

Flow Cytometric Crossmatch for Kidney Transplantation

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Serum samples from 49 patients with panel reactive antibodies of greater than 15% and 17 patients who have related donor pairs were collected at the Department of Transfusion Medicine, Faculty of Medicine Siriraj Hospital. Crossmatching was performed by three methods, flow cytometry crossmatch (FCXM), the standard National Institutes of Health (NIH), and the antihuman globulin (AHG) microlymphocytotoxicity. 28.9% Spell out of both T- and B-cell crossmatch was positive by FCXM and negative by NIH and AHG. When the T-cell and B-cell crossmatches were negative by FCXM, they were negative by both NIH- and AHG method. There was significant difference of the crossmatch result between FCXM and NIH and between FCXM and AHG ($p < 0.0001$). In addition, FCXM was about 4-16 and 8-32 times more sensitive than AHG- and NIH method, respectively. In conclusion, the result of FCXM is clear and this method is more sensitive than NIH- and AHG method. FCXM should be used together with the NIH- and AHG method for kidney transplantation.

Keywords: Flow cytometry, Crossmatch, Kidney transplantation

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Kidney transplantation is a well established, successful treatment for end-stage renal disease. Patients on the waiting list can be maintained on dialysis, but many deaths occur while waiting. The risk of mortality was decreased when they received a kidney transplant⁽¹⁾. Pretransplant crossmatching has become a routine in recipient selection⁽²⁾. High risk group of rejection (a second transplant and a panel reactive antibodies of greater than 10%) has a higher probability of rejecting a retransplant than those who do not have panel reactivity, even though patients from both groups transplanted with negative crossmatches against the donor^(3,4). The essential of more sensitive crossmatch methods to detect low levels of preformed anti-donor antibodies that might be missed by the standard National Institutes of Health (NIH) crossmatch tests remains at issue in renal transplantation. The low levels of antibodies which may affect graft outcome stem from observations that sensitized patients experience a higher incidence of delayed graft function and primary nonfunction than non-sensitized patients^(5,6). Promotion of long-term organ

graft survival is the goal of every transplantation team, and so the development of new sensitive techniques for the detection of donor-specific presensitization in potential recipients is paramount⁽⁷⁾. In 1983, Garovoy et al introduced the flow cytometry crossmatch (FCXM) assay which was a highly sensitive crossmatch technique. The FCXM assay can be performed rapidly on a large number of cells (5,000-10,000 cells) within a fraction of a minute and provides objective evaluation of patient serum antibodies to HLA-specificities of donor target cells. It can detect weak positive reactions (false-negative crossmatches) better than the use of a light microscope, and can detect dead cell better than a technologist using a microscope to visualize the incorporation of a vital dye (Eosin-Y)⁽⁷⁻¹²⁾. However, the FCXM assay has not been widely used as the standard NIH- and AHG method.

The purpose of this study was to compare crossmatching between FCXM and the standard NIH and AHG method.

Material and Method

Sample

Sera from 49 patients with panel reactive antibodies of greater than 15%, who were awaiting for

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renal transplantation at the Department of Transfusion Medicine, Faculty of Medicine Siriraj Hospital were used for antibody screening and crossmatching. Several lots of sera from some patients were used for crossmatching in the present study. Sera from 17 patients who had living-related donors were also studied. In addition, known anti-HLA-A2, A9, A11.1, B7, B40 at dilution of 1:1 to 1:128 were used to study the sensitivity for the NIH, AHG and FCXM crossmatch. The negative control was serum from a normal healthy nontransfused male donor of blood group AB. There was no antibodies reaction with panel lymphocytes in the negative control serum. The positive control consisted of pooled sera from patients, each had antibodies reaction with > 90% of panel lymphocytes.

Lymphocytes from 46 blood donors and 17 living-related donors at the Department of Transfusion Medicine, Faculty of Medicine/Siriraj Hospital were isolated to perform crossmatch with the above sera. Lymphocytes from donors with known HLA specificity HLA-A2, HLA-A9, HLA-A11.1, HLA-B7, HLA-B40 were also studied with the corresponding antisera.

Method

T- and B-cells preparation

Fifty ml of ACD blood samples (donors and patients) were collected at the Department of Transfusion Medicine, Faculty of Medicine/Siriraj Hospital.

Blood sample was centrifuged and buffy coat was obtained. Lymphocytes were separated from buffy coat by using Isoprep (Nycomed, Norway). Separation of B-cells and T-cells was performed utilizing the nylon wool column technique. The dead cells were eliminated by separation with 40% Percoll (Pharmacia, Sweden).

Dithiothreitol treatment of sera for cross-match (DTT treatment)

Treatment of sera with DTT was performed by mixing one part stock (50 mMol) DTT with 9 parts of serum to give the final concentration of DTT at 5 mMol.

Standard NIH lymphocytes microcyto-toxicity test

The HLA microcytotoxicity test is the fundamental test used for defining both the HLA antigens and antibodies. In this test a suspension of lymphocyte (enriched T-cells for HLA class I and B-cells for HLA class II) was incubated with sera in a Terasaki

tray. Rabbit serum was added as a source of complement and all dead cells were determined by the uptake of a vital dye (Eosin-Y). Crossmatch was positive when the test result showed at least 20% dead cells above the background.

AHG complement dependent cytotoxicity test

Preparation of sera for AHG crossmatch technique was performed in the same way as NIH technique, except that the medium 199 buffer was added to wash the cells after being incubated with patient sera. Serum was removed by flicking the tray with a quick motion. The wash step was repeated twice. The optimized antihuman globulin reagent (antihuman kappa light chain goat serum) in a dilution 1:100 was added. Thereafter, rabbit complement and Eosin-Y were added respectively. Crossmatch was positive when the test result showed at least 20% dead cells above the background.

FCXM T-cells

Twenty microliters of serum was incubated with T-cells suspension for 30 min at room temperature. Excess serum was washed out 3 times with Phosphate Buffered Saline (PBS). The second antibodies, Phycoerythrin labeled anti-CD3 monoclonal antibody (Becton Dickinson, San Jose, CA) and goat F(ab')₂ antihuman IgG-FITC antibody (Organon Teknica, West Chester, PA) were reacted with cells for 20 min at room temperature. The cells were washed two times with PBS containing 0.1% sodium azide (PBS-Az) and then fixed in 0.5% paraformaldehyde. The sample was kept at 4 °C in the dark until analyzed in a flow cytometer (1024 channel, linear scale) (Becton Dickinson, San Jose, CA).

FCXM B-cells

After coating of B-cells surface with goat F(ab')₂ antihuman IgG antibody (Organon Teknica, West Chester, PA), to improve sensitivity and specificity of anti-B cell antibodies, these cells were resuspended in PBS-Az and incubated with serum for 30 min at 37 °C. Thereafter, the cells were washed with PBS-Az. The second antibodies, Phycoerythrin labeled anti-CD19 monoclonal antibody (Becton Dickinson, San Jose, CA) and goat F(ab')₂ antihuman IgG-FITC antibody were reacted with the cells for 20 min at room temperature. The cells were washed with PBS-Az again, and fixed in 0.5% paraformaldehyde. The sample was kept at 4 °C in the dark until analyzed in a flow cytometer.

Interpretation of flow cytometry

Positive flow cytometry crossmatch results were defined as mean channel shift to the right of either the T-peak or B-peak values in test sera above 2SD of normal controls when compared with control sera.

Statistical analysis

Mc Nemar Chi square test was used for statistical comparisons. P values of 0.05 or less were considered significant.

Results

Positive and negative results of T- and B-cell by FCXM are shown in Fig. 1 and 2. The mean channel shift of the negative T-cell was 270.96, while the mean channel shift of the negative B-cell was 517.96. This showed the higher background of B-cell than T-cell.

The results of 83 crossmatches for T-cell are shown in Table 1. 16 Spell out of 83 (19.3%) crossmatches were positive by all the three crossmatch methods and 43 out of 83 (51.8%) were negative by all

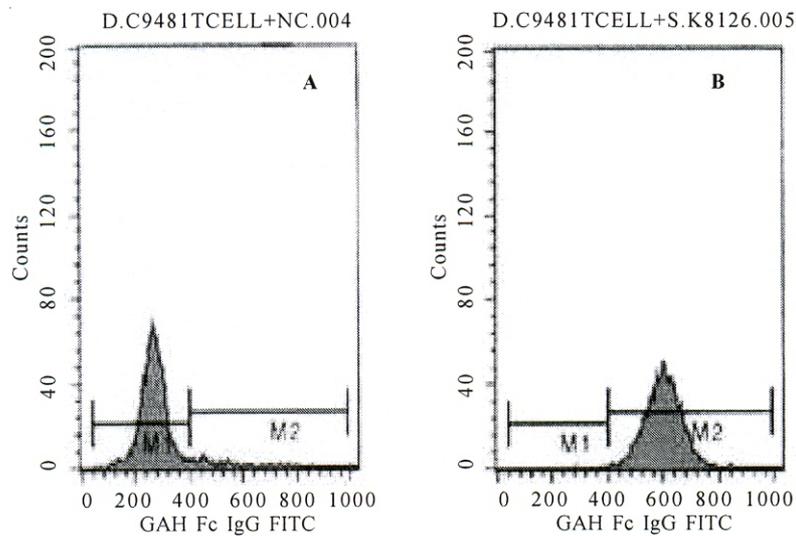


Fig. 1 T-cell of flow cytometric crossmatch; Negative (A) and Positive (B)

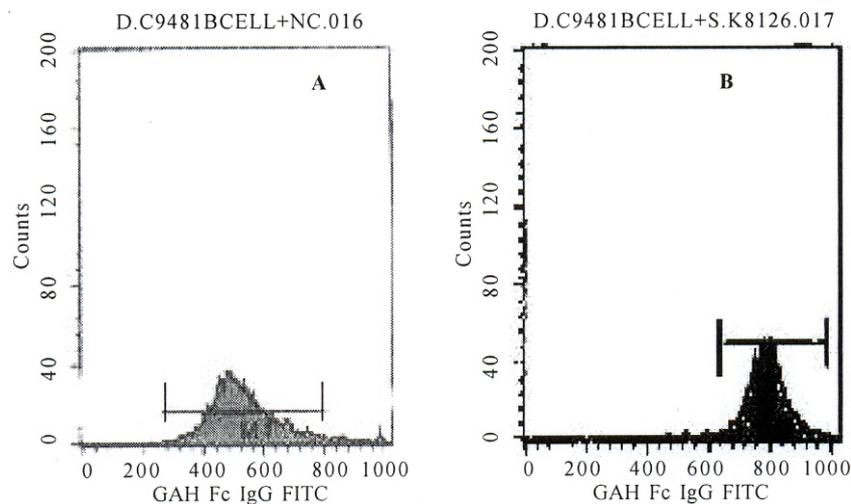


Fig. 2 B-cell of flow cytometric crossmatch; Negative (A) and Positive (B)

the three different methods. However, 24 out of 83 (28.9%) crossmatches were positive by only FCXM. One case showed IgM antibody that was detected by AHG the method.

The results of 76 crossmatches for B-cell are shown in Table 2. 26 Spell out of 76 (34.2%) crossmatches were positive by all the three crossmatch methods and 27 out of 76 (35.5%) were negative by all the three different methods. 1 out of 76 (1.3%) of the crossmatch was positive by AHG and FCXM methods but negative by NIH. However, 22 out of 76 (28.9%) of the crossmatches were positive by the FCXM only. Five cases of the B-cell crossmatch showed IgM antibody, in which two cases were positive by NIH and AHG and three cases were positive by AHG only.

There was a significant difference of the crossmatch results between FCXM and NIH ($p < 0.0001$) and between FCXM and AHG ($p < 0.0001$).

The sensitivities of FCXM, AHG and NIH methods are compared in Table 3. The dilution (1:1-1:128) of anti-HLA-A2, A9, A11.1, B7, B40 were tested against A2, A9, A11.1, B7, B40 cells respectively, and the FCXM gave the highest sensitivity in all panels tested. The AHG is one dilution more sensitive than the NIH.

Discussion

The microcytotoxicity test has been used universally for crossmatching in kidney transplantation

since it was first used for crossmatching in 1964⁽¹³⁾. The standard microcytotoxicity test has largely eliminated hyperacute rejection but the fact that many early acute rejections and failures still occur, especially in presensitized patients suggests that low levels of immunization are not detectable by the standard test. Garovoy et al⁽¹⁴⁾ were the first to introduce FCXM as a more sensitive test. They showed that the test was 30-250 times more sensitive by titration study of HLA antibodies. In Thailand, FCXM for kidney transplantation had been performed by Chiewsilp et al⁽¹⁵⁾. It was shown that FCXM was about 2 times and 4 times more sensitive than AHG and NIH respectively. However, the antibody to donor B-cell was better detected by AHG than FCXM and the sensitivity of FCXM was not so high for B-cell crossmatch. This may be because there are immunoglobulins on the B-cell surface and the fluorochrome conjugated anti-human IgG antibody also recognizes surface immuno-globulins on B-cell. Therefore, the results were difficult to interpret⁽¹⁶⁾. Improvement was instituted in the present study, B-cells were tested by goat F(ab')₂ antihuman IgG antibody and the background was reduced. However, the background of the B-cell cross-match was not reduced to the level of a flow cyto-metric T-cell crossmatch (Fig. 1, 2). In the present study, FCXM could detect the false negative results of T- and B-cell crossmatch occurring in the NIH and AHG methods (Table 1, 2). The

Table 1. Comparison of the three T-cell crossmatch methods

Patterns of T-cell reaction			N (%)
NIH	AHG	FCXM	
+	+	+	16=19.3%
-	-	+	24=28.9%
-	-	-	43=51.8%
Total			83

Table 2. Comparison of the three B-cell crossmatch methods

Patterns of B-cell reaction			N(%)
NIH	AHG	FCXM	
+	+	+	26=34.2%
-	+	+	1=1.3%
-	-	+	22=28.9%
-	-	-	27=35.5%
Total			76

Table 3. Comparative sensitivity of FCXM analysis and standard cytotoxicity crossmatch techniques

HLA antiserum	FCXM crossmatch	Cytotoxic crossmatch	
		NIH	AHG
A2 (TP4415-37)	1:1 to 1:32	1:1 to 1:4	1:1 to 1:8
A9 (TP3702-33)	1:1 to 1:128	1:1 to 1:16	1:1 to 1:32
A11.1 (TP2456-35)	1:1 to 1:8	1:1 to 1:1	1:1 to 1:2
B7 (TP6194-37)	1:1 to 1:128	1:1 to 1:4	1:1 to 1:8
B40 (TP12345-33)	1:1 to 1:32	1:1 to 1:2	1:1 to 1:4

discrepancy between FCXM and standard NIH and AHG was highly significant in both T- and B-cell ($p < 0.0001$). When the higher serial dilutions of antisera were used; the more sensitivity of FCXM assay was found in all HLA antisera tested (Table 3). It was found that FCXM was about 4-16 and 8-32 times more sensitive than AHG and NIH, respectively (Table 3).

Several authors reported a poor outcome of kidney transplantation in patients with a positive FCXM^(2,17-19). Kotb et al⁽⁹⁾ reported that the incidence of first year rejection was significantly greater in patients who had a positive B-cell FCXM than in patients with a negative B-cell FCXM. FCXM is increasingly being used as a crossmatch procedure in addition to the standard microcytotoxicity (NIH) or AHG. Using FCXM, it can easily distinguish between IgG and IgM antibodies or between anti-T and B-cell. In addition, it is less affected by poor cell viability and takes less time to perform. However, the disadvantage of FCXM is that more cells, serum, and expensive equipment are needed. In addition, the technique varies between the Labs. In the present study B-cell cross-match procedure is based on the coating of B-cell surface immunoglobulins with an unconjugated polyvalent antihuman immunoglobulin antibody in order to improve sensitivity and specificity of anti-B-cell antibodies⁽¹⁶⁾. Since most patients receive cadaver donor kidneys, and rarely find antigens matched for A, B and DR, the poorly matched transplants were always selected for transplantation. The sensitive crossmatching should be performed to minimize early graft rejection and to maximize the graft survival. Therefore, a procedure that is able to predict acute graft rejection should also improve long term graft survival.

In conclusion, FCXM is significantly more sensitive than the NIH and AHG methods. It should be used in combination with cytotoxic assay, to aid in better recipient-donor pair selection.

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การตรวจความเข้ากันได้เพื่อการเปลี่ยนไตโดยวิธี โฟลไซโตเมทรี

กฤษฎดา โคกตาทอง, ศศิจิต เวชแพศย์, โกวิท พัฒนาปัญญาสัจย์, ศศิธร เพชรจันทร์

ได้ทำการศึกษาวิธีที่ใช้ทดสอบการเข้ากันได้กับผู้ป่วยเปลี่ยนไตโดยใช้น้ำเหลืองของคนไข้ที่มีปฏิกิริยากับ Panel cell มากกว่า 15% จำนวน 49 ราย และใช้น้ำเหลืองของคนไข้ที่ได้รับบริจาคไตจากญาติจำนวน 17 ราย มาทำการทดสอบความเข้ากันได้โดยเปรียบเทียบวิธีตรวจวัดเซลล์ด้วยโฟลไซโตมิเตอร์ (FCXM), วิธีตรวจมาตรฐาน National Institutes of Health (NIH) และวิธี Antihuman globulin microlymphocytotoxicity test (AHG) พบว่า 28.9% ของการตรวจความเข้ากันได้ทั้ง T และ B cell ให้ผลบวกเฉพาะวิธี FCXM โดยให้ผลลบกับวิธี NIH และ AHG และเมื่อตรวจความเข้ากันได้ให้ผลลบโดยวิธี FCXM ก็จะได้ผลลบโดยวิธี NIH และ AHG ผลการตรวจเปรียบเทียบวิธี FCXM กับวิธี NIH และ AHG มีความแตกต่างอย่างมีนัยสำคัญ ($p < 0.0001$) ในการเปรียบเทียบความไวโดยใช้ซีรัมที่มีความเข้มข้นต่างกัน พบว่า FCXM มีความไวกว่าวิธี AHG ประมาณ 4-16 เท่าและไวกว่าวิธี NIH 8-32 เท่า สรุปการศึกษาพบว่าวิธี FCXM อ่านผลได้ชัดเจนและมีความไวสูงกว่าวิธี NIH และ AHG ควรนำไปใช้ร่วมกับวิธี NIH และ AHG ในการตรวจความเข้ากันได้ก่อนการเปลี่ยนไต