Method of Lymphocytotoxic Crossmatch Test for Feline Renal Transplantation

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ABSTRACT. The optimal condition for methods of lymphocytotoxic crossmatch test for feline renal transplantation was investigated. On separation of viable lymphocytes from whole blood, the best results were obtained when Ficoll-diatrizoate with 1.078 of a specific gravity at 20°C was centrifuged with $800 \times g$ for 30 min at 4°C. A nylon wool column was used to separate T and B cells from lymphocyte fraction. The ratio of T cells in nylon wool effluent cells was 95%, while the ratio of B cells in adherent cells was 41%. Lymphocytotoxic crossmatch tests were performed by using the effluent cells as T cells and the adherent cells as B cells, at 37°C (warm) and 4°C (cold). The ratio of B cells in adherent cells was low, however, the result was utilized as a matching test before transplantation by combining with the T cell result. The trypan blue stain method made it easier than the eosin stain method to distinguish living and dead cells. The lymphocytotoxic crossmatch tests were performed on 15 pairs of healthy cats, and only one pair showed doubtful positive against anti-B cell cold antibodies. During acute rejection after renal transplantation in two pairs which were negative on any anti-lymphocyte antibodies before the transplantation, the anti-T cell warm antibodies became positive in both pairs, and the anti-T cell cold antibodies became positive on one of the two pairs.—**KEY WORDS:** anti-lymphocyte antibody, feline, lymphocytotoxic crossmatch test, renal transplantation, trypan blue stain.

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Renal transplantation is applied as a treatment for end-stage renal failure in human. Although effective immunosuppressive drugs such as Cyclosporine (Cys) and FK 506 have been developed recently, allograft rejection in recipients remains the most important problem in this field. Types of rejection include hyperacute rejection, accelerated acute rejection, acute rejection and chronic rejection in human renal allograft [17]. It is considered that humoral immunity such as the antibodies against erythrocytes and leukocytes is involved in the hyperacute and accelerated acute rejections, and that the major histocompatibility complex (MHC) is involved in the acute and chronic rejections [17]. Therefore, several matching tests are routinely performed before human renal transplantation: determination of blood group to confirm the antibodies against erythrocytes, lymphocytotoxic crossmatch test to detect antibodies against lymphocytes, and serological test or mixed lymphocyte reaction (MLR) test to match the type of MHC [13]. It is considered that antibodies against lymphocyte are induced by blood transplantation, pregnancy, allograft and unknown causes [20]. The type of antibodies against lymphocyte is related with the severity of rejection. A warm antibody against T cell triggers the most severe rejection, and a cold antibody against T cell and a warm antibody against B cell cause moderate reaction, while a cold antibody against B cell results in no reaction [13, 20]. Therefore, the lymphocytotoxic crossmatch tests to detect anti-donor lymphocyte antibodies are performed in both warm (37°C) and cold (4°C) condition with each T and B lymphocyte of the donor in human [20].

Many cases of irreversible chronic renal failure by kidney fibrosis are seen in feline clinical practice, and renal transplantation has recently been applied in these cases [1,

2, 9, 11]. Only limited matching tests for an allograft have been conducted in feline renal transplantation in these studies. It was always confirmed that no antibody against erythrocytes was detected between recipient and donor, but not confirmed for antibodies against lymphocytes. If the recipient has antibodies against lymphocytes of the donor, a hyperacute rejection from which recovery is impossible may be also induced in the recipient in cats [13]. Therefore, antibodies against lymphocytes should be also confirmed before feline renal transplantation. Anti-lymphocyte antibodies have not been investigated well in cats to date. However, it was reported that the antibodies against lymphocyte were detected in one male cat with no history of blood transplantation, one of twenty pregnant cats [15], and 22% of 59 cats with multiple skin transplantation [3, 21]. In these studies, a lymphocytotoxic crossmatch test for human was applied to cats, and suitable method for the cat has been little studied till now.

The purpose of this study is to establish an optimal method for the feline lymphocytotoxic crossmatch test as a matching test for renal transplantation.

MATERIALS AND METHODS

Lymphocyte collection: Ficoll-diatrizoate density gradient centrifugation was used to collect lymphocytes [19]. The blood drawn from the donor cat was put into tubes with acid citrate dextrose (CPD solution; VT-100SC, Terumo, Tokyo). The blood was diluted by addition of an equal volume of PBS. The diluted blood was layered on Ficoll-diatrizoate (Lymphoprep TM, Nycomed, Norway) with a specific gravity of 1.078 at 20°C and was centrifuged at $800 \times g$ for 30 min. The suitable temperature of Ficoll-

diatrizoate and centrifugation to collect a greater number of viable lymphocytes was examined between 4°C and 20°C. The mononuclear cell layer was washed 3 times by PBS with 0.1% bovine serum albumin, then suspended in 1 ml of culture medium. Platelets were removed by adding 100 units/ml of thrombin (Bovine thrombin, Sigma, St. Louis) at the first washing of cells [7]. The culture medium was RPMI 1640 supplemented with 5 mM L-glutamine, 25 mM HEPES buffer, 10^{-5} M 2-ME, 1% antibiotic (Penicillin and Streptomycin) and 10% heat-inactivated fetal calf serum (FCS). The components of separated cells were examined by morphological classification using May-Giemsa stain, and the viability of cells was examined by trypan blue stain [10].

Separation and differentiation of T and B cells: Separated mononuclear cells were fractionated into nylon wool effluent cells and nylon wool adherent cells with nylon wool column (Nylon wool column for mouse T cell separation, Wako, Osaka) [6, 18]. Effluent cells were washed and resuspended into culture medium, and used as T cell suspension. Adherent cells were collected by crumpling and washing the nylon wool in cold culture medium, and the cells were washed and resuspended into culture medium and used as B cell suspension. The components of effluent cells and adherent cells were examined by May-Giemsa stain. B cells were determined by using flow cytometry (FACS CALIBUR, Becton-Dickinson, NJ) with FITC-conjugated anti-mouse B cell antibodies (RA3-6B2, Cedarlane, Ontario). It was reported that this anti-B cell antibody was sensitive to feline B cell, and 99% of lymphocytes not bound to this antibody were T cells which can be recognized with pan-T lymphocyte antibodies [5, 12].

Lymphocytotoxic crossmatch test procedure: One or 3 μ l of recipient serum was put into wells of micro-tissue culture plates, and 1 or 3 μ l of donor T or B cell suspension was added and mixed. Four μ l of liquid petrolatum was added and incubated at 4°C or 37°C for 30 min. Then 5 or 15 μ l of rabbit complement for T or B cell (Rabbit Standard or Rabbit Low-Tox-H, Cedarlane, Ontario) was added, respectively. They were incubated at 25°C for 60 min, and living or dead cells were determined by the eosin stain method [14] or trypan blue stain method [10]. Wells with 1 μ l of serum and cell suspension and 5 μ l of complement solution were used for the eosin stain method, and wells with 3 and 15 μ l were used for the trypan blue stain method. Scoring of cytotoxicity was according to Hara *et al.* [3] (Table 1). It was confirmed that human lymphocytes were reacted with anti-human leukocyte antigen serum (ANTI-HLA-ABC SERA, Dainippon, Tokyo) and anti-human B cell serum (CL8701, Cedarlane, Ontario) [4] as a positive control, and human lymphocyte was not reacted with FCS as a negative control [15]. The results were read with both the eosin stain method and trypan blue stain method in order to know which stain made it easier to distinguish living from dead cells.

The staining procedures were as follows. For the eosin staining, 3 μ l of 5% eosin solution was added to cell

Table 1. Evaluation and cytotoxicity score

Evaluation	Score	Ratio of dead cells
Negative	1	under 10%
Doubtfully negative	2	11–20%
Doubtfully positive	4	21–40%
Positive	6	41–80%
Strongly positive	8	over 80%

Table 2. Results of lymphocytotoxic crossmatch test

Pair No.	T-warm	T-cold	B-warm	B-cold
No. 1 (A vs B) ^{a)}	1 ^{b)}	1	1	1
No. 2 (A vs C)	1	1	2	1
No. 3 (A vs D)	1	1	1	1
No. 4 (B vs A)	2	1	1	1
No. 5 (B vs C)	1	1	2	1
No. 6 (B vs D)	2	1	2	1
No. 7 (C vs A)	1	2	1	2
No. 8 (C vs B)	1	1	1	1
No. 9 (C vs D)	2	1	1	1
No. 10 (D vs A)	1	1	1	1
No. 11 (D vs B)	1	2	2	2
No. 12 (D vs C)	1	1	1	1
No. 13 (F vs E)	1	1	1	4
No. 14 (G vs C)	1	1	1	1
No. 15 (H vs D)	1	1	1	1
No. 16 (G' vs C) ^{c)}	6	2	ND ^{d)}	2
No. 17 (H' vs D) ^{c)}	8	6	ND	ND

a) Lymphocytotoxic crossmatch test in serum of A cat versus lymphocyte of B cat.

b) Score of cytotoxicity test.

c) The test was done during acute rejection after renal transplantation.

d) Not done.

suspension under the petrolatum after reaction with complement, and 8 μ l of 50% buffered formaldehyde was then added. After keeping overnight at room temperature, the wells were covered with microscope cover glass and observed with phase contrast microscope. The ratio of dead cells which stained with eosin was calculated in relation to the total number of cells. For the trypan blue staining, the cell suspension was removed from the well after reaction with complement and mixed with an equal volume of 0.5% trypan blue solution. Then the mixture was put into a Neubauer haemocytometer chamber, and the ratio of dead cells which stained with trypan blue was calculated in relation to the total number of cells in the chamber.

Application of lymphocytotoxic crossmatch test to natural cats and experimental cats with renal transplantation: Eight healthy cats (A~H), domesticated or stray, mongrels of both sexes and 1 to 5 years old in age, were used in this study. They were paired in 15 different combinations (Nos. 1–15, Table 2). Lymphocytotoxic crossmatch tests of the 15 pairs were conducted under the optimal conditions determined in this study. Renal transplantation was performed in two pairs (Nos. 14 and 15) which were confirmed to be negative in lymphocytotoxic crossmatch test. Lymphocytotoxic

Table 3. Number of viable lymphocytes in separated mononuclear cells

Specific gravity	Temperature (°C)	Viable cell rate in SMC ^{a)} (%)	Number of SMC ($\times 10^7/\text{ml}$)	Number of lymphocytes in SMC ($\times 10^7/\text{ml}$)	Number of viable lymphocytes in SMC ($\times 10^7/\text{ml}$)
1.078 (n=3)	20	92.7 ± 3.1^b	$1.73 \pm 0.71^*$	1.54 ± 0.10	$1.43 \pm 0.15^*$
1.080 (n=3)	4	97.7 ± 1.2	$2.27 \pm 0.21^*$	1.61 ± 0.11	$1.58 \pm 0.31^*$
1.082 ^{c)} (n=3)	4	97.7 ± 0.9	2.47 ± 0.12	1.60 ± 0.21	1.56 ± 0.05

a) Separated mononuclear cells.

b) Mean $\pm$ standard deviation.

c) 1.080 at 20°C.

* Significant differences between 20°C and 4°C ($p < 0.05$).

Fig. 1. Ratio of B cells in nylon wool effluent cells. a: Without FITC RA3-6B2. b: With FITC RA3-6B2. *: M1 cells show ratio of cells conjugated with FITC RA3-6B2, namely, ratio of B cells in nylon wool effluent cells is 1.58%.

crossmatch tests between serum of recipient and lymphocytes of donor were performed again during acute allograft rejection following transplantation (Nos. 16 and 17).

RESULTS

Lymphocyte collection: As Table 3 shows, on comparing the temperature of the procedure in lymphocyte collection, numbers of separated mononuclear cells and viable lymphocytes at 4°C were significantly greater than at 20°C. The specific gravity of Ficoll-diatrizoate rose from 1.078 at 20°C to 1.080 g/ml at 4°C. When Ficoll-diatrizoate with a specific gravity of 1.082 at 4°C was used for lymphocyte collection, more viable lymphocytes were not obtained than with a specific gravity of 1.080 at 4°C.

Separation and differentiation of T and B cells: When feline separated 2×10^7 mononuclear cells were passed through the column, the count of nylon wool effluent cells was $4.3 \pm 0.5 \times 10^6$ (n=3), and the mean rate of cell recovery was 21.7%. As for the components of effluent cells,

lymphocytes accounted for $97.5 \pm 0.7\%$, neutrophils $0.3 \pm 0.2\%$, monocytes $1.6 \pm 0.5\%$ and eosinophils $0.5 \pm 0.4\%$, while the ratio of B-cells in effluent cells was 1.58% (Fig. 1). Adherent cells consisted of $93.2 \pm 1.3\%$ lymphocytes, $1.1 \pm 0.9\%$ neutrophils, $5.2 \pm 0.6\%$ monocytes and $0.7 \pm 0.5\%$ eosinophils, while the ratio of B cells in adherent cells was 41.06% (Fig. 2).

Comparison of eosin and trypan blue stain method in lymphocytotoxicity test: The lymphocytotoxicity reading results of human separated mononuclear cells against anti-human leukocyte antigen serum, anti-human B cell serum and FCS with both the eosin stain method and trypan blue stain method showed actual agreement. The death of feline cells was far easier to determine in the trypan blue method than the eosin method (Fig. 3).

Application of lymphocytotoxic crossmatch test to natural cats and experimental cats with renal transplantation: As shown in Table 2, all of 15 pairs were negative or doubtful negative against anti-T cell cold and warm antibodies. However, one pair (No. 13) presented doubtful positive against anti-B cell cold antibodies. Two pairs (Nos. 16 and

Fig. 2. Ratio of B cells in nylon wool adherent cells. a: Without FITC RA3-6B2. b: With FITC RA3-6B2. *: M1 cells show ratio of cells conjugated with FITC RA3-6B2, namely, ratio of B cells in nylon wool adherent cells is 41.06%.

Fig. 3. Discrimination of dead (arrows) and live (arrowheads) cell by eosin stain method (a) and trypan blue stain method (b) in a cat.

17) treated with renal transplantation presented positive and strong positive against anti-T cell warm antibodies during acute rejection. Moreover, one pair (No. 17) also presented positive against anti-T cell cold antibodies at the same time.

DISCUSSION

The optimal method was studied for the feline lymphocytotoxic crossmatch test to detect anti-lymphocyte antibodies as a transplantation matching test. Over 95% cell survival rate in separated lymphocytes is needed for lymphocytotoxic crossmatch test [14]. Ficoll-diatrizoate density gradient centrifugation is carried out at 20°C in

human, but cell viability was often below 95% at 20°C in cats. Therefore, centrifugation at 4°C was tried to maintain sufficient cell viability. When Ficoll-diatrizoate with a specific gravity of 1.078 g/ml at 20°C was cooled to 4°C, its specific gravity became 1.080, and both separated mononuclear cells and viable lymphocytes increased in number. However, when Ficoll-diatrizoate with a specific gravity of 1.082 at 4°C was used, the number of viable lymphocytes did not increase. Therefore, the optimal condition to collect viable lymphocytes in cats seemed to be obtained by adjusting Ficoll-diatrizoate to 1.078 at 20°C and using at 4°C.

When the nylon wool column for mouse T cell separation

was used to separate T and B cells from the separated mononuclear cells in cats, the recovery of nylon wool effluent cells from separated mononuclear cells was 21.7%, and the contamination of B cells was 1.58%. These results agreed with the result of using separated mononuclear cells from mouse spleen cells [8]. The results showed very high T cell purity of effluent cells, whereas B cell purity of adherent cells was low (41%) in this study. From the results of cell differentiation, about half of adherent cells were T cells. There was no problem when effluent cell suspension was used as T cell for the lymphocytotoxic crossmatch test. However, one must realize that there are many T cells contained when adherent cell suspension is used for B cell. A cold antibody against B cell is not related to allograft rejection, so the antibody against B cell can be disregarded in the cold condition. On the other hand, a warm antibody against B cell causes acute rejection, so the antibody against B cell is important in the warm condition. If effluent cells are negative and adherent cells are positive in the results of crossmatch test, the positive reaction of adherent cells can be explained as due to antibodies against B cell. If both effluent cells and adherent cells are positive, it is difficult to explain whether the positive reaction of adherent cells is due to antibodies against B cell or not, but a positive result of antibodies against T cell means a transplantation contraindication, so the result of B cell crossmatch test is not needed.

The eosin stain method has been used in human lymphocytotoxic crossmatch test [7, 14], but in cats the dead cells were not clearly stained with eosin, and the counting of feline lymphocytes was difficult due to cell aggregation at the margin of the well after keeping overnight. Hence, an attempt was made to use the trypan blue stain method to identify dead cells. As a result, the trypan blue stain method made it easy to distinguish injured cells, and almost the same results as for the eosin stain method were obtained. Moreover, the trypan blue method is simple to perform, and takes little time, so it is deemed suitable for the lymphocytotoxic crossmatch test in cat.

From the results of this study and other reports [3, 15, 21], some cats have iatrogenic or natural anti-lymphocyte antibodies. Therefore, it is necessary to survey cats having anti-lymphocyte antibodies widely, and to confirm the effects of the antibodies on transplantation in cats. This study may well be utilized for the investigation of anti-lymphocyte antibodies in cats.

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