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# Applications of Flow Cytometry in Solid Organ Allogeneic Transplantation

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## 1. Introduction

The implementation of flow cytometry assays in evaluation of transplant recipients began in 1983 when Garavoy et. al. applied this technology to study the in vitro production of HLA antibodies and observed that flow cytometric technology identified serum HLA antibody levels so low that the complement dependent cytotoxicity assays could not detect them. Since even low levels of antibodies have been associated with early rejection episodes and graft loss, antibody detection by flow cytometry has become a routine technique for the study of donor and recipient compatibility, especially for renal allograft candidates and patients submitted to desensitizing pre-emptive therapies. Nowadays, flow cytometric based assays have become some of the most valuable tools to monitor allograft recipients both pre and post transplantation, with the detection and characterization of HLA-specific alloantibody being the principal application in organ transplantation.

## 2. Flow cytometry crossmatch

Flow cytometry crossmatch (FCXM), in contrast to standard complement-dependent cytotoxicity (CDC), measures alloantibody levels by quantifying the fluorescence intensity staining of anti-immunoglobulin (anti-Ig) reagents following the incubation of donor lymphocytes with patient serum, which is a more demanding and less well-defined application. The FCXM is typically performed by first incubating donor lymphocytes from peripheral blood, lymph node, or spleen with the recipient's serum, followed by a second incubation with a fluorescein-conjugated, anti-human- IgG (or anti-human-IgM) antibody. Phycoerythrin conjugated anti-CD3 antibody is also added to measure the anti-Ig-fluorescence on both T and non-T cells. Staining intensity produced by the patient's serum compared against that of a normal control serum provides the FCXM score. More recently, pretreatment of target cells with the proteolytic enzyme Pronase, a method which eliminates nonspecific binding of IgG to Fc receptors on B-cells, has been reported to further increase the sensitivity of the assay (Vaida et al, 2001).

## Target cells

Although B lymphocytes also express HLA Class I antigens, FCXM refers to T lymphocytes, which are the target of choice to measure cytotoxic alloantibodies directed against donor's HLA Class I antigens. T cells abundantly express HLA Class I antigens, but not Class II (T lymphocytes from organ donors are in general not activated), and produce very low levels of nonspecific fluorescence. IgG antibodies against other surface components are rarely found, so that a positive reaction is, as a rule, a good indicator for HLA Class I antibodies. However, the expression of individual HLA specificities is variable (Kao et al, 1988) and the signal-to-noise ratio is less favorable (Cook et al, 1985). As mentioned above, alloantibodies against Class I antigens, HLA-DR or DQ can be detected on B cells as well. However, the B-cell FCXM is not as reliable as the T-cell FCXM, and it remains difficult to interpret. The low sensitivity of the test in this case is attributed to the fact that B cells are in general less reliable than T cells as antibody targets, despite the use of a B-cell-specific marker (such as CD19 or CD20) when testing donor peripheral blood lymphocytes, whereas the background fluorescence of B cells is considerably higher than that of T cells (Scornik et al, 1985). In addition, a positive test may or may not be due to antibodies, because aggregated IgG or immune complexes can also bind to B cells.

*The T-cell FCXM:* Because of the higher sensitivity of FCXM, often in cases where the standard complement-dependent cytotoxicity (CDC) is negative, antidonor antibodies are detected by the FCXM. Although the concentration of these antibodies is probably not sufficiently high to produce a hyperacute rejection of the graft, it has been shown that patients with a positive T-cell FCXM and a negative CDC, especially those who lost a previous graft or had broadly reactive HLA antibodies before the transplant, have a higher risk for graft loss (Garavoy et al, 1983; Talbot et al, 1992; Barteli et al, 1992; Kerman et al, 1990; Ogura et al, 1993, 1994; Mahoney, 1990; Cook, 1987), which is attributed to the fact that previous transplants induce greatly expanded memory clones that may mediate graft rejection if they recognize antigens in the new transplant (Scornik et al, 1992). On the contrary, it seems that in nonsensitized, first renal transplant candidates, graft survival is as good as with negative FCXM results (Talbot et al, 1992; Scornik et al, 1994). Thus, the greatest value of the FCXM is in the pretransplant evaluation of patients at high immunological risk (retransplant and sensitized patients); a negative result puts them in the same outcome probability bracket as first transplant candidates, whereas a positive result makes the decision to transplant rather risky.

*The B-Cell FCXM:* The role of anti-HLA Class I antibodies in transplantation has been controversial (Braun, 1989). There is growing evidence that in the absence of Class I antibodies low-titer DR or DQ antibodies, as measured by cytotoxicity, are not detrimental for graft survival (Karuppan et al, 1990), albeit high-titer antibodies can produce hyperacute graft rejection (Scornik et al, 1992), although they do not always do so (Taylor et al, 1987; Panajotopoulos et al, 1992). A published report concluded that the mere presence of B-cell antibodies is not a risk factor, but that when the antibody concentration was above an arbitrary level, there was a decreased graft survival. The majority, but not all, of these cases were also positive by cytotoxicity (Lazda, 1994). Thus, it is not clear whether the B-cell FCXM contributes additional information to the cytotoxicity technique and, given the technical problems in using B-cell FCXM and its poor correlation with cytotoxicity, its clinical relevance remains to be proven.

## Result evaluation

The variance between patient and control sera staining intensity can be expressed either as a difference or shift (patient's serum minus control serum) or as a ratio (patient's serum divided by control serum). To account for the variability of the test, the distinction between a positive and a negative FCXM result is usually performed by testing a number of normal sera, with cutoff point the mean of all normal results plus 2 or 3 standard deviations (Garavoy et al, 1983; Talbot et al, 1992; Berteli et al, 1992; Scornik et al, 1989). This cutoff, however, may not be sufficient to account for the variability of the test and, to avoid false-positive results, it is often necessary to use a relatively high cutoff and sacrifice some sensitivity to gain specificity. Current literature lacks unanimity on FCXM, with several publications not providing their cutoff points and others reporting various shifts above normal control staining intensity (Cicciarelli et al, 1992; Kerman et al, 1990; Ogura et al, 1993; 1994; Mahoney et al, 1990). The distinction between weakly positive and negative results is also particularly problematic with the FCXM (Scornik, 1995).

## Clinical application

The choice of one or the other is usually based on the individual preference of a laboratory as it considers the relative advantages and disadvantages of the assays for clinical use. The FCXM is more sensitive, quantitative, and objective than CDC. On the other hand, CDC benefits from an experience of over 30 years of use and, being a microtechnique, is better suited for testing multiple specimens. In practice, whereas many centers consider FCXM as a *sine qua non* in order to proceed to transplant, other centers consider the FCXM to be "too sensitive" and the exclusion of a patient from being transplanted based on a positive result too conservative. In essence, even though the sensitivity of FCXM is significantly better compared to CDC based assays, its specificity (especially that of the B-cell FCXM) for HLA antibodies is low. Indeed, it is now well known that except for donor directed HLA antibodies, clinically irrelevant autoantibodies, non-HLA antibodies and even non-HLA specific immunoglobulins that bind to surface Fc receptors can also lead to positive crossmatches. In these cases, positive crossmatches not due to HLA antibodies are not actually false positive reactions, but they are clinically inconsequential. Nonetheless, a positive T lymphocyte FCXM in many centers was formerly always ascribed to HLA class I antibodies and a positive B cell crossmatch was considered due to class II HLA antibodies. The development of solid-phase antibody detection assays, particularly those utilizing flow cytometry or a Luminex platform, in the last years permitted the clinical determination of the cause of a positive FCXM, ie whether the positive FCXM was in fact due to clinically relevant (HLA class I and/or class II) antibodies. The implementation of these assays revolutionized allogeneic transplantation, in regard to interpretation of a positive FCXM. In solid phase antibody detection assays, clusters of class I or class II molecules (specifically, the entire class I or class II set of proteins expressed by a single individual) or, as of recently, individual HLA alleles, are adhered to microparticles that bind only to HLA specific antibodies. Thus, when a FCXM is positive but flow based solid phase antibody detection documents absence of HLA antibodies in the same serum, the positive crossmatch should be interpreted as clinically irrelevant.

In kidney transplantation, in parallel with donor HLA typing, the initial screening of potential recipients' sera with donor cells to exclude patients who have clear reactivity against that donor can only be done by CDC, unless the number of potential recipients is significantly reduced by some other selection criteria. Once the recipient is identified, the

final crossmatch is generally performed in most laboratories by CDC, whereas FCXM is done as an additional test in presensitized or retransplant candidates, although some programs perform it routinely in all patients. As long as the turn-around time of the FCXM is faster than CDC, the decision whether to transplant or not can be made based on the FCXM alone when the FCXM results are negative as well as when FCXM is positive in sensitized patients. In the remaining cases where FCXM is positive in patients who are not clearly sensitized, it is prudent to base the final decision on the results of all tests (FCXM, T-cell CDC, and B-cell CDC) (Scornik, 1995). Indeed, over the last two decades a positive FCXM has been shown to be a test conveying patients a higher risk not only for early antibody-mediated rejection and graft loss (Nelson et al., 1996; Talbot et al., 1992; Graff et al, 2010), but also for increased late renal allograft loss (Graff et al, 2009) as well as decreased survival (Ilham et al., 2008; Lindemann et al., 2010).

FCXM is a practical alternative to CDC in heart and liver transplantation because of its faster turnaround time, a lower incidence of ambiguous results due to poor cell viability, and the direct detection of IgG antibodies, which avoids the false-positive reactions caused by IgM autoantibodies. Although preformed anti-donor antibodies can cause early heart transplant rejection and compromise patient survival (Singh et al, 1983; Ratkovec et al, 1992), pretransplant crossmatch is performed only occasionally in heart transplantation. The liver is less susceptible to antibody induced damage (Fung et al, 1987), although recent reports indicate that preformed antibodies do represent a higher risk for rejection (Takaya et al, 1992; Nikaein et al, 1994). A positive crossmatch during or after the transplantation alert the clinicians for suspected early rejection episode. Antibodies detected by the FCXM but not by CDC also appear to be of significance in heart (Shenton et al, 1991) and liver (Ogura et al, 1994) transplantations. The procedures outlined for heart and liver are likely to apply to other solid organ transplants, such as lung and pancreas, as well albeit there is little information about the effect of anti-donor antibodies in these clinical settings.

### **Future prospectives**

Due to its superior performance characteristics, an increasing number of transplant programs perform the FCXM prospectively as a test complementary to CDC. Yet, a systematic evaluation of its capabilities and limitations is still missing. The main weak points of the technique that still warrant further elucidation are the variability of the background fluorescence for T and, especially, B cells, the changing behaviour of target cells according to their source or integrity, and the possibility of using standards or positive controls that can serve as reliable references for antibody binding are factors that still need to be studied in detail. In all cases, a complete understanding by the laboratory personnel of the technical principles and the clinical implications of the test is a prerequisite in order to provide a meaningful result and consult to the clinicians.

### **3. Flow cytometry HLA specificity**

Flow cytometry is used before and after transplantation to assess the specificity of anti-HLA antibodies. Single HLA antigen beads are used to determine specific HLA antibodies in high Panel Reactive Antibody (PRA) containing sera. In this technique, nucleated leucocytes are added to monoclonal antibodies that are labelled with a molecule that fluoresces. Cells with surface antigens that bind to the antibody become fluorescent. The flow cytometer detects the fluorescent cells by detecting the light emitted from them as they pass through a laser

beam. The antibody screen consists of testing the serum of the patient with a panel of 30 or more cells. The objective is to detect alloantibodies and to determine their HLA specificity so that donors carrying the target HLA antigens can be excluded beforehand. Flow cytometry has been used to screen for antibodies by using cell panels from 5-10 individuals (Scornik et al, 1984). This panel size is not sufficient to identify individual HLA antigen specificities but it is a sensitive way to determine the presence or absence of alloantibodies. The flow cytometry screen has been simplified by mixing several target cells in one tube (Cicciarelli et al, 1992), although this procedure has not become widely accepted. Given the increasing use of the FCXM at the time of transplantation, there is a growing need to perform antibody screens with the same degree of sensitivity. Some laboratories test larger cell panels, which is obviously an option for selected cases but not for routine use. Flow cytometry antibody screening with fewer (ex. 10) panel cells permits its application in all organ transplant candidates when first evaluated, whereas further testing is then decided according to the initial results. The later approach provides highly accurate and fast information and substantially reduces repeat or duplicate testing. With the use of the flow cytometry antibody detection assays described above donors expressing any of those antigens would be avoided, increasing the likelihood that, when a donor is crossmatched with the particular candidate recipient, the final result (predicted by "virtual crossmatching") will be negative.

#### 4. Posttransplant monitoring

Antibodies produced after transplantation can mediate acute rejection, although not all rejection episodes involve detectable antibodies. After the organ transplant, detection of antidonor antibodies can confirm a suspected diagnosis of rejection and the need for immediate antirejection therapy, indicate bone marrow toxicity during immunosuppressive therapies, and help in the differentiation of infections from transplant rejection (Shanahan, 1997). A variety of cell surface markers and activation antigens can be used depending on the clinical condition and the organ transplanted. Posttransplant antibody monitoring is not routinely necessary but it may be helpful in some cases of heart (Suciu-Foca et al, 1991; Smith et al, 1991) or kidney (Scornik et al, 1989; Martin et al, 1987; Zhang et al, 2005, Reed et al, 2006) transplantation. Flow cytometry is very useful because results with frozen donor cells are more consistent than when using the CDC technique and because differences between the pretransplant and posttransplant specimens can be detected with higher sensitivity. Results are usually not affected when patients have already been treated with OKT3 (OKT3 in the patient's serum kills T lymphocytes in the CDC test). In the presence of OKT3, the fluorescent CD3 reagent for flow cytometry staining should be replaced by another T-cell marker for satisfactory T-cell cluster separation. Finally, immunophenotyping of lymphocyte subsets with the use of flow cytometry, i.e. the analysis of cell surface markers using fluorochrome-conjugated monoclonal antibodies (ex., CD3+, CD4+, CD8+ T cells, CD19+ B cells, and CD3-, CD56+ NK cells) can be used for assessing the immune status of the patient and monitor effectiveness of immunotherapies.

#### 5. ATG/ALG therapy monitoring

One of the mainstays of therapy against the rejection process in transplantation, either as a prophylaxis or as treatment, is the use of anti-lymphocyte globulin (ALG) and anti-thymocyte globulin (ATG). Polyclonal heterologous products act principally by reducing the level of peripheral blood T-lymphocytes. The majority of transplant centres administer

ATG/ALG in an empirical fashion according to a fixed dose regimen. Due to the idiosyncratic response of individual patients, a fixed dose regimen may undersuppress some patients or result in oversuppression, which may, in turn, lead to acute renal allograft rejection or opportunistic infections.

The standard methods for monitoring the efficacy of ATG therapy are the determination of serum IgG levels (McAlack et al, 1979) and the detection of circulating T-cell levels with the use of E-rosettes employing sheep erythrocytes (Cosimi et al, 1976). The reproducibility of the E-rosette assay system was questioned when flow cytometric measurement of monoclonal antibody-labelled T lymphocytes was introduced (Cosimi et al, 1981). Flow cytometric analysis of monoclonal antibody-labelled T lymphocytes proved to be more accurate, more reproducible, and easier to perform (No authors listed, 1985) than the E-rosette assay, with the optimal threshold T-cell count for such treatment being approximately 50 T cells/ $\mu$ L (Shenton et al, 1994). Flow cytometry with the use of three fluorescent markers provides an excellent, rapid and reproducible way of measuring such cells. The lymphocytes are identified using right-angle light side scatter (RALS) vs anti-CD45 (PerCP) plots, with the use of anti-CD3-FITC markers for the determination of the percentage of T cells and the inclusion of Leu-M3-PE (anti-CD14) for the removal of monocytes from the gate. By spiking the labelled sample with fluorescent beads the number of T cells/ $\mu$ L can be calculated.

## 6. Conclusion

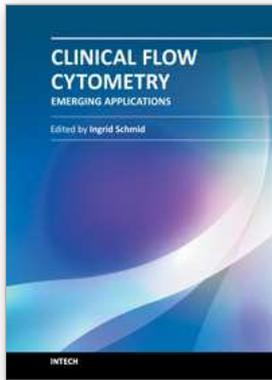
Flow cytometric based assays have become amongst the most valuable tools to monitor allograft recipients both pre and post transplantation nowadays. Their principal applications in solid organ transplantation are the FCXM and the detection and characterization of HLA-specific alloantibodies. Whereas they have been in clinical use for more than a decade, the accumulating experience of the laboratories and the clinicians have generated expectations for even more exciting applications in the years to come.

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"Clinical Flow Cytometry - Emerging Applications" contains a collection of reviews and original papers that illustrate the relevance of flow cytometry for the study of specific diseases and clinical evaluations. The chapters have been contributed by authors from a wide variety of countries showing the broad application and importance of this technology in medicine. Examples include chapters on autoimmune disease, cancer, and the evaluation of new drugs. The book is intended to give newcomers a helpful introduction, but also to provide experienced flow cytometrists with novel insights and a better understanding of clinical cytometry.

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