Cytotoxic Antibody Detection by Means of Flow-Cytometric Cross-Match

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ABSTRACT

Background. Complement-dependent lymphocytotoxicity (CDC-XM) and flow-cytometric (FCXM) cross-match are analyzed individually for each donor and recipient pair, because these techniques have fundamental differences for the evaluation of histocompatibility. Lately, cytotoxic flow-cytometric cross-match (cFCXM) has been developed as an alternative to both CDC-XM and FCXM techniques. We evaluated the limits of cFCXM with the use of different positive serum dilutions.

Methods. CDC-XM, FCXM, and cFCXM tests were performed with the use of commercially available negative and positive serum samples and lymphocytes from healthy donors.

Results. Complement-dependent cell death was successfully detected with the use of cFCXM. Complement-dependent cell death ratios in cFCXM were similar those in CDC-XM. With cFCXM, not only complement-dependent cell death but also IgG binding could be detected within a single assay. At higher concentrations of the positive serum, IgG–fluorescein isothiocyanate (FITC) mean fluorescent intensity (MFI) values detected with the use of cFCXM were less than those of conventional FCXM. Correspondingly, for dead cells, MFI values of IgG-FITC were less than those of live cells in higher positive serum concentrations in the cFCXM assay. Moreover, our results demonstrated that in cFCXM analysis, the decreasing ratio of dead cells at increasing positive serum dilutions was not in parallel with the same decrease in IgG-FITC MFI values.

Conclusions. The cFCXM technique detects complement-mediated cytotoxic cell death with the additional ability to show IgG binding in the same tube and therefore may reduce the necessary bench time and workload.

PREFORMED antibodies (Abs) are strictly associated with early graft loss [1]. Complement-dependent lymphocytotoxicity (CDC-XM) and flow-cytometric (FCXM) cross-match are 2 distinct commonly used tests to detect preformed donor-specific Abs in tissue-typing laboratories. Because these 2 techniques are able to reveal different properties, making them unique, they are separately analyzed for each donor and recipient pair. The most prominent advantage of CDC-XM is its ability to detecting cytotoxic Abs. On the other hand, low Ab levels below the detection limit of CDC-XM are thought to be clinically important and responsible for rejection [2]. FCXM is more sensitive than CDC-XM and can detect these low level Abs but without discriminating the complement-fixing and noncomplement-fixing properties of the Abs. It is also

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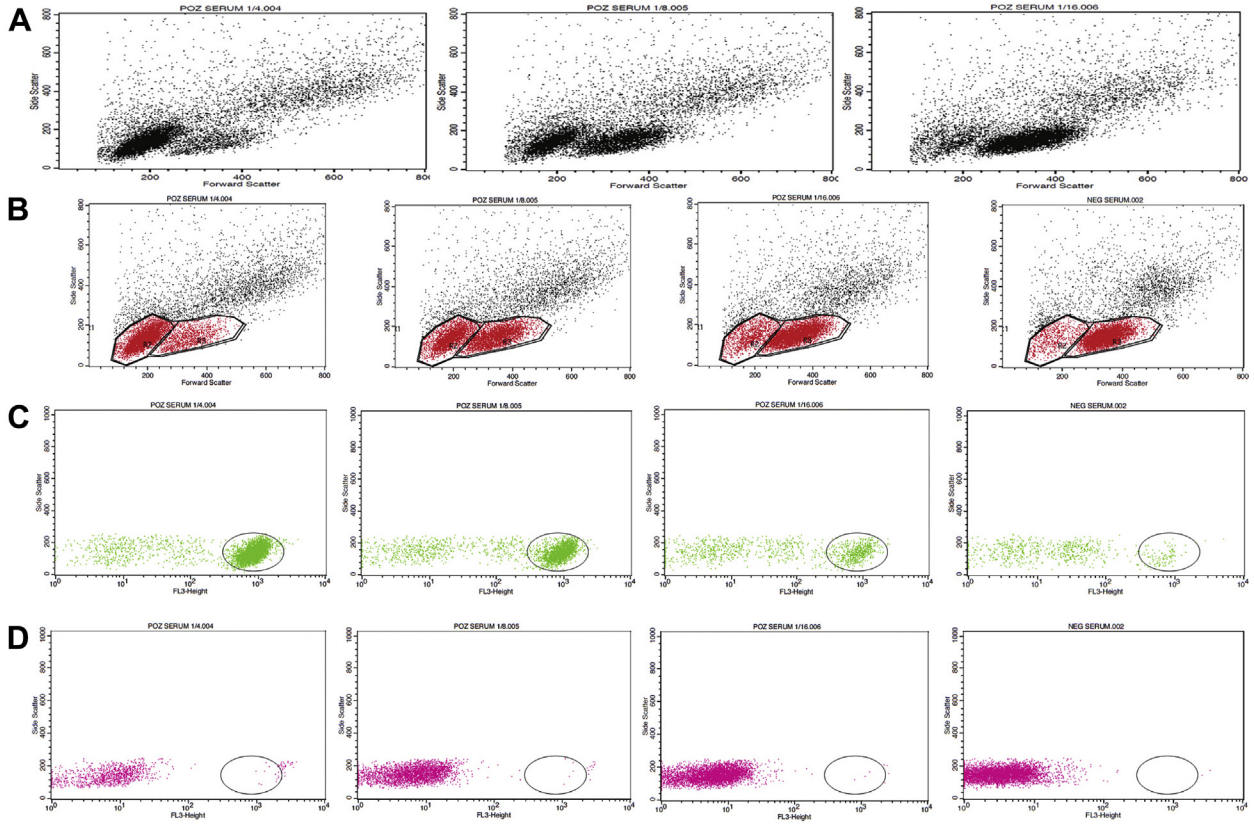

Fig 1. (**A**, **B**) The cells killed by complement-dependent cytotoxicity formed homogenous clusters on the FSC/SSC plots so that the percentage of dead cells could be calculated without using a fluorescent dye. (**C**) 7AAD positivity of dead cells. (**D**) 7AAD positivity of live cells.

thought that FCXM may cause "false positive" results due to detection of clinically irrelevant Abs rather than cytotoxic Abs [3,4]. Recently, a new technique has been described as cytotoxic flow-cytometric cross-match (cFCXM) and can detect simultaneous cytotoxicity and lymphocyte antibody binding in a single assay [5–10]. cFCXM, if standardized, because it exhibits the advantages of both CDC-XM and FCXM, might be a useful analysis tool in clinical laboratories. By using different dilutions of the pooled positive serum which is tested for its IgG subclass composition and Luminex-based panel reactive antibody (PRA) level, we designed the present study to evaluate the limits of cFCXM.

METHODS

Lymphocytes were isolated from heparinized peripheral blood from healthy donors by means of the standard Ficoll-Hypaque density-gradient centrifugation technique. Negative control serum was a commercially available product. Pooled positive serum samples were prepared by mixing sera from 3 adult renal transplant patients with high PRA levels detected with the use of Luminex. The positive sera were analyzed with the use of enzyme-linked immunosorbent assay–IgG subclass and Luminex PRA analysis methods. IgG subclass testing was performed using Novex IgG Subclass Human ELISA Kit (Invitrogen).

CDC-XM, FCXM, and cFCXM

Propidium iodide and acridine orange fluorescent dyes are used in combination at the analysis of CDC-XM test. As a percentage to total cells, the dead cells ratio was determined. FCXM tests were performed according to standard procedures and analyzed with the use of FACScalibur (Becton Dickinson). The cFCXM technique was as follows.

Complement-dependent cytotoxicity was determined with the use of 7-aminoactinomycin-D (7AAD) dye (Biolegend) and its fluorescence intensity was analyzed at channel 3 of the FACScalibur. The lymphocyte-Ab binding was determined with the use of anti–IgG-fluorescein isothiocyanate (FITC) (Jackson Immunoresearch Laboratories). Cell suspensions were incubated with 50 μL positive serum serially diluted at 1/2 to 1/128 dilution ratios in polypropylene tubes at room temperature for 30 minutes. Forty microliters of the rabbit complement (Sigma) was added to the centrifuged and gently resuspended cells at each tube. The cells were incubated at room temperature for 60 minutes. The cells were then washed 3 times with 0.5 mL cell wash solution and centrifuged for 5 minutes at 1,500 rpm. Hundred sixty microliters of FITC-conjugated goat F(ab)2 anti–human IgG (1/40 diluted) was added to the resuspended cell pellet and incubated in the dark at 4°C for