Comparison of Cytotoxic Flow Cytometric Cross Match With Complement Dependent Lymphocytotoxicity and Flow Cytometric Cross Match in Renal Transplant Patients

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ABSTRACT

Cytotoxic flow cytometric crossmatch (cFCXM), identified by detecting complement-mediated cytotoxic cell death in addition to the capability of showing the alloantibodies binding onto lymphocytes at the same time, can reduce the necessary time and workload in evaluating alloantibodies. More data from clinical samples are needed for cFCXM to be accepted by tissue typing laboratories. In this study, we compared cFCXM with complement-dependent lymphocytotoxicity and standard flow cytometric crossmatch in 41 renal pretransplant patients. A comparison of the obtained data was performed using Spearman's correlation test. We found that cFCXM showed no statistically significant differences with complement-dependent lymphocytotoxicity and flow cytometric crossmatch. We believe that cFCXM can be used in clinical laboratories in the near future following intra-laboratory validation.

IN TISSUE typing laboratories, complement-dependent lymphocytotoxicity crossmatch (CDCXM) and flow cytometric crossmatch (FCXM) are 2 commonly used distinguished tests to detect preformed donor-specific antibodies, which are associated with early graft loss [1]. Because these 2 techniques have distinct advantages and disadvantages, they both are performed for each donor and recipient pair in clinical practice [2–4]. As a modified FCXM technique, cytotoxic flow cytometric crossmatch (cFCXM), which can simultaneously detect both complement dependent cytotoxicity and alloantibody binding onto lymphocyte in a single assay, has recently been described [5–9]. Previous studies have shown that cFCXM is a promising assay that exhibits valuable advantages [10–12]. Because of the invaluable nature of the clinical data, we compared cFCXM with CDCXM and FCXM assays in 41 different renal transplant patients in this current study.

MATERIALS AND METHODS

Forty-one different serum samples obtained from individuals who had been preparing for organ transplantation in the Department of Nephrology and Transplantation Unit, Faculty of Medicine, University of Medical Sciences were included in the study. Lymphocytes were isolated from heparinized peripheral blood from healthy donors using the standard Ficoll-Hypaque density gradient centrifugation technique. Negative and positive control serum samples verified during previous studies were included. The study was approved by the Tekirdağ Namık Kemal University Ethical Committee and informed consent was obtained from all patients and healthy volunteers.

cFCXM, CDCXM, and Standard FCXM Tests

Propidium iodide and acridine orange fluorescent dyes were used in combination in the analysis of the CDCXM test. The dead cell ratio was determined as a percentage of the total number of cells. FCXM tests were performed according to standard procedures and analyzed using a BD FACSCalibur (BD Biosciences, Sparks, Md, United States). The cFCXM technique is described below; the complement-dependent cytotoxicity was determined using 7 aminoactinomycin D (7AAD) dye (Biolegend, San Diego, Calif, United

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Table 1. Comparison of cFCXM With CDCXM for Cytotoxic Cell Death and With FCXM for MFI Values

Techniques		CDCXM (n = 41)	FCXM (n = 41)
cFCXM	r	.649*	.849*

Abbreviations: CDCXM, complement-dependent lymphocytotoxicity crossmatch; cFCXM, cytotoxic flow cytometric crossmatch; FCXM, flow cytometric crossmatch; MFI, median fluorescence intensity; r, correlation efficiency.
*Correlation is significant at the .01 level.

States) and its fluorescence intensity was analyzed using channel 3 of the BD FACSCalibur (BD Biosciences). The lymphocyte-Ab binding was determined with anti-IgG-fluorescein isothiocyanate (Jackson Immunoresearch Laboratories, West Grove, Pa, United States). Cell suspensions were incubated with serum samples in polypropylene tubes at room temperature for 30 minutes. Forty microliters of rabbit complement (Millipore Sigma, Darmstadt, Germany) was added to the centrifuged and gently resuspended cells in each tube. The cells were incubated at room temperature for 60 minutes. The cells were then washed 3 times with .5 mL of cell wash solution and centrifuged for 5 minutes at 1500 rpm. One hundred and sixty microliters of fluorescein isothiocyanate–conjugated goat F(ab)2 anti-human IgG (1/40 fold diluted) were added to the resuspended cell pellet and incubated in the dark at 4°C for 30 minutes. The cells were washed twice with .5 mL of cell wash solution. Twenty microliters of 7AAD staining solution was added to the washed and resuspended cells. The mixture was incubated in the dark at 4°C for 15 minutes. The cells were resuspended with 0.4 mL of FACS flow solution and transferred into flow tubes for analysis using the BD FACSCalibur. The cytotoxicity ratio was calculated as percentage of 7AAD positive cells within all cells and it was compared to the cytotoxic cell death percentage detected in CDCXM. The Ab binding score was determined as the median fluorescence intensity (MFI) value on the histogram. The MFI values reflecting IgG alloantibody binding were compared to the simultaneously studied cFCXM and FCXM. A comparison of the obtained data was performed using Spearman's correlation test and SPSS (IBM, Armonk, NY, United States).

RESULTS

The current study confirmed that cells that die because of complement treatment form a differentially located homogenous cluster separate from the live cells on a forward scatter (FSC)/side scatter (SSC) plot. This finding was considered both an independent parameter and an internal control parameter in cFCXM. The cytotoxic cell death percentages obtained from the CDCXM and cFCXM analyses were detected both by 7AAD positivity and by gating at FSC/SSC plot were similar in the 41 serum samples. The correlation efficiency between CDCXM and cFCXM analyses was found to be .649, with $P < .01$ (Table 1). In a corresponding finding, the IgG-fluorescein isothiocyanate MFI values at cFCXM analysis were considerably similar to those of FCXM. The correlation efficiency between cFCXM and FCXM analyses was found to be .849 with $P < .01$ (Table 1).

DISCUSSION

The cFCXM test was designed to demonstrate complement-mediated cell death in the flow cytometry platform in addition to showing the IgG alloantibodies. Hence, not only complement dependent cell death but also IgG alloantibodies can be detected in a single tube and single assay using the cFCXM technique. Combining the CDCXM and FCXM assays gives the cFCXM the potential to reduce the required time and workload involved in evaluating immunological risks in transplant patients. Our previous study, performed using serially diluted positive sera, and other studies have shown that complement-dependent cytotoxicity and the IgG alloantibody bound onto lymphocytes can be technically detected using cFCXM [6,10,13,14]. Moreover, more than 90% of the consistencies were previously reported among cFCXM, CDCXM and FCXM tests [8]. Another previous study using clinical samples reported that the correlation efficiencies between CDCXM and cFCXM were .522 for B-cell crossmatch and .670 for T-cell crossmatch, although the researchers did not evaluate the correlation between MFI values of cFCXM and standard FCXM [15]. Similarly, we found a correlation efficiency of r = .649 between CDCXM and cFCXM for the total lymphocyte pool in our study. Moreover, there was a high correlation (r = .849) between MFI values detected for IgG alloantibodies in cFCXM and conventional FCXM assays in our study. Another prominent

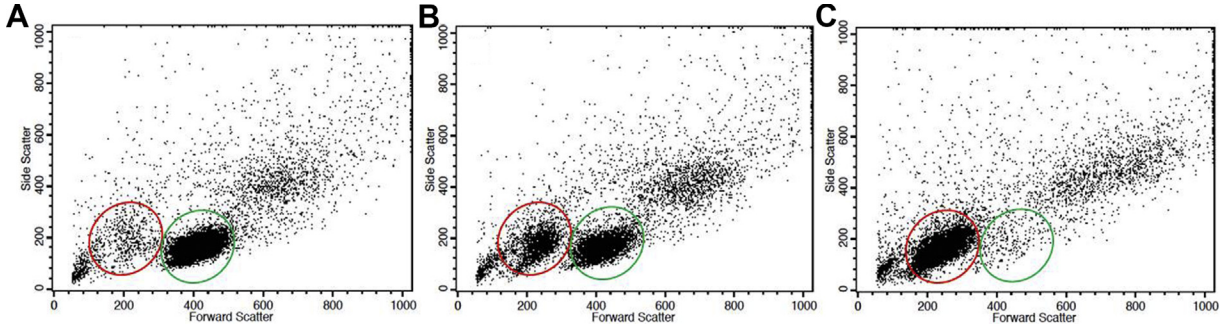

Fig 1. The complement-dependent dead lymphocyte percentage can be calculated by forming separate clusters on the FSC/SSC plots in cFCXM assay without using a fluorescent dye. The left circle (green) indicates the dead lymphocyte zone and the right circle (red) indicates the live lymphocyte zone. **(A)** cFCXM by negative serum; **(B)** cFCXM by 1:4 diluted positive serum; **(C)** cFCXM by positive serum.

result of our study is that the dead cells resulting from the complement treatment form a distinct cluster on FSC/SSC plot of flow cytometry in the cFCXM assay (Fig 1). Dead and live cell segregation in the FSC/SSC plot presents an opportunity to calculate the complement-dependent cytotoxicity on the FSC/SSC plot without using fluorescent dyes. This finding was considered both an independent parameter and an internal control parameter in cFCXM. The other important point is that the cFCXM assay may provide information about the cytotoxic Abs ratio within the total alloantibody pool in a sample. This makes the cFCXM assay an alternative to the C1q assay and a novel parameter for pretransplant immunological assessment. We believe that the cFCXM assay will be an important tool for pretransplant donor-specific alloantibody evaluation by accumulation of data from more clinical samples.

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