection. These rejections, which manifested several days following the transplantation, were not of the "hyperacute" type but were coined "accelerated" graft rejection. It was postulated that these rejection episodes could occur in patients whose pre-transplant titer of anti-HLA antibodies was not detected in the standard crossmatch. Subsequently, several modifications to the original complement-dependent cytotoxic crossmatch were developed to enhance the sensitivity of the assay (i.e., additional wash steps, extended incubation times and,

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FC = flow cytometry

most significantly, the addition of a secondary antibody — anti-human globulin) [2]. Currently, the AHG-CDC is considered the gold standard technique exhibiting reproducible and significant reduction of hyperacute as well as accelerated rejection and enhanced graft survival.

Nonetheless, some patients still experienced "accelerated" vascular rejection and graft loss due to alloantibodies, indicating the need for a more sensitive method. In 1983, Marvin Garovoy applied FC as the means to test for the presence of anti-donor antibodies and developed the flow cytometry crossmatch assay [3] [Figure 1]. Since even low levels of antibody may impact significantly on overall graft survival, it is not surprising that a prospective FCXM demonstrated improved prognosis especially in high risk patients, namely, patients in need of re-grafting or patients with a significant history of alloimmunization events such as multiple transfusions or pregnancies. Thus, patients whose crossmatches were negative both by cytotoxicity and FCXM experienced less graft loss and fewer episodes of rejection than patients whose crossmatches were cytotoxicity negative but FCXM positive [4–6]. Subsequent studies by other investigators at centers performing multiple transplants [7–9] substantiated the initial reports. In fact in many transplant centers, including ours, a negative cytotoxicity crossmatch in the presence of a positive FCXM is a contraindication for transplantation in high risk patients.

Panel reactive antibody

Serum screening for alloreactive antibodies against a random cell panel provides an estimate of the degree of allo-sensitization expressed as the percentage of panel reactive antibody. The PRA can vary between 0% (non-sensitized) and 80–100%, the latter indicating a high degree of sensitization. Patients with high PRA values are more likely to have pre-formed anti-donor-specific anti-HLA antibodies, i.e., a greater likelihood that a final crossmatch will be positive with any given donor. In addition, a detailed analysis of PRA reactivity can determine the specificity of the HLA antibody, information that can predict a positive crossmatch and eliminate unnecessary labor. Such information is most helpful in directing the organ to the most appropriate recipient, thereby expediting organ allocation, especially in the case of kidney donors who are prospectively HLA typed.

Most laboratories perform PRA testing by a CDC or AHG-CDC method, utilizing a panel of several (between 30 and 60) HLA-typed cells as targets. As can be expected, the sensitivity differences between cytotoxicity and FC procedures —which are used for the final crossmatch — result in an underestimation of the levels of alloantibody present in a given patient's serum and in-
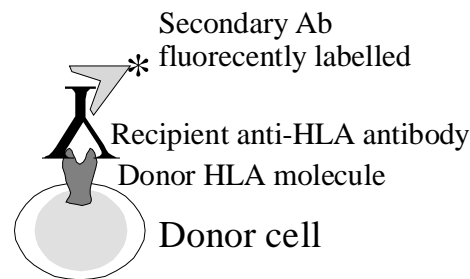
AHG = anti-human globulin
CDC = complement-dependent cytoxicity
FCXM = flow cytometry crossmatch
PRA = panel reactive antibody

Figure 1. Flow cytometry crossmatch. Donor cells are incubated with recipient serum, and anti-HLA antibody, if present, will bind to the donor HLA molecule. Fluorescein-conjugated, goat anti-human antibody is used as the reporter fluorescent dye to detect the binding of alloantibody. This secondary antibody can detect either IgG or IgM antibodies. Simultaneously, a phycoerythrin-conjugated anti-CD20 (or CD19) and a PerCP-conjugated anti-CD3 monoclonal antibody can be added to distinguish B cells from T cells.

creases the frequency of unanticipated positive final crossmatches. The time spent in performing final crossmatches for recipients who could or should be ruled out can lead to increased cold ischemia time for the organ and impact the overall outcome of transplantation. The need was therefore apparent to develop a serum-screening test prior to transplantation, with sensitivity equal to that of the final FCXM. Yet, the logistics of designing and performing a flow cytometric PRA as a routine test are not trivial. Following the first attempts by Ciccarelli et al. [10] and Shroyer et al. [11], additional modifications were introduced by Bray [12]. Hence, six pools of four cells each, selected according to the cross-reactive group antigens they expressed, are used in the assay. The particular clustering into cross-reactive groups allows for accurate %PRA determinations and occasionally permits the assignment of individual specificities. Although Bray's approach significantly improved flow cytometric PRA, collecting the required pool of volunteers for a continual panel is quite cumbersome and over time proved impractical. Also, the effort involved in performing the assay on a large scale is too costly and labor intensive.

New developments in PRA detection

Recently, two new approaches to perform routine %PRA analysis were introduced. Both assays use purified soluble HLA antigens that were immobilized onto a solid surface. One method implements ELISA strategy [13] and the other is based on FC and is termed FlowPRA [14]. The FlowPRA assay utilizes microparticle beads coated exclusively with either class I or class II HLA molecules purified from well-characterized, HLA-typed B lymphoblastoid cell lines. Two types of beads are available: unstained beads coated with class I antigen and red-stained beads coated with class II antigens. The test involves incubation of the patient's sera with the microparticles, washing, and staining with a secondary anti-human IgG antibody conju-

gated to a green fluorescent dye. The two beads can be run simultaneously to test for the presence of class I and/or class II antibody [Figure 2]. Analysis is performed by flow cytometry. The sensitivity of the microparticle assay far exceeds that of non-cytometric assays, but more importantly, the specificity of any antibodies detected will unequivocally be anti-HLA. Data from our laboratory have shown a good correlation with AHG-CDC.

The microparticle assay offers several advantages over current technology. Large-scale routine evaluations can now be performed with the same level of sensitivity as the final crossmatch, but with the advantage of reduced logistical considerations compared with cell pools. Subsequently, the PRA values obtained can better reflect a patient's potential to produce a negative FC crossmatch. A version of the microparticle FlowPRA allows the determination of anti-HLA antibody specificities, a major advantage that will assist in appropriate organ allocation.

One of the advantages of FCXM is its ability to simultaneously analyze T cells and B cells. However, while a positive FCXM T cell crossmatch is routinely a contraindication to transplantation [15], how to interpret, and more importantly how to proceed when the FCXM is exclusively B cell positive is controversial. Until recently one could argue that a T cell-negative/B cell-positive result may indicate either the presence of anti-class II antibodies, low levels of anti-class I antibodies (due to the higher number of class I molecules on the surface of B lymphocytes), or non-HLA antibodies, since lymphocytes express a variety of cell surface molecules in addition to HLA antigens. The case report presented below illustrates the potential use of the microparticle FlowPRA in resolving this dilemma.

A 26-year-old Caucasian male patient was consistently FCXM T cell negative/B cell positive against four successive potential donors with whom he was crossmatched. Importantly, the recipient denied any sensitization event in his history. When a zero antigen mismatched cadaveric donor became available for this patient, it was decided to transplant if the T cell FCXM was negative. Upon analysis, both AHG-CDC and FCXM were T cell negative/B cell positive. Following transplantation, and in an attempt to remove any antibodies that might be deleterious, three sessions of plasmapheresis were performed.

To characterize the donor reactive antibody, pre- and post-transplant sera from the recipient were evaluated using the class I and class II coated microparticles. The test results indicated that while the class I beads did not fluoresce, numerous anti-class II antibodies were apparent. Thus, anti-class I activity can effectively be ruled out as an explanation for the B cell-positive FCXM. The positive result obtained with class II coated microparticles indicated that, although denied by the recipient, previous sensitization event(s) with class II antigens had occurred. On the other hand, the mere fact that recipient sera bind to class II coated beads is not a definite indication that the
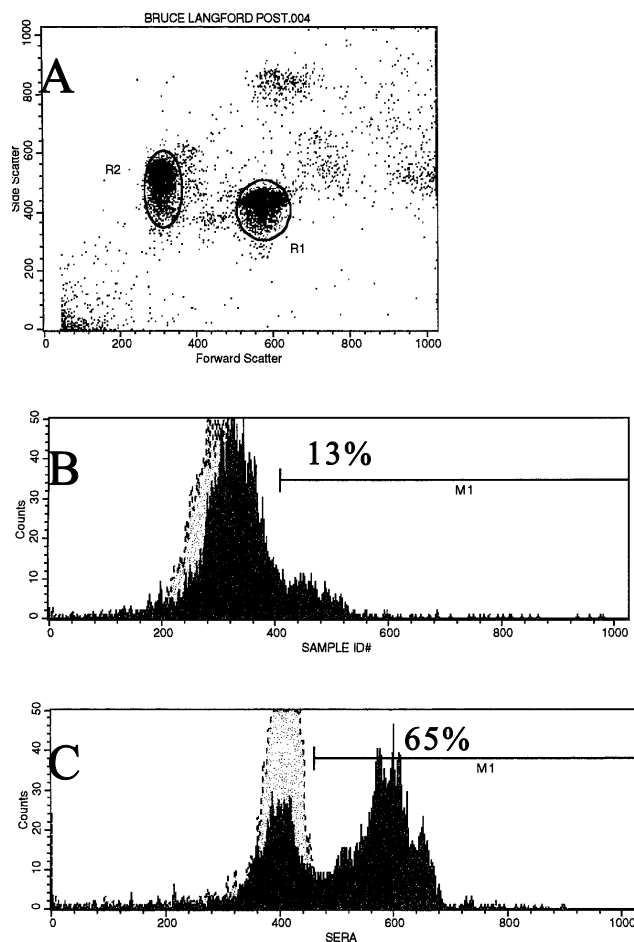

Figure 2. Example of the FlowPRA. HLA class I and class II coated microparticles can be run simultaneously to identify the presence of anti-HLA antibodies. **[A]** Delineation of the forward scatter vs. side scatter of the bead mixture. The class I (R1) and class II (R2) beads are easily separable by light scatter properties, but can be assessed by their fluorescence staining (class II beads are labeled red). **[B]** Histograms of anti-HLA class I reactivity of a patient. Dark histogram = overlay on top of a negative control (gray) histogram. The patient's class I PRA, as determined by the peak to the right of the cursor, is 13%. **[C]** Histogram generated from the class II microparticles. The patient's results are shown by the dark histogram, the gray area representing the overlay on negative control. Class II PRA, to the right of the cursor, is 65%.

B cell-positive crossmatch is due to donor-specific anti-class II reactivity. In order to test this possibility, further analysis of HLA class II antigens by high resolution DNA typing was carried out. No DR or DQ allele differences were detected between the donor and the recipient (the initial zero antigen mismatch was based on serological typing). Currently, 18 months post-transplant, the patient has not experienced any episodes of rejection and both the pancreas and kidney are functional.

In the recent past, absorption — which is labor intensive and time consuming — was the only mechanism to help determine whether anti-B cell reactivity was due to HLA class I or class II antibodies or to non-HLA determinants that are clinically irrelevant to the outcome of the

transplant. The above case illustrates an easier resolution of such previously problematic crossmatches.

Comparison between cytotoxicity and FCMX and PRA assays

Other than its higher sensitivity, FC offers many other advantages. Cytotoxicity assays require viable cells and simultaneously detect IgM and IgG antibodies. In FC assays, based on the design of the assay, the class of anti-HLA antibodies to be detected is determined by the secondary antibody used (i.e., IgG versus IgM antibodies). Since immunoglobulins of the IgG class are thought to be responsible for early graft loss, it is imperative to detect them within a short time. Indeed, cytotoxicity assays can differentiate between IgG and other immunoglobulin types, but additional time- and labor-consuming manipulations (use of DTT, DTE or 2-mercaptoethanol) are necessary. In contrast to cytotoxicity assays, FC allows to gate out (disregard) dead and dying cells, a feature that reduces the need for high initial cell viability (>85% for CDC assays) and requires less time to perform. Initially developed as a single-color immunofluorescence assay, the FCXM has been redesigned over the past several years, culminating in its current three-color configuration [8,16]. This approach permits the simultaneous detection of anti-HLA alloantibodies that bind to either T cells (CD3-positive cells) and B cells (CD19/CD20-positive cells), eliminating the need for physical separation of the different cell populations; or the requirement for selective cell tagging and use of fluorescence microscopy. This information can facilitate the interpretation of the crossmatching results due to the differential expression of HLA molecules, since T cells display predominantly HLA class I molecules while B cells express both class I molecules (at a higher density than T cells) and HLA class II molecules. As was discussed extensively in the previous section, the use of FlowPRA microparticles will extend the definition to either class I or class II antibodies, exclusively.

Controversies

Final crossmatch by FC has become a standard method in many transplant laboratories and represents the most sensitive test available for anti-donor antibody. Data from our laboratory [17–19] as well as numerous others have demonstrated the clinical utility of the FCXM in predicting early graft failure and increased episodes of rejection. For re-transplant and highly sensitized individuals, a negative FCXM predicts graft survival equal to that of non-sensitized patients [20–22].

Despite publications supporting the clinical significance of FC, opponents claim that FC is "too sensitive" and inappropriately deny individuals a transplant. For example, Christiaans et al. [23] reported no difference in the number of rejection episodes or in one year graft survival among renal recipients transplanted with positive or negative FCXM donor kidneys. However, such studies are victims of the misconception that each positive FCXM

necessarily represents an anti-HLA reactivity. Analyzing an assay in which antibodies bind to cell surface molecules will produce only one conclusive result — the presence or absence of recipient antibodies against a particular donor cell surface protein. The nature of that cell surface molecule remains elusive. This predicament is the Achilles heel of FCXM interpretation. We therefore contend that the routine FXCM is not too sensitive, rather it is not specific enough. The case presented herein is only one example.

Two major issues should be addressed when deliberating the clinical need for a more sensitive evaluation of organ recipients. One, as just mentioned, refers to the antibodies detected in previous studies, anti-HLA antibodies. Until the introduction of the microparticle FlowPRA assay (or ELISA-based screens), most studies did not directly tackle the issue. A B cell-positive FCXM was falsely regarded a priori as indicating the presence of donor-specific anti-HLA class II antibodies [24].

The second, perhaps more crucial, issue involves the biological role of those antibodies and their interplay with current immunosuppression. It is evident that certain individuals, under appropriate immunosuppression, can have a good transplant outcome in the face of a positive FCXM. Therefore, other aspects of the clinical/immunological status of the patient should be considered. It has been shown that the gender and race of the patient are important factors in graft and patient survival (UNOS database). Likewise, the patient's immunological makeup, prior sensitization events, and the class of immunoglobulins are all significant considerations when determining the importance of FC results versus AHG-CDC. Additional attention is warranted regarding living donations among spouses. Specifically, Terasaki et al. [25] demonstrated that the graft survival in wife-to-husband transplantation was equal to that of husband-to-wife if the female recipient had never been pregnant, but the 3 year graft survival dropped from 87% to 76% if the female recipient had had a previous pregnancy. We believe that the final decision whether to perform a transplantation is determined by the clinical judgment of the transplant physician, who weighs the risks and benefits of transplantation. However, it is also our opinion that such prerogative assessment should be based on all available information, including the highest sensitivity and specificity screening data such as FlowPRA or ELISA-based assays.

Future directions

Based on our recent experience with FlowPRA, we use the micoparticles to screen new patients at our center for the presence of anti-HLA antibodies. Apart from the higher sensitivity, we are now able to obtain better accuracy in assigning anti-HLA antibody specificity. We do not encounter problems due to autoantibodies and eliminate the detection of any IgM antibodies. We suggest that the lack of specificity with FCXM is due to non-HLA antibodies reacting with donor T or B lymphocytes. Thus, the

application of solid-phase screening methods (both ELISA and FC) to routine evaluations of patients' sera presents an opportunity to provide data that were previously difficult to generate. Specifically, a negative FlowPRA screening will allow us to confidently report a positive final FCXM as irrelevant reactivity since it is not due to HLA antibodies. Therefore, a patient with 0% antibody by FlowPRA (providing that no sensitization events had occurred in the meantime) can most probably proceed to transplantation without having to wait for prospective crossmatch results. This consideration is especially valuable in reducing ischemia time for cadaveric renal transplants. A similar concept was recently published by Kerman et al. [26] and Matas and Sutherland [27], calling for elimination of final crossmatch in non-sensitized patients (based on cytotoxicity assays). We want to stress, though, that currently only the solid-phase assays provide the required sensitivity and specificity for the assignment of 0% anti-HLA antibodies. (FlowPRA methodology has the additional advantage of distinctly identifying anti-class I from anti-class II antibodies).

FlowPRA can also be used for post-transplant monitoring of anti-HLA antibody development as a diagnostic tool for upcoming rejection events. Although this notion had already been addressed in the past, with partial success, we believe it was due to sensitivity and specificity limitations of the methods employed. Christiaans et al. [28] and Kimball et al. [29] recently reported that the presence of anti-donor antibodies post-transplantation is an indication of poor outcome in kidney recipients. We have data to substantiate the same conclusion in cardiac transplant recipients (unpublished data). Evidently, due to as yet undefined reasons, some patients are more prone to develop cellular or humoral rejection episodes, and belong to groups with a higher or lower risk to develop post-transplant immunological complications. Although the cellular component of rejection cannot be monitored using FC, the humoral component is considered more prominent in long-term chronic allograft dysfunction. Preliminary data from our laboratory indicate that using information gained by monitoring for %FlowPRA pre- and post-transplantation, together with the routine surveillance measures, will facilitate a strategy to sort heart recipients based on risk classification.

We propose that similar measures can be taken for all solid organ transplantation. The formulation of such algorithms will enable prospective and ongoing stratification of allograft recipients into risk categories for the purpose of designing distinctly tailored management protocols. Patients qualifying for the low risk group may benefit from rapid tapering of immunosuppression, as may individuals with moderate risk from a more frequent schedule of surveillance so that rejection episodes could be treated at the very onset with minimal permanent damage to the organ. And finally, patients at high risk could possibly be candidates for more aggressive initial immunosuppression regimens, more specific immunomodulatory protocols, and additional strategic interventions.

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