Simultaneous Detection of Antibody Binding and Cytotoxicity in Flow Cytometry Crossmatch for Renal Transplantation

Dong Il Won,[1]* Hee Du Jeong,[1] Yong Lim Kim,[2] and Jang Soo Suh[1]

[1]Department of Laboratory Medicine, Kyungpook National University Hospital, Daegu, Korea

[2]Division of Nephrology, Department of Internal Medicine, Kyungpook National University Hospital, Daegu, Korea

Background: The anti-HLA antibody can be detected using either a complement-dependent lymphocytotoxicity (CDC) assay or a flow cytometry crossmatch (FCXM) in renal transplantation. Discordant results are often obtained because the two methods detect different reaction phases between the donor lymphocytes and the recipient sera. This study was intended to confirm that antibody binding and cytotoxicity to the lymphocytes can be detected simultaneously in a single FCXM assay, cytotoxic FCXM.

Methods: In the cytotoxic FCXM, the antibody binding to the lymphocytes was measured using anti-IgG-FITC, and the cytotoxicity using 7-aminoactinomycin D (7-AAD) after adding complement. For an evaluation of two test parameters, the cytotoxicity test moiety (dead-cell percentage) was compared with the anti-human globulin (AHG)-CDC, and the antibody-binding test moiety (sample/control fluorescence ratio) with the conventional FCXM in 77 positive and 30 negative crossmatches.

Results: In the cytotoxic FCXM, both antibody binding and cytotoxicity could be assessed in a single anti-IgG-FITC/7-AAD plot. Regarding the correlation between the presence of HLA antibodies and the test result, the cytotoxicity parameter (r = 0.55) appeared to be more suitable than that of the AHG-CDC (r = 0.50) but the antibody-binding parameter (r = 0.83) was worse than that of the conventional FCXM (r = 0.93). The sensitivity of both parameters of the cytotoxic FCXM was not significantly different from each conventional counterpart (P = 0.33 and P = 0.22, respectively).

Conclusions: The simultaneous detection of Ab binding and cytotoxicity was possible by the cytotoxic FCXM with the test efficiencies similar to the conventional counterparts. If this new assay is improved through the further studies to optimize the critical assay variables, this may be used as an alternative to the conventional assays to acquire more information on the characteristics of the recipient's HLA alloantibodies. © 2006 International Society for Analytical Cytology

Key terms: transplantation; crossmatch; HLA antibody; complement-dependent lymphocytotoxicity; flow cytometry crossmatch

Preformed HLA antibodies (Abs), which play a major role in the early loss of an allograft, can have a low titer and be generally undetectable even in the most sensitive complement-dependent lymphocytotoxicity (CDC) assays. However, these Abs are readily detectable using a flow cytometry crossmatch (FCXM) (1–3), because the FCXM method is up to 50 times more sensitive than that of the CDC (4). Renal transplant recipients showing a negative CDC but a positive FCXM are also likely to experience early accelerated rejection episodes and graft loss (5).

However, there is a great deal of controversy regarding the clinical significance of Abs detected only by a "too sensitive" FCXM in renal transplantation. These Abs have not been always associated with decreased renal allograft survival (6,7). Normal or irrelevant IgG can bind to the Fc receptors on the T- and B-cell surface, particularly when the sera contains IgG in an aggregated or complexed form and causes a false-positive result (8,9).

The CDC technique is also influenced by many factors, and can show false-positive and false-negative test reactions (10). False-positive reactions can occur because of autoantibodies, non-HLA-specific lymphocytotoxic Abs, and antilymphocyte Abs infused for therapy. The CDC can-

Grant sponsor: Kyungpook National University Hospital.

*Correspondence to: Dong Il Won, M.D., Department of Laboratory Medicine, Kyungpook National University Hospital, 50 Samduk-Dong 2-Ga, Jung-Gu, Daegu, 700-721, South Korea.

E-mail: wondi@knu.ac.kr

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Table 1
Characteristics and Tested Number of HLA Allosera in This Study[a]

Serum ID	% PRA	HLA specificity	Number of sera tested (total 77)				
			Undiluted sera	Diluted sera			
				5[b]	10[b]	20[b]	30[b]
SY	96	Polyspecific	3	6	16	15	1
JH	98	Polyspecific	3	2	2	2	
UY	57	A24, B37, 44	2	3	3	1	
HJ	91	Polyspecific			4	4	4
PW	93	Polyspecific			2	2	2

% PRA, percentage of panel reactive antibodies.
[a]The HLA specificity of sera was determined by CDC. The % PRA was determined by flow cytometry.
[b]1/dilution of sera.

not detect a noncomplement-fixing or low-level complement-fixing HLA Abs (11).

In the HLA crossmatches, cases with a positive FCXM and a negative CDC are often met. In these cases, two separately performed tests must initially be investigated for probable technical errors so as to accept the two discrepant results as being true. If two test procedures are performed in a single assay, an in vitro serial process such as an Ab binding and complement-mediated cytotoxicity can be assessed simultaneously. In particular, this may make it possible to distinguish between two populations of Ab-bound lymphocytes, one that reaches the complement-mediated cell death and another that remains vital without complement-activation. Therefore, a new method is required to be developed, which enables the simultaneous detection of Ab binding and cytotoxicity to the lymphocytes in a single FCXM assay. This study was intended to confirm that Ab binding and cytotoxicity to the lymphocytes can be detected simultaneously in a single FCXM assay, using already known techniques to improve the sensitivity of the National Institutes of Health (NIH)-CDC (10). This new FCXM was named the cytotoxic FCXM.

MATERIALS AND METHODS

Cell and Sera Preparation

Heparinized blood was obtained from healthy donors ($N = 20$). Mononuclear cells were isolated from the peripheral blood using the standard Ficoll-Hypaque technique. HLA allosera were obtained from the five renal transplant candidates who had complement-fixing HLA alloantibodies of a high panel reactive antibody percentage, which was evaluated by the anti-human globulin (AHG)-CDC and flow cytometry (FC), respectively (Table 1). For positive crossmatches, each lymphocyte donor was paired with one serum among these HLA allosera, which was confirmed to have complement-fixing HLA alloantibodies specific against the donor by a positive AHG-CDC crossmatch. This serum was diluted 1:5, 1:10, 1:20, or 1:30 with RPMI appropriately so as to have strong, weak, or no cytotoxic reactivity. All the diluted sera were also regarded as being HLA allosera directed against the donor regardless of their high dilution. In this manner, 77 crossmatches were designed to give a true positive reaction. The negative control sera ($N = 30$) were obtained from the nontransfused healthy male donors (blood group AB), and 30 crossmatches using these sera were designed to give a true negative reaction. In each crossmatch, we compared the results obtained by three different methodologies below.

Cytotoxic FCXM

The Ab binding to the lymphocytes was detected using anti-IgG-FITC in a similar manner to that of the conventional FCXM. Complement-mediated lymphocyte death was detected using 7-aminoactinomycin D (7-AAD). The 7-AAD penetrates only the damaged cell membranes and stains the DNA. Its fluorescence can be measured in the FL3 channel of a flow cytometer.

Twenty-microliter volumes of a cell suspension (3×10^6 cells/mL RPMI) were incubated with 50 μL of the test serum in FACS tube at room temperature for 60 min. After incubating with the serum, 200 μL of the rabbit complement (Biotest, Dreieich, Germany) was added and the cells incubated at room temperature for 120 min. The cells were then washed four times with 2 mL of phosphate-buffered saline (PBS), and the supernatant was discarded. The cells were then resuspended for staining. Twenty microliters (at a 1:40 dilution) of fluorescein isothiocyanate (FITC)-conjugated goat $F(ab)_2$ anti-human IgG, Fcγ specific (Jackson Immunoresearch Laboratories, West Grove, PA) was added to the cell pellet. Twenty microliters of the pretitered phycoerythrin (PE)-conjugated anti-CD3 (Dinona, Seoul, Korea) was added to the above mixture so as to identify T cells. The mixture was incubated in the dark at 4°C for 30 min. The cells were washed once, and then resuspended. Five microliters of 7-AAD (Becton Dickinson, San Jose, CA) was added to the cell pellet. The mixture was incubated in the dark at 4°C for 10 min. The cells were resuspended after adding 130 μL of PBS and were ready for FC analysis.

The fluorescence was evaluated using an FACSCalibur Flow Cytometer (Becton Dickinson). The T cells were gated on the forward scatter/side scatter (SSC) plot and the CD3-PE/SSC plot (Fig. 1A). Of the two test parameters, the cytotoxicity (the first parameter) was measured as the percentage of dead cells (dead-cell %), which was the percentage of events in the upper two quadrants in the anti-IgG-FITC/7-

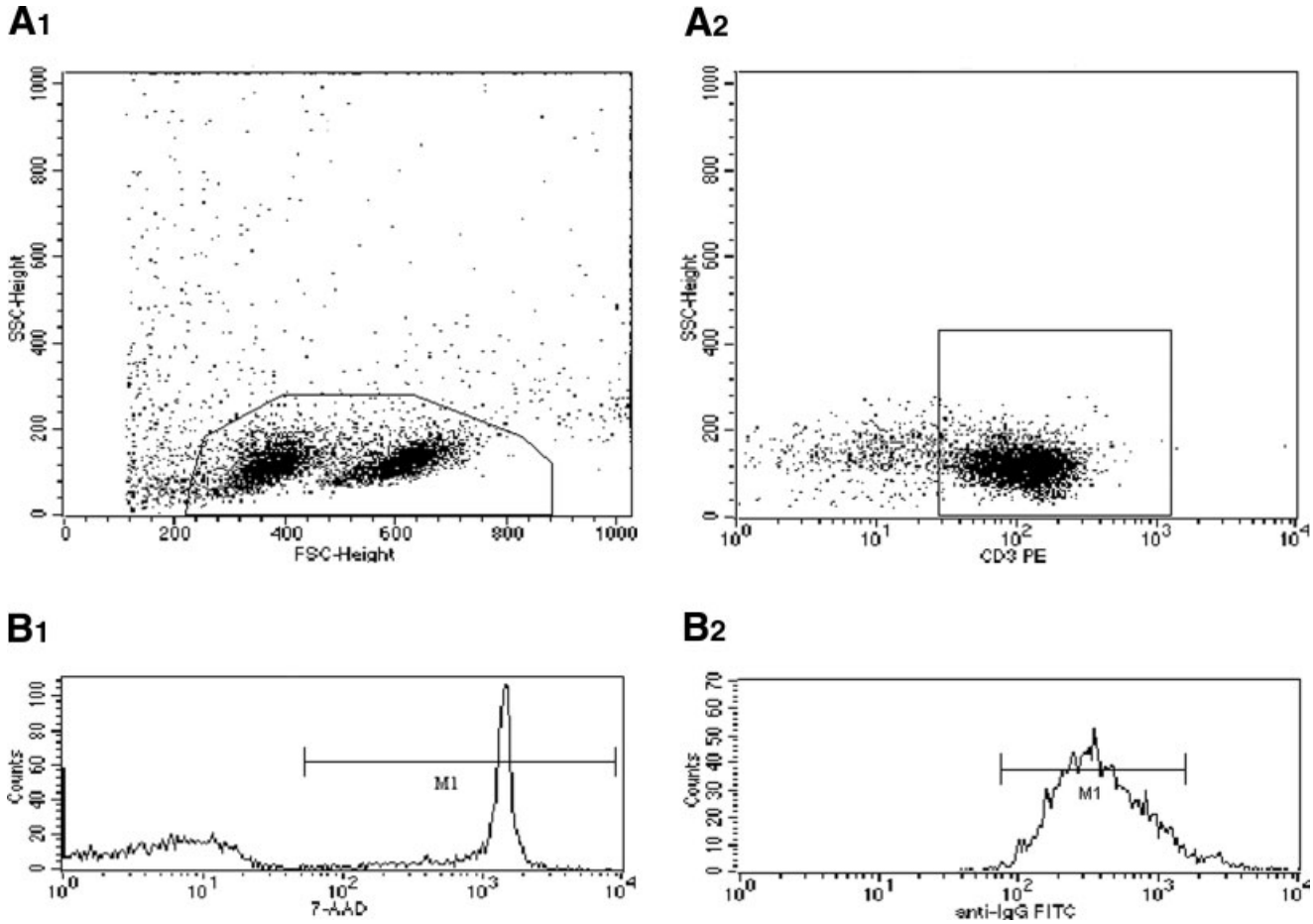

FIG. 1. Analyses of two test parameters of cytotoxic FCXM. A lymphocyte gate was set on the FSC/SSC plot (**A1**), and a T-cell gate on the CD3-PE/SSC plot (**A2**). The dead-cell % was determined on the 7-AAD histogram of the T cells (**B1**), and the MFI on the anti-IgG-FITC histogram of the T cells (**B2**). FSC, forward scatter characteristics; SSC, side scatter characteristics; 7-AAD, 7-aminoactinomycin D; MFI, mean fluorescence intensity.

AAD plot (Fig. 2) or shifted to the right of the cutoff point on the 7-AAD histogram (Fig. 1B1). The appropriate cutoff point in the plot or histogram was defined for each test cell as compared with the negative controls. The Ab binding (the second parameter) was measured as the mean fluorescence intensity (MFI) ratio (sample MFI/control MFI). Each MFI was determined as a geometric mean of the peak on the anti-IgG-FITC histogram of the T cells (Fig. 1B2).

The cutoff definition for the qualitative determination of two measured parameters was previously found after the statistics of mean and standard deviations using multiple negative sera. At each batch, four negative control sera were used, and its mean dead-cell % was somewhat different between the batches, mainly dependent on the donor lymphocytes. Therefore, the criteria for the positive cytotoxicity was defined as more than twice the mean dead-cell % of negative controls of its own batch, not as a constant value. The median and standard deviation of the dead-cell % of all the negative controls during this study were 6.1 and 2.9%, respectively. Thus, the universal cutoff for the cytotoxicity is supposed to be $6.1 \times 2 = 12.2\%$. A positive MFI ratio was defined as more than 2.0, a constant value.

For evaluation of the two test parameters of the cytotoxic FCXM, the cytotoxicity test moiety was compared with the AHG-CDC crossmatch, and the Ab-binding test moiety with the conventional FCXM.

AHG-CDC Crossmatch

CDC crossmatch was performed by the AHG-mediated crossmatches. One microliter of a nylon-purified T-lymphocyte suspension and 1 μL of the serum (dilution range from 1:1 to 1:32) were incubated at room temperature for 60 min. The cells were washed three times with 5 μL of RPMI to remove the excess Abs. One μL (at a 1:100 dilution) of AHG (anti-human κ chain, Helena Laboratories, Beaumont, TX) was then added for 2 min before adding 5 μL of rabbit complement. The mixture was incubated at room temperature for an additional 120 min. Eosin-Y was added to each well. Finally the cells were fixed with formalin. The microtest trays were analyzed by phase contrast microscopy. The readout of the assay was done in two different manners: (1) the titer of AHG-CDC was determined as the highest dilution level, showing a dead-cell % higher than 10–20% above the

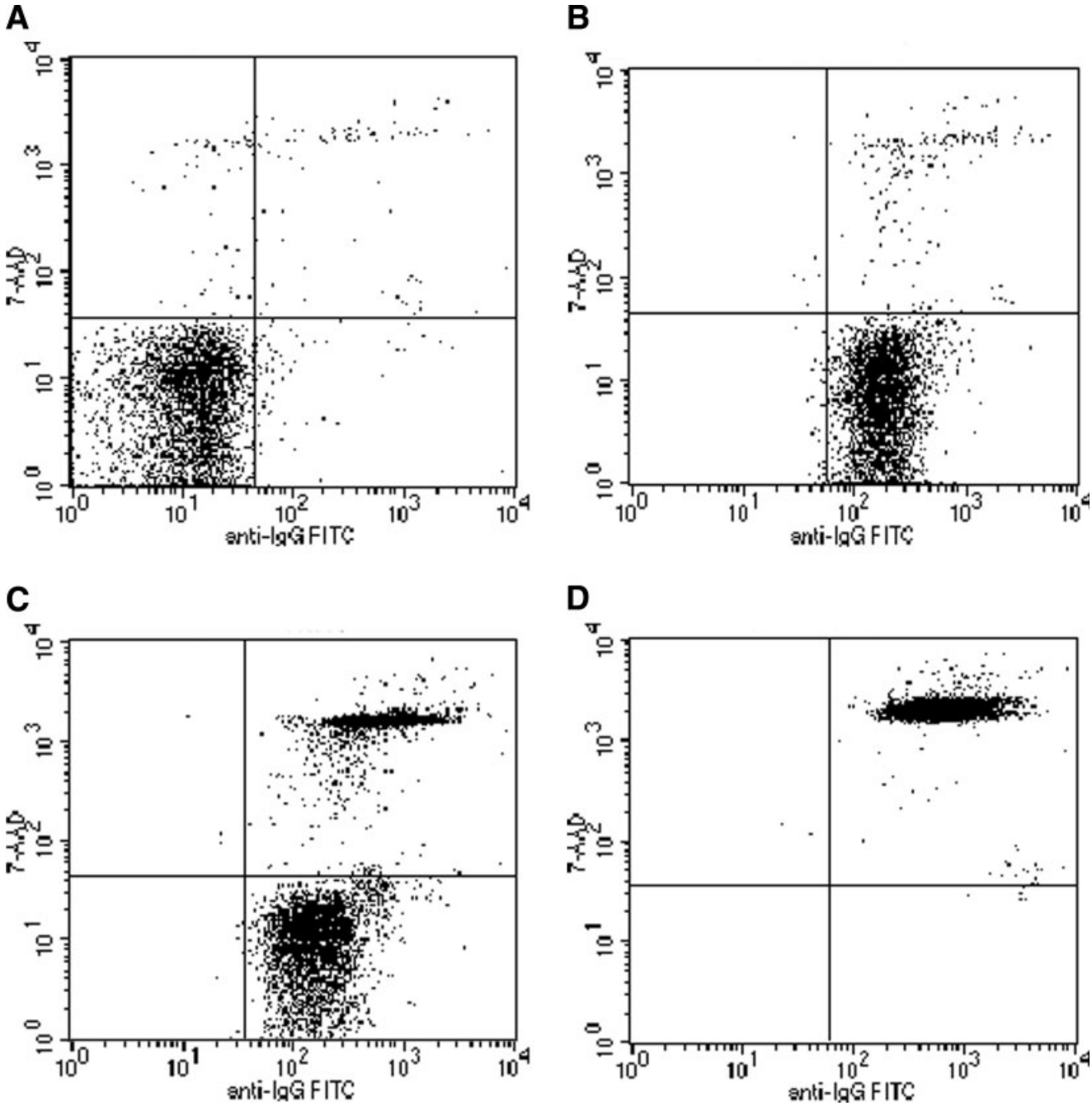

FIG. 2. Anti-IgG-FITC/7-AAD plot of representative examples of T-cell cytotoxic FCXM using the negative sera and various HLA allosera. The quadrant setting was defined by the negative controls. Neither Ab binding nor cytotoxicity was detected in the negative sera (**A**). Ab binding without cytotoxicity was detected in the so called "CYNAP" sera (**B**). In the HLA allosera of the positive AHG-CDC, Ab binding was detected in all T cells, but cytotoxicity was detected in some T cells (**C**) or in all T cells (**D**). 7-AAD, 7-aminoactinomycin D.

background of the negative controls; (2) the dead-cell % was determined as a mean of two different experts at the well of titer 1:1 made in duplicate. Because an exact counting of the cells in the wells is difficult, the readout was subjectively classified into 16 levels of the dead-cell % for the direct comparison with the dead-cell % by cytotoxic FCXM: 2, 5, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 85, 90, 95, and 100%.

Conventional FCXM

Twenty-microliter volumes of a cell suspension were incubated with 50 μL of the test serum in FACS tube at 37°C for 30 min. The cells were then washed four times with 2 mL of PBS, and the supernatant was discarded. The cells were then resuspended for staining. Twenty microliters (at a 1:40 dilution) of anti-IgG-FITC was added to the

Table 2
*Quantitative Results of the Two Parameters of the Cytotoxic FCXM When Compared with That of Its Conventional Counterpart**

	Cytotoxicity (dead-cell %)		Ab binding (MFI ratio)	
	Cytotoxic FCXM	AHG-CDC	Cytotoxic FCXM	Conventional FCXM
Negative sera ($N = 30$)	6.8 ± 2.9	4.2 ± 2.1	1.0 ± 0.4	1.0 ± 0.1
HLA allosera ($N = 77$)	37.9 ± 36.9	37.9 ± 39.1	22.6 ± 26.9	54.1 ± 82.9
paired *t*-test	$P = 0.458$		$P < 0.0001$	

*Data are mean ± standard deviation.

cell pellet. Twenty μL of the pretitered anti-CD3-PE was added to the above mixture. The mixture was incubated in the dark at 4°C for 30 min. The cells were washed once, and then resuspended after adding 130 μL of PBS, and were ready for FC analysis. A positive MFI ratio was defined as more than 2.0.

Statistical Analysis

Statistical analyses were performed using CELLQuest (Becton Dickinson), Microsoft Excel (Microsoft, Redmond, WA), and SPSS (SPSS, Chicago, IL). The sensitivity of the cytotoxic FCXM was calculated as the probability of a positive result in the presence of known HLA Abs. The specificity was calculated as the probability of a negative cytotoxic FCXM in the absence of HLA Abs. A comparison of the conventional, cytotoxic FCXM and AHG-CDC was carried out using a paired *t*-test, correlation coefficient, and Fisher's exact test. A comparison of the sensitivity was carried out using a McNemar test. Statistical significance was defined as $P < 0.05$.

RESULTS

In the cytotoxic FCXM, Ab binding and cytotoxicity could be assessed in a single anti-IgG-FITC/7-AAD plot. This plot helped to visually discriminate the so called "CYNAP" (cytotoxicity negative-adsorption positive) and complement-fixing cytotoxic Abs (Fig. 2).

The cytotoxicity parameter (dead-cell %) of the cytotoxic FCXM was not significantly different from that measured using the AHG-CDC in a total of 107 cases of crossmatches (Table 2, Fig. 3), although direct comparison is thought to be inaccurate because an exact cell counting in the wells is difficult in the AHG-CDC. As expected, the dead-cell % by the cytotoxic FCXM showed a positive correlation with the AHG-CDC titer, i.e. the highest dilution level producing a positive reaction (Fig. 4). This suggests that the dead-cell % by the cytotoxic FCXM reflects the titer of the AHG-CDC approximately.

However, the addition of complement increased the MFI for the anti-IgG-FITC of the negative controls. Therefore, this decreased the separation between the negative control and the positive samples (i.e., decreased signal to noise ratio). As a result, the Ab-binding parameter (MFI ratio) by the cytotoxic FCXM (22.6 ± 26.9) was significantly lower than that by the conventional FCXM (54.1 ± 82.9) in the HLA allosera (Table 2, Fig. 5).

Table 3 shows the qualitative interpretations of the three HLA crossmatch techniques (cytotoxic FCXM, conventional FCXM, and AHG-CDC) in 107 crossmatches (HLA allosera 77 and negative control sera 30). The cytotoxicity moiety of the cytotoxic FCXM appeared to be more sensitive than that of the AHG-CDC (61 and 55%, respectively). But, the Ab-binding moiety of the cytotoxic FCXM was less sensitive than that of the conventional FCXM (91 and 96%, respectively). As a whole, the sensitivity of both cytotoxic FCXM parameters was not significantly different from that of its conventional counterpart ($P = 0.33$ and $P = 0.22$, respectively). There were no instances of false-positive results except for one case in the Ab-binding moiety of the cytotoxic FCXM. The MFI ratio of that case was 2.6, which is a relatively low value. As expected, the best-correlated technique with the presence of the HLA Ab was the conventional FCXM ($r = 0.96$). There were no instances where the two parameters of the cytotoxic FCXM were negative when the AHG-CDC was positive.

In the cytotoxic FCXM, dead-cell % appeared to be amplified immediately after reaching a certain level of MFI ratio (Fig. 6). In ROC curve analysis, the corresponding MFI ratio to cytotoxicity cutoff was 6.4, showing the maximum sensitivity and specificity for the positive cytotoxicity.

DISCUSSION

The correlation coefficient (r) of the dead-cell % was higher (0.55) in the cytotoxic FCXM rather than that in the AHG-CDC (0.50). Indeed, the method of the cytotoxic

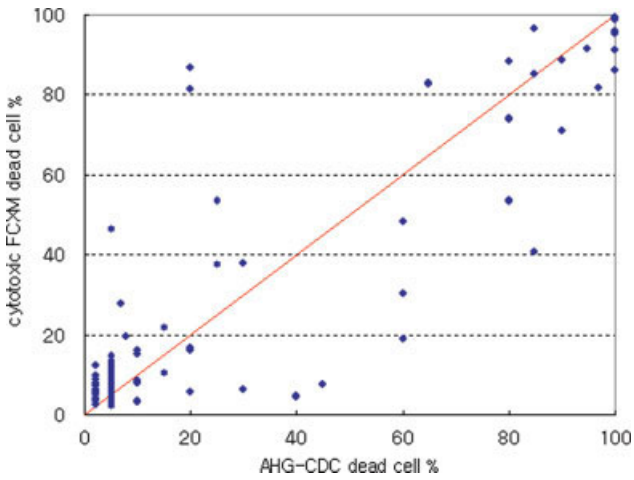

FIG. 3. Dead-cell % measured by the AHG-CDC vs. by the cytotoxic FCXM. The diagonal reference line ($y = x$) is plotted as a solid line. There was a good correlation except for a few cases ($r = 0.947$, $P < 0.0001$). The dead-cell % by the AHG-CDC was determined at the well of titer 1:1. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

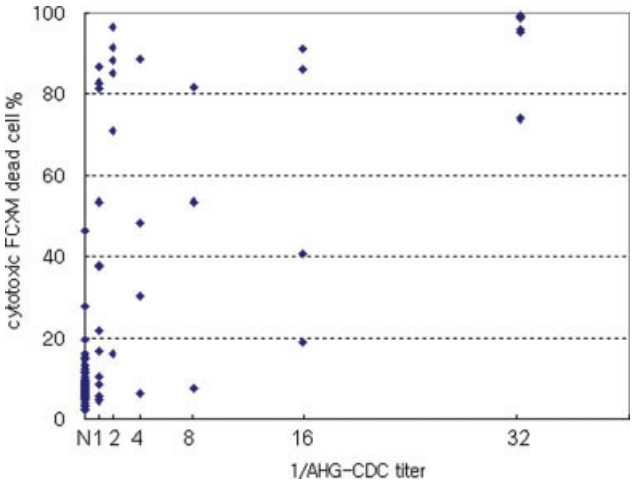

FIG. 4. Titer of the AHG-CDC vs. dead-cell % by the cytotoxic FCXM. The titer of the AHG-CDC is the highest dilution level producing a positive reaction. There was a positive correlation between the two ($r = 0.745$, $P < 0.0001$). N, negative. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

FCXM in this study was largely based on the standard protocol of the NIH-CDC. Although NIH-CDC is known to be less sensitive than that of the AHG-CDC, the sensitivity of the cytotoxicity moiety of the cytotoxic FCXM of this study was higher than that of the AHG-CDC (61 and 55%, respectively). The higher number of cells analyzed (~5,000 T cells) in the cytotoxic FCXM than that in the AHG-CDC (~3,000 lymphocytes) appears to improve the accuracy of the qualitative interpretation. In addition, doubling both the serum/cell ratio and the complement incubation time, as proposed by Zachary et al. (10), also appears to promote complement-mediated cytotoxicity. Regarding the dead-cell %, a few discordant results were observed in the HLA allosera at a low or borderline level. At this level, in the cytotoxic FCXM, a percentage more than twice the mean of four negative controls could be objectively interpreted as a positive reaction. However, in the AHG-CDC, a percentage that did not exceed at least 10% more than the negative control was interpreted as being negative. In this study of 77 cases with HLA allosera, 11 cases were positive by the cytotoxicity moiety of the cytotoxic FCXM and negative by the AHG-CDC. Of these 11 cases, 7 cases showed cytotoxic FCXM dead-cell % less than 15%. This percentage is usually interpreted as being negative in the AHG-CDC. This observation is concordant with that of Stefoni et al. (12), who described that FC allowed a better detection of weak positive reactions (false-negative crossmatches) than did the NIH-CDC.

In the HLA allosera, the MFI ratio by the cytotoxic FCXM significantly decreased (22.6 ± 26.9) when compared with that of the conventional FCXM (54.1 ± 82.9). This was because of the increased background fluorescence of the T cells when complement was added, probably because of the nonspecific binding of immunoglobulin. This decreased the separation between the negative controls and the test samples (i.e., signal-to-noise separation or MFI ratio), thereby decreasing the test sensitivity.

In the cytotoxic FCXM, Ab binding and cytotoxicity could be assessed simultaneously in an anti-IgG-FITC/7-AAD plot. This made it possible to visually discriminate between the so called "CYNAP" and complement-fixing cytotoxic Abs. Therefore, the cytotoxic FCXM was useful to obtain more information on the whole characteristics of the recipient's HLA alloantibodies than that of the separately performed conventional assays. In addition, regarding the qualitative determination, the sensitivity of both parameters in the cytotoxic FCXM was similar to each conventional counterpart.

In a conventional HLA crossmatch, the CDC-negative and FCXM-positive cases can be interpreted as follows: (1) noncomplement-fixing HLA Abs; (2) sublytic concentration of HLA Abs; (3) excess irrelevant IgG binding to Fc receptor of lymphocytes because sera contain IgG in an aggregated or complexed form (8,9). In this study with the HLA allosera being confirmed to have complement-fixing Ab, the cytotoxicity parameter became negative sooner but the Ab-binding parameter remained positive at the higher dilutions (Fig. 6). A representative example is illustrated in Figure 7, depicting the different effects of the serial dilutions on Ab binding and cytotoxicity. On the assumption that the tested sera had only complement-fixing HLA alloantibodies, these findings further confirm that even the complement-fixing Ab cannot exert cytotoxicity until it is above a certain level. Fuller et al. (13) also had found that CYNAP reactions are not the result of low affinity alloantibodies or generally caused by noncomplement-fixing HLA alloantibodies. The ROC curve analysis based on the qualitative status of the cytotoxicity parameter revealed that the corresponding MFI ratio to cytotoxicity cutoff was ~6.4. Therefore, the HLA Ab level of the MFI ratio from 2.0 (cutoff for Ab binding, which was previously defined with negative sera) to 6.4 could be considered to be a "positive but sublytic" level. The correspond-

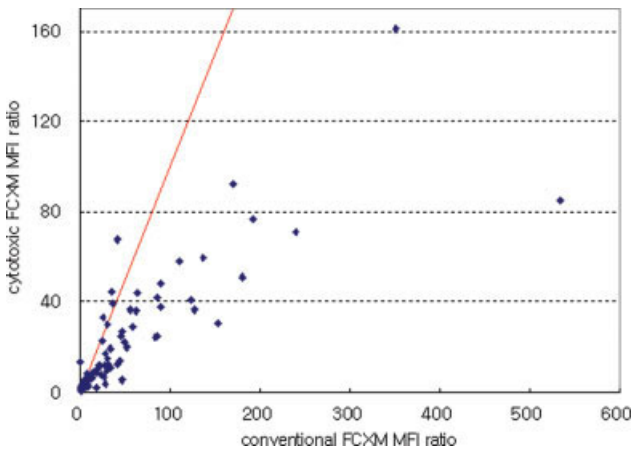

FIG. 5. MFI ratio by the conventional vs. by the cytotoxic FCXM. There was a positive correlation ($r = 0.872$, $P < 0.0001$). However, MFI ratio by the cytotoxic FCXM (22.6 ± 26.9) was significantly lower than that of the conventional counterpart (54.1 ± 82.9) in the HLA allosera. The diagonal reference line ($y = x$) is plotted as a solid line. MFI, mean fluorescence intensity. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Table 3
*Sensitivity and Specificity Analysis of the Two Parameters of the Cytotoxic FCXM When Compared with That of Its Conventional Counterpart**

	Number of crossmatches							
	True positive	False negative	False positive	True negative	Total	Sensitivity/ specificity (%)	*r*-value	γ^2
Cytotoxicity (dead-cell %)								
Cytotoxic FCXM	47	30	0	30	107	61/100	0.55	33[a]
AHG-CDC	42	35	0	30	107	55/100	0.50	27[a]
Ab binding (MFI ratio)								
Cytotoxic FCXM	70	7	1	29	107	91/97	0.83	74[a]
Conventional FCXM	74	3	0	30	107	96/100	0.93	93[a]

*Number of negative sera = 30; number of HLA allosera = 77.
[a]$P < 0.0001$.

ing MFI ratio to cytotoxicity cutoff in the conventional FCXM is expected to be higher because the MFI ratio obtained by the cytotoxic FCXM was lower than that of its conventional FCXM.

In the FCXM for renal transplantation, the HLA class II Abs are usually detected using B-cell markers. In this study, only the T-cell crossmatches were of interest. The effect of Abs to donor HLA class II antigens has been controversial (14). It is hypothesized that Ab binding and cytotoxicity to B cells can be assessed, if a B-cell marker is substituted for a T-cell marker and a larger number of donor lymphocytes is processed.

The method of the cytotoxic FCXM in this study was based on the modified NIH-CDC, a sort of NIH-long test. In a few cases, cells died in a smaller percentage by the cytotoxic FCXM than that by the AHG-CDC, although the increased MFI ratio did not exclude the presence of the HLA Ab in these cases fortunately. Furthermore, the choice to perform first the complete NIH-long and subsequently the next surface staining had a negative impact on the Ab-binding parameter because of the increased background signals.

To increase the test sensitivity of the cytotoxicity parameter, it can be suggested that a CDC-augmenting AHG reagent be used before adding complement as in the AHG-CDC, provided an assay is devised to minimize the interaction between a CDC-augmenting AHG and anti-IgG-FITC. Otherwise, instead of cell death, the complement product deposition can be detected with monoclonal Abs against complement, as proposed by Wahrmann et al. (11).

To increase the test sensitivity of the Ab-binding parameter, a modified assay step sequence can be suggested, in which anti-IgG-FITC staining is done before adding complement. We tried this sequence. Disappointedly, this sequence apparently decreased the dead-cell % when compared with that of the AHG-CDC, although the anti-IgG-FITC staining was enhanced. Perhaps anti-IgG-FITC used in this assay did not play another role as a potential CDC-augmenting AHG but inhibited complement binding profoundly. In turn, the inhibition of complement binding by anti-IgG-FITC appeared to be partially alleviated by the addition of a CDC-augmenting AHG (data not shown). These findings are due to the characteristics of anti-IgG-FITC used in this study, which was a $F(ab)_2$ fragment and Fcγ specific. As described by Fuller et al. (13), the AHG reagent specific against the light chain and expressing an intact Fc domain only augments CDC and overrides CYNAP, and the anti-IgG reagent blocks the C1q receptor site located within the Fc domain. To increase the test sensitivity of both parameters, a wide range of variables will be evaluated in a future study.

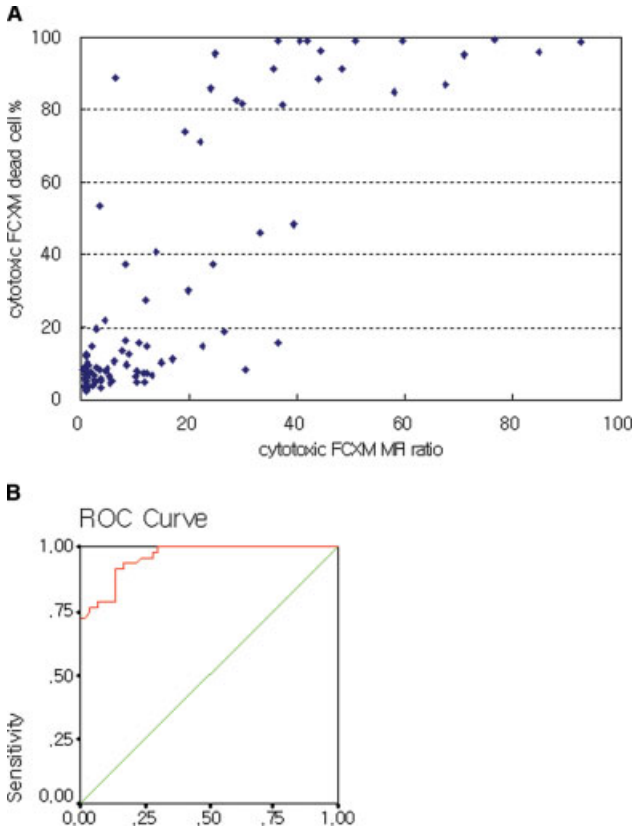

FIG. 6. The correlation between the two parameters simultaneously obtained in a single assay, the cytotoxic FCXM. **A**: the plot of MFI ratio vs. dead-cell %. **B**: ROC curve analysis of MFI ratio depending on the state of cytotoxicity. The corresponding MFI ratio to the cytotoxicity cutoff was 6.4, showing a maximal sensitivity of 0.915 and a specificity of 0.867. The area under the curve was 0.960. The diagonal segments are produced by ties. MFI, mean fluorescence intensity. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

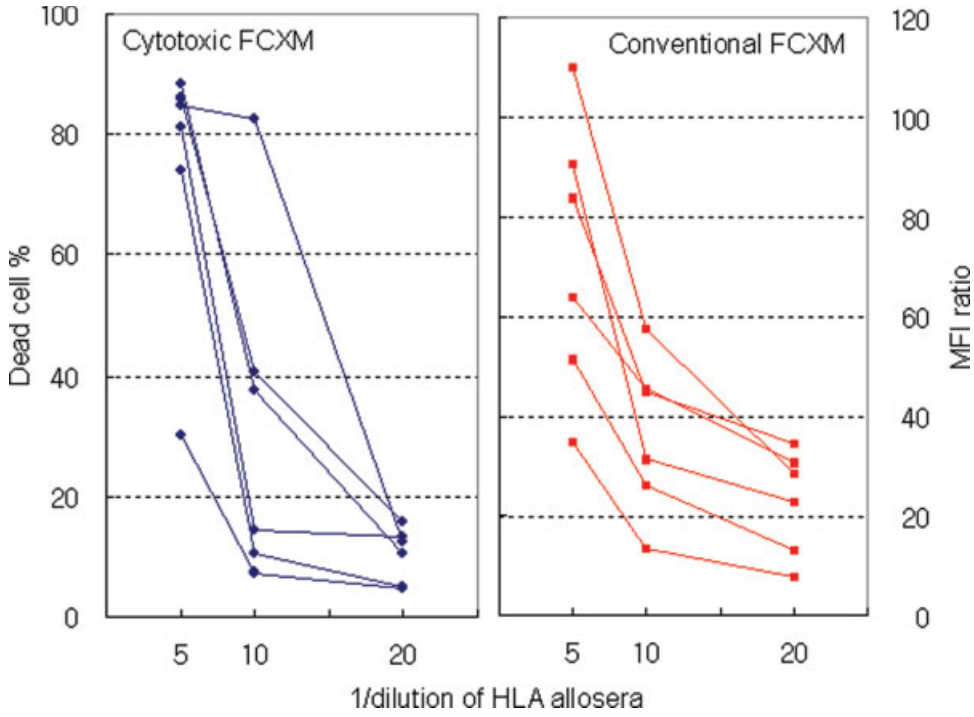

FIG. 7. The different effects of serial dilutions of an HLA alloserum on Ab binding and cytotoxicity. A polyspecific HLA alloserum (serum ID SY) was tested against six donors. At a dilution 1:20, regarding the cytotoxicity parameter of cytotoxic FCXM, two donors became negative and the rest also were near the universal cutoff 12.2%, but regarding the MFI ratio of conventional FCXM, all donors remained positive and still far from the cutoff 2.0. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Attempts have been made to measure the extent of cell death by the complement-fixing cytotoxic Ab in HLA crossmatches using FC (12,15–17). Although Schonemann et al. (17) used anti-IgG-FITC/propidium iodide plots, those were used to demonstrate the effect of the complement-fixing IgG Abs on light scattering. This study is believed to be the first trial in measuring the level of Ab binding and cytotoxicity simultaneously.

This method has a few defects in that it takes more time to perform and in a few cases, two parameters of the cytotoxic FCXM cannot detect the HLA Abs reaction as sensitively as that of the separately performed conventional counterpart. In a few cases, the cytotoxicity was positive only in the AHG-CDC. However, there were more positive cases only in the cytotoxic FCXM. The cytotoxicity moiety of the cytotoxic FCXM was more sensitive rather than that of the AHG-CDC. Moreover, the Ab-binding parameter could sufficiently detect the HLA Abs at the level of the positive AHG-CDC.

Compared with the conventional methods, the advantages we documented are: (1) HLA alloantibodies, complement-fixing, and nonfixing can be detected simultaneously, and the mechanisms of cell death can be identified, which is Ab-dependent or not; (2) the determination of positivity is objective; (3) a high number of lymphocytes is assessed; (4) detection of weak positive reaction is enhanced; (5) 7-AAD has minimal spectral overlap with FITC and PE fluorescence emissions than that of propidium iodide or ethidium bromide used in other studies (12,15–17) to detect the dead cells. This allows other fluorescence to be measured more accurately in three-color FC.

Despite many advantages, however, further studies are needed to obtain more reliable and clinically applicable tests, and we hope this report may contribute to an ongoing discussion on a still highly controversial FCXM.

In conclusion, the simultaneous detection of Ab binding and cytotoxicity was possible by the cytotoxic FCXM with the test efficiency similar to the conventional counterparts. If this new assay is improved through the further studies to optimize the critical assay variables, this may be used as an alternative to the conventional assays to acquire more information on the characteristics of the recipient's HLA alloantibodies.

LITERATURE CITED

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