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Comparison of the Pharmacological Efficacies of Immunosuppressive Drugs Evaluated by the ATP Production and Mitochondrial Activity in Human Lymphocytes

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The lymphocyte immunosuppressant sensitivity test (LIST) using patient peripheral lymphocytes can predict the therapeutic efficacy of immunosuppressive drugs used in renal transplantation. We have evaluated the pharmacological efficacy of drugs by using the LIST with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, which measures the cellular mitochondrial activity. The LIST with the MTT assay requires a relatively large amount of blood. As such, we developed a new assay for examining drug sensitivity with a CellTiter-Glo assay, which measures the amount of cellular ATP to help increase the assay's sensitivity and reduce the amount of blood needed. Renal transplant recipients generally receive either cyclosporine or tacrolimus, in addition to mycophenolate mofetil and methylprednisolone, as an immunosuppressive therapy to prevent acute rejection. We evaluated the pharmacological efficacy of these immunosuppressive agents with both the MTT and CellTiter-Glo assays using the peripheral blood mononuclear cells of 21 healthy volunteers. Furthermore, we also examined the relationship between these immunosuppressive agents' pharmacological efficacy and the results of the MTT and CellTiter-Glo assays. The IC_{50} values for cyclosporine, tacrolimus, mycophenolic acid, and methylprednisolone were significantly correlated between the MTT and CellTiter-Glo assays. The amount of blood cells required for LIST with the CellTiter-Glo assay was able to be reduced to 25% of the amount required for the previously established LIST with the MTT assay procedure. We concluded from these observations that the LIST with the CellTiter-Glo assay should be used instead of the MTT assay for carrying out individualized immunosuppressive therapy in renal transplantation patients.

Key words: CellTiter-Glo assay; Lymphocyte immunosuppressant sensitivity test (LIST); 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay; Peripheral blood mononuclear cells (PBMCs)

INTRODUCTION

The lymphocyte immunosuppressant sensitivity test (LIST) with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay has been used to predict the pharmacological efficacy of immunosuppressive agents for the prevention of acute rejection episodes in renal transplant recipients (7,8). The LIST with MTT assay can estimate the IC_{50} values of immunosuppressive agents against the mitogen-induced proliferation of peripheral blood mononuclear cells (PBMCs) of patient origin (6–8). The merit of LIST with the MTT assay is that the assay does not require the use of a radioisotope. However, a relatively large amount (16 ml) of blood, as well as dimethyl sulfoxide use for the cell lysis, is necessary for this procedure. To overcome these inconveniences, we developed a new LIST using the CellTiter-Glo assay as a novel means to evaluate the pharmacological efficacy of immunosuppressive drugs.

The mechanistic basis of the MTT assay relates to the cellular mitochondrial reducing activity, which converts MTT to colorimetric MTT-formazan, in growing cells (4). In contrast, the CellTiter-Glo assay procedure analyzes the number of viable cells based on the cellular ATP level (9). Thus, the cell growth assay using the CellTiter-Glo method should be more sensitive than that using the MTT assay. Indeed, LIST with a CellTiter-Glo

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assay can be performed with 75% less blood cells compared to the amount of blood cells required for the MTT assay. In addition, the LIST with the CellTiter-Glo assay is easier to carry out compared to the MTT assay, and the CellTiter-Glo assay does not require dimethyl sulfoxide.

Renal transplant recipients receive immunosuppressive therapy to prevent acute rejection episodes. These immunosuppressive therapies are composed of either cyclosporine or tacrolimus, in addition to mycophenolate mofetil and methylprednisolone. In this study, we compared the pharmacological efficacies of these immunosuppressive drugs by LIST procedures using the CellTiter-Glo assay with those using the MTT assay with PBMC specimens obtained from 21 healthy subjects. Furthermore, we also evaluated the relationship between the pharmacological efficacies between the LIST with the MTT and CellTiter-Glo assays.

MATERIALS AND METHODS

Materials

Cyclosporine and tacrolimus were kindly provided by Novartis Pharma K.K. (Basel, Switzerland) and Astellas Pharma Inc. (Tokyo, Japan), respectively. The MTT (M2128-1G) and methylprednisolone (M0639-250MG) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The Ficoll-Paque (17-1440-02) was from Amersham Pharmacia Biotech Inc. (Little Chalfont, Buckinghamshire, UK). Roswell Park Memorial Institute (RPMI) 1640 medium (11875-093), fetal bovine serum (26140-087), and Hank's balanced salt solution (14170-112) were purchased from Gibco Laboratories (Rockville, MD, USA). CellTiter-Glo (G7571) was obtained from Promega K.K. (Madison, WI, USA). Mycophenolic acid (132-11001) and dimethyl sulfoxide (043-07216) were obtained from Wako Chemical Co. (Osaka, Japan). Concanavalin A (J-103) was obtained from J-OIL MILLS, Inc. (Tokyo, Japan).

Subjects

This study was approved by the Ethics Review Board of the Medical Faculty of Niigata University. After informed consent was obtained, venous blood (20 ml) was taken from 21 healthy volunteers (13 males and 8 females). The mean ± SD age of these subjects was 31.6 ± 6.8 years old, and the median was 30.0 years old. The range of age was from 24 to 46 years old. These subjects had no history of taking immunosuppressive agents, including glucocorticoids.

Isolation of PBMCs

Venous blood (20 ml) was taken from the healthy volunteers using a vacuum blood collection tube with heparin (Terumo, Tokyo, Japan). The isolation and culture of the PBMCs were carried out according to the method described previously (3,6–8). Briefly, 5 ml of heparinized blood were loaded onto 4 ml of Ficoll-Paque and centrifuged at $900 \times g$ for 20 min at room temperature. The buffy coat containing PBMCs was taken and rinsed three times with Hank's balanced salt solution. PBMCs, including lymphocytes, were suspended in RPMI 1640 medium containing 10% fetal bovine serum. The PBMCs obtained as mentioned above were divided to carry out each LIST procedure with the CellTiter-Glo or MTT assay. The percentage of PBMCs used for the CellTiter-Glo and MTT assay procedures was about 20% and 80%, respectively. These PBMCs were adjusted to a cell density of 1×10^6 cells/ml for the LIST with MTT assay. The cell density of the LIST with CellTiter-Glo assay was 5×10^5 cells/ml.

LIST With the MTT Assay

PBMCs for the MTT assay were suspended in RPMI 1640 medium containing 10% fetal bovine serum to a cell density of 1×10^6 cells/ml. Concanavalin A, as a T-cell mitogen, was added at a final concentration of 5.0 μg/ml. Subsequently, immunosuppressive drugs were added individually at a range of concentrations into the clear microplates with 96 flat-bottomed wells (Becton Dickinson, Franklin Lakes, NJ, USA). After a 96-h incubation in an atmosphere of 5% CO_2 at 37°C, the LIST procedure using the MTT assay was performed as described previously (6–8). In brief, 10 μl of 5 mg/ml MTT solution was added, and then the cultures were incubated for a further 4–5 h. The cultures were centrifuged at $375 \times g$ for 5 min to obtain precipitated formazan, and aliquots of the supernatant were removed. Dimethyl sulfoxide was added, followed by shaking for 10 min to dissolve the formazan crystals, and the absorbance of the solution was measured at 550 nm. The percentage of blastogenesis was plotted against the immunosuppressive agent concentrations, and the IC_{50} values were determined from the dose–response curves.

LIST With CellTiter-Glo Assay

Each of the 88 μl PBMC suspensions (as separated above) at 5×10^5 cells/ml was plated in 96-well white opaque plates (Becton Dickinson). Ten microliters of concanavalin A were added at a final concentration of 5.0 μg/ml. Subsequently, 2 μl of immunosuppressive drug solution was added individually at a range of concentrations into a white opaque microplate with 96 flat-bottomed wells. The total volume of the CellTiter-Glo assay was 100 μl in each well of the opaque microplate. After a 96-h incubation in a 5% CO_2 atmosphere at 37°C, both the incubated microplate and the CellTiter-Glo agents stayed at room temperature for 10 min. Then, 100 μl of the CellTiter-Glo reagent was added to each well, including the PBMCs, using the opaque plate. The contents were mixed on a shaker for 2 min. The luminescence was then recorded by a luminometer (Promega K.K). The percentages of cell

proliferation in the presence of serial concentrations of immunosuppressive drugs were plotted, and the IC_{50} values of the drugs were determined by the same procedure as with the MTT assay.

Statistical Analysis

The correlation coefficients between the IC_{50} values of cyclosporine, tacrolimus, mycophenolic acid, or methylprednisolone estimated by the CellTiter-Glo assay and those estimated by the MTT assay were evaluated with the Kendall and Spearman test. The differences in the IC_{50} values of immunosuppressive drugs obtained by the MTT or CellTiter-Glo assay between the male and female volunteers was analyzed by Wilcoxon's signed-ranked test (two-side). The nonparametric data are shown as mean and SD for clarity. These data analyses were performed using the PASW statistics base 18.0 software package (SPSS Japan Inc., IBM, Tokyo, Japan) and EXCEL 2010 (Microsoft, Redmond, WA, USA).

RESULTS

The pharmacological effects of cyclosporine, tacrolimus, mycophenolic acid, and methylprednisolone on the mitogen-induced PBMC growth in vitro were evaluated in the PBMCs obtained from the 21 healthy volunteers by the LIST using both the MTT and CellTiter-Glo assays. The amount of blood required to estimate the IC_{50} values for these immunosuppressive agents by the LIST with the MTT assay was approximately 15 ml, while the amount required for the CellTiter-Glo assay was approximately 5 ml.

The typical dose–response curves for cyclosporine obtained by the LIST with the MTT and CellTiter-Glo assays against the mitogen-stimulated proliferation of PBMCs are shown in Figure 1A. Similarly, typical

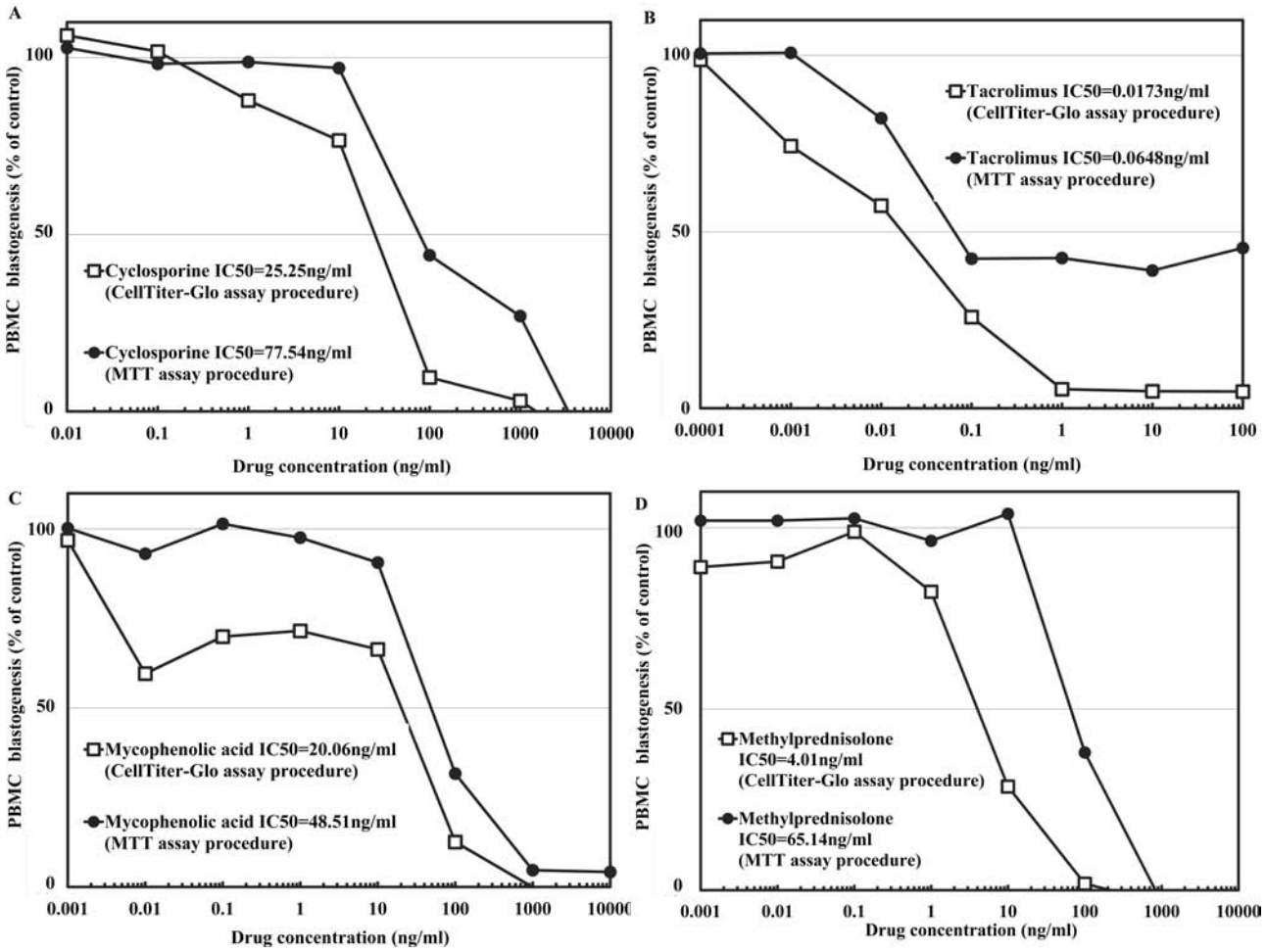

Figure 1. Representative lymphocyte immunosuppressant sensitivity test (LIST) determined by MTT and CellTiter-Glo assays. Typical dose–response curves for cyclosporine (A), tacrolimus (B), mycophenolic acid (C), and methylprednisolone (D) on concanavalin A-stimulated blastogenesis of peripheral blood mononuclear cells (PBMCs) from one healthy subject estimated by the LIST, followed by MTT and CellTiter-Glo assay procedures.

dose–response curves for tacrolimus, mycophenolic acid, and methylprednisolone are shown in Figure 1B, C, and D, respectively.

The mean ± SD of the cyclosporine IC_{50} values, as estimated by the MTT assay, was 239.8 ± 439.3 ng/ml, and the median was 77.5 ng/ml. The IC_{50} range showed a huge variation from 0.01 to 1581.9 ng/ml between the subjects (Table 1). The mean ± SD of the cyclosporine IC_{50} values estimated by the CellTiter-Glo assay was 18.8 ± 17.5 ng/ml, and the median was 16.2 ng/ml. The IC_{50} range also showed a wide variation between the subjects from 0.01 to 64.6 ng/ml (Table 1). Furthermore, the median of the cyclosporine IC_{50} values determined by the MTT assay was 4.78 times greater than that determined by the CellTiter-Glo assay. There was a significant difference in the pharmacological efficacy of cyclosporine (IC_{50} values) estimated by the MTT or CellTiter-Glo assays in these subjects ($p < 0.01$) (Table 1). The correlation between the cyclosporine IC_{50} values estimated by the MTT assay and those estimated by the CellTiter-Glo assay was also significant ($r = 0.511$ by the Kendall test, $p = 0.001$; $r = 0.683$ by the Spearman test, $p = 0.001$) (Fig. 2A).

The IC_{50} value of tacrolimus estimated by the MTT assay was 47.9 ± 218.2 ng/ml (mean ± SD), and the median was 0.065 ng/ml. The IC_{50} range showed a large variation among the subjects, from 0.0001 to 1000 ng/ml. The IC_{50} value of tacrolimus estimated by the CellTiter-Glo assay was 0.011 ± 0.02 ng/ml (mean ± SD), and the median was 0.00012 ng/ml. The IC_{50} range showed a wide variation between the subjects, from 0.0001 to 0.068ng/ml (Table 1). Furthermore, the median of the tacrolimus IC_{50} values evaluated by the MTT assay was 541.7 times greater than that evaluated by the CellTiter-Glo assay. There was also a significant difference in the pharmacological efficacy of tacrolimus (IC_{50} values) estimated by the MTT or CellTiter-Glo assays in these subjects ($p < 0.01$) (Table 1). The correlation between the tacrolimus IC_{50} values estimated by the MTT assay and those estimated by the CellTiter-Glo assay was also significant ($r = 0.555$ by Kendall test, $p = 0.001$; $r = 0.697$ by Spearman test, $p < 0.001$) (Fig. 2B).

The IC_{50} value of mycophenolic acid estimated by the MTT assay was 51.8 ± 51.8 ng/ml (mean ± SD), and the median was 47.2 ng/ml. The IC_{50} range showed a huge variation, from 0.001 to 229.1 ng/ml among the subjects (Table 1). The IC_{50} value of mycophenolic acid estimated by the CellTiter-Glo assay was 15.2 ± 32.4 ng/ml (mean ± SD), and the median was 0.0027 ng/ml. The IC_{50} range also showed a variation between the subjects, from 0.001 to 123.7 ng/ml. Furthermore, the median of the mycophenolic acid IC_{50} value determined by the MTT assay was 17,481.5 times greater than that determined by the CellTiter-Glo assay. There was also a significant difference in the pharmacological efficacy of mycophenolic acid (IC_{50} values) estimated by the MTT assay and CellTiter-Glo assay in these subjects ($p < 0.01$) (Table 1). The correlation between the mycophenolic acid IC_{50} values estimated by the MTT assay and those estimated by the CellTiter-Glo assay was also significant ($r = 0.359$ Kendall test, $p = 0.029$; $r = 0.508$ by Spearman test, $p = 0.019$) (Fig. 2C).

The IC_{50} value of methylprednisolone estimated by the MTT assay was 46.8 ± 54.4 ng/ml (mean ± SD), and the median was 21.2 ng/ml. The IC_{50} range showed a huge variation, from 0.42 to 166.4 ng/ml, among the subjects (Table 1). The IC_{50} value of methylprednisolone estimated by the CellTiter-Glo assay was 4.84 ± 12.4 ng/ml (mean ± SD), and the median was 0.31 ng/ml. The IC_{50} range also showed a variation between the subjects, from 0.001 to 46.8 ng/ml (Table 1). The median of the methylprednisolone IC_{50} value determined by the MTT assay was 68.4 times greater than that determined by the CellTiter-Glo assay. There was a significant difference in the pharmacological efficacy of methylprednisolone (IC_{50} values) estimated by the MTT or CellTiter-Glo assays in these

Table 1. Immunosuppressive Agents' Pharmacological Effects Estimated by the MTT and CellTiter-Glo Assays in the PBMCs of 21 Healthy Subjects

		IC_{50} Value (ng/ml)			
Immunosuppressive Agent	Procedure	Mean (SD)	Median	Minimum	Maximum
Cyclosporine	MTT assay	239.8 (439.3)	77.5*	0.01	1581.9
	CellTiter-Glo assay	18.8 (17.5)	16.2	0.01	64.6
Tacrolimus	MTT assay	47.9 (218.2)	0.065*	0.0001	1000
	CellTiter-Glo assay	0.011 (0.02)	0.00012	0.0001	0.068
Mycophenolic acid	MTT assay	51.8 (51.8)	47.2*	0.001	229.1
	CellTiter-Glo assay	15.2 (32.4)	0.0027	0.001	123.7
Methylprednisolone	MTT assay	46.8 (54.4)	21.2*	0.42	166.4
	CellTiter-Glo assay	4.84 (12.4)	0.31	0.001	46.8

PBMCs, peripheral blood mononuclear cells; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.
*$p < 0.01$ median IC_{50} Wilcoxon's signed-rank test.

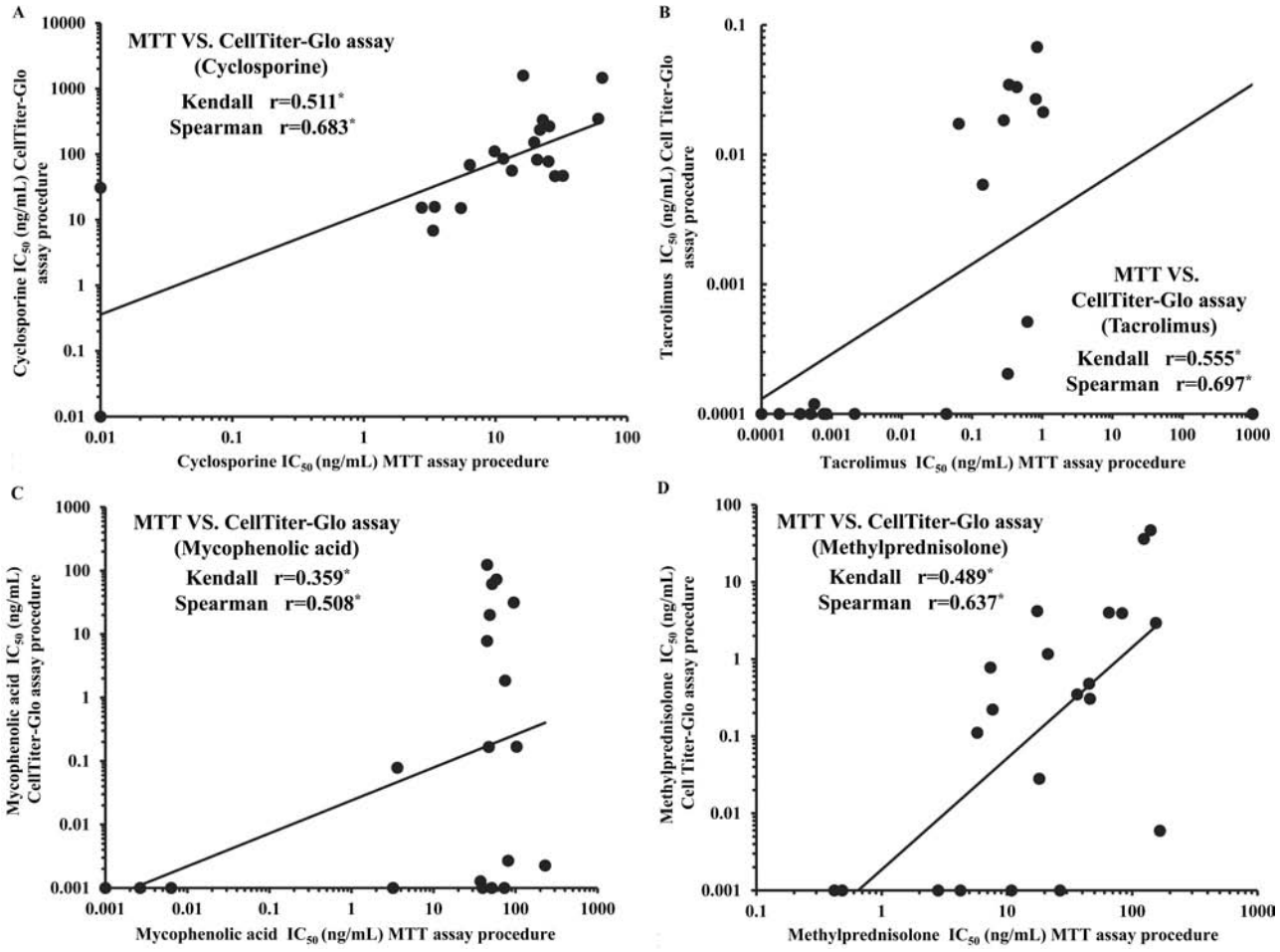

Figure 2. Correlation between immunosuppressant drug IC_{50} by MTT and CellTiter-Glo assays. (A) The relationship between the cyclosporine IC_{50} values obtained by the LIST procedure with the MTT and CellTiter-Glo assays in the PBMCs of 21 healthy subjects. A significant correlation was observed between these values by the Kendall and Spearman coefficient correlation ($r=0.511$ by Kendall test, $p=0.001$; $r=0.683$ by Spearman test, $p=0.001$). (B) The relationship between the tacrolimus IC_{50} values obtained by the LIST with the MTT and CellTiter-Glo assays in the PBMCs of 21 healthy subjects. A significant correlation was observed between these values by the Kendall and Spearman coefficient correlation ($r=0.555$ by Kendall test, $p=0.001$; $r=0.697$ by Spearman test, $p=0.001$). (C) The relationship between the mycophenolic acid IC_{50} values obtained by the LIST with the MTT and CellTiter-Glo assays in the PBMCs of 21 healthy subjects. A significant correlation was observed between these values by the Kendall and Spearman coefficient correlation ($r=0.359$ by Kendall test, $p=0.029$; $r=0.508$ by Spearman test, $p=0.019$). (D) The relationship between the methylprednisolone IC_{50} values obtained by the LIST procedure with the MTT and CellTiter-Glo assays in the PBMCs of 21 healthy subjects. A significant correlation was observed between these values by the Kendall and Spearman coefficient correlation ($r=0.489$ by Kendall test, $p=0.002$; $r=0.637$ by Spearman test, $p=0.002$).

subjects ($p<0.01$) (Table 1). The correlation between the methylprednisolone IC_{50} value determined by the MTT assay and those determined by the CellTiter-Glo assay was also significant ($r=0.489$ by Kendall test, $p=0.002$; $r=0.637$ by Spearman test, $p=0.002$) (Fig. 2D).

As described above, the IC_{50} values of all the immunosuppressive agents examined in this study estimated by the MTT and CellTiter-Glo assays significantly correlated with each other. Thus, the pharmacological efficacies of cyclosporine, tacrolimus, mycophenolic acid, and methylprednisolone were able to be evaluated with not only the MTT assay but also the CellTiter-Glo assay. Accordingly, the LIST with the CellTiter-Glo assay should be used instead of the MTT assay to carry out individualized immunosuppressive therapy in renal transplantation, since the LIST with the CellTiter-Glo assay required approximately 80% less blood compared to the LIST with the MTT assay. Furthermore, all of the immunosuppressive agents' pharmacological efficacies determined by the LIST with MTT assay were greater than those determined by the CellTiter-Glo assay. There was no significant difference in these pharmacological efficacies (IC_{50} values)

estimated by the MTT or CellTiter-Glo assay between the male and female (data not shown) subjects. Furthermore, there was no significant correlation between the ages of the subjects and the IC_{50} values of the calcineurin inhibitors estimated either by the MTT or CellTiter-Glo assays (data not shown).

DISCUSSION

Renal transplant recipients are administered immunosuppressive drugs to prevent acute rejection. Immunosuppressive therapies are normally composed of either cyclosporine or tacrolimus in addition to mycophenolate mofetil and methylprednisolone. LIST has been used to predict the pharmacological efficacy of the immunosuppressive agents for renal transplant recipients (7,8). In this study, the pharmacological efficacies of these immunosuppressive agents were compared using the MTT and CellTiter-Glo assays. The IC_{50} values for all of these agents were significantly correlated between these two assays. Furthermore, the IC_{50} values for all of these immunosuppressive agents estimated by the LIST with the MTT assay were significantly lower than those estimated by the LIST with the CellTiter-Glo assay.

Cyclosporine and tacrolimus are both calcineurin inhibitors, and therefore these immunosuppressive agents have similar pharmacological efficacies. We previously reported that the pharmacological efficacies of calcineurin inhibitors against the proliferation of concanavalin A-stimulated PBMCs in vitro were correlated in the PBMCs of renal transplant recipients (6). The median tacrolimus IC_{50} value estimated by the LIST with the MTT assay was almost equal to the median of the tacrolimus IC_{50} value estimated by the ^{3}H-thymidine assay procedure. However, the median of the cyclosporine A IC_{50} value estimated by the MTT assay was 33.0 times greater than that estimated by the ^{3}H-thymidine assay (6). The larger cyclosporine A IC_{50} values estimated by the MTT assay might have resulted from the specific effects of cyclosporine A on the mitochondrial membrane potential.

The LIST with the MTT assay does not require the use of a radioisotope; however, it is necessary to use a relatively large amount of venous blood (usually about 16 ml) from patients. This problem may be improved by changing the assay method to another one that requires less blood. In the case of pediatric renal transplant recipients or hemodialysis patients, for instance, it would be difficult to obtain a sufficient amount of blood to evaluate the pharmacological efficacy of immunosuppressive drugs using the MTT assay. Moreover, despite the smaller amount of blood required to carry out the LIST, the ATP assay exhibits higher sensitivity compared with the MTT assay (5). Furthermore, the CellTiter-Glo assay analyzes the number of viable cells based on cellular ATP level, providing a more accurate assessment of the impact of the drug (9).

The MTT assay is a rapid colorimetric assay, in which the MTT reagent is reduced in active mitochondria, which occurs only in living cells. Thus, the MTT assay quantifies both growing (viable) and proliferating cells (4). The viabilities of Daudi and CCRF-CEM cells were previously compared between the ATP and MTT assays, and the ATP assay was demonstrated to be much more sensitive than the MTT assay (5). Zhelev et al. reported determining the cytotoxicity of phenothiazines using the CellTiter-Glo assay luminescent cell viability assay, using ATP bioluminescence as a marker of cell viability, as well as a marker of mitochondrial activity. The CellTiter-Glo assay has also been reported to be able to monitor the ATP in lymphocytes (9). Moreover, Friberg et al. reported that calcium-induced swelling in isolated mitochondria from the hippocampus is depressed by cyclosporine A, but not by tacrolimus (2). Both cyclosporine A and tacrolimus prevented the increase in DNA synthesis, lactase production, and ATP levels seen in response to mitogen stimulation (2). The CellTiter-Glo assay is able to determine the number of viable PBMCs based on the cellular ATP level. Crouch et al. reported that cellular ATP monitoring using the CellTiter-Glo assay gave a significant correlation between the proliferating PBMC number and measured luminescence intensity based on the cellular ATP level (1).

In our previous study, the PBMC density and total volume required for the assay were 1×10^{6} cells/ml and 200 µl, respectively, to carry out LIST with the MTT assay. In this new LIST procedure using the CellTiter-Glo assay, the density and total volume required were 5×10^{5} cells/ml and 100 µl, respectively, and thus 50% of the total volume of blood and its cell concentration, which equates to a 75% reduction in the number of cells required. Thus, the LIST procedure with the CellTiter-Glo assay can help reduce the amount of blood required from patients, compared to the MTT assay.

In summary, in this study, the IC_{50} values for all of the immunosuppressive agents examined by the MTT and CellTiter-Glo assays significantly correlated with each other. Furthermore, the IC_{50} values of the drugs estimated by the LIST with the MTT assay were significantly higher than those estimated by the LIST with the CellTiter-Glo assay. Thus, the data also suggest that the LIST procedure with the CellTiter-Glo assay is much more sensitive for estimating the pharmacological efficacy of immunosuppressive drugs, compared with the LIST using the MTT assay. In the previous studies, the pharmacological effects of immunosuppressive drugs on PBMC proliferation have been evaluated by either the thymidine uptake or MTT assay. The present observations give new insights into the

evaluation of the pharmacological efficacy of immunosuppressive drugs using the CellTiter-Glo assay, which requires a much smaller amount of blood compared to the generally used method.

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