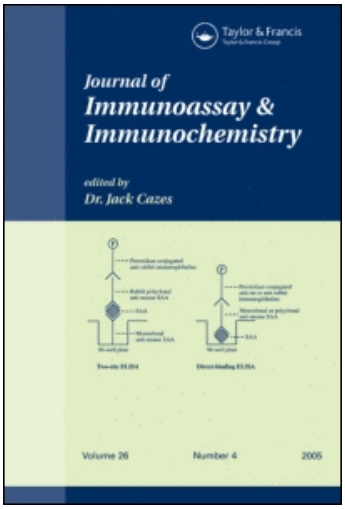

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Correlation Between Pharmacological Efficacy of Cyclosporine A and Tacrolimus, Evaluated by Lymphocyte Immunosuppressant-Sensitivity Test (LIST) with MTT Assay Procedure in Renal Transplant Recipients

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Abstract: The dose of calcineurin inhibitors in renal transplantation has been adjusted, based on the therapeutic drug monitoring data. However, the data do not always correlate with clinical drug efficacy. In vitro response of peripheral-blood mononuclear cells to immunosuppressive drugs is reported to correlate with the recipient-response to therapeutic efficacy of the drug. We report, here, usefulness of a lymphocyte immunosuppressant sensitivity test for the estimation of individual drug sensitivity in renal transplant recipients. The LIST we have developed includes MTT assay procedures

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without the use of radioisotope-labeled compounds, which is convenient for general hospital use. Utilizing this procedure, we compared the pharmacological efficacy between cyclosporine A and tacrolimus in 41 renal transplant recipients.

Keywords: Cyclosporine A, Tacrolimus, Peripheral blood mononuclear cells, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Lymphocyte immunosuppressant-sensitivity test (LIST)

INTRODUCTION

Treatment with a combination of basiliximab, calcineurin inhibitors, glucocorticoids, and purine-synthesis inhibitors has recently been used for prevention of acute rejection after renal transplantation. The dose of cyclosporine A or tacrolimus has been adjusted based on the results of therapeutic drug monitoring. However, the data of therapeutic drug monitoring do not always correlate with clinical efficacy of the immunosuppressive drugs. Therefore, estimation of the efficacy of immunosuppressive agents is necessary from the point of view of, not only pharmacokinetics, but also pharmacodynamics of the drugs. We have reported that, in peripheral-blood mononuclear cells of chronic renal failure patients, the variation of the pharmacological efficacy of prednisolone in vitro is larger than that in peripheral-blood mononuclear cells of healthy subjects. In contrast, the effect of methylprednisolone against peripheral-blood mononuclear cells-blastogenesis is almost the same in chronic renal failure patients and healthy subjects.[1] We have established, through these studies, a lymphocyte immunosuppressant sensitivity test (LIST) to be carried out for renal transplant recipients. The LIST generally uses ^{3}H-thymidine for the evaluation of peripheral-blood mononuclear cells-blastogenesis. However, radioisotope-labeled compounds are not suitable for general hospital use. Mosmann reported that the data obtained by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay procedure correlated with those obtained by a ^{3}H-thymidine incorporation procedure.[2] The MTT assay procedures do not require radioisotope-labeled compounds for the cell proliferation assay. Therefore, we have established a procedure for LIST with the MTT assay procedure. In our previous studies, we have compared pharmacological efficacy between cyclosporine A and tacrolimus by the LIST with ^{3}H-thymidine assay procedure.[3,4] Cyclosporine A concentrations giving 50% inhibition of peripheral-blood mononuclear cells-blastogenesis (IC50) and tacrolimus IC50s significantly correlated in peripheral-blood mononuclear cells of chronic renal failure patients.[3,4] However, the pharmacological efficacy of calcineurin-inhibitors evaluated by the LIST with MTT assay procedure has not been compared to that evaluated by the LIST with ^{3}H-thymidine assay procedure in renal transplant recipients so far to our knowledge. Therefore, in the present study we compared the pharmacological efficacy between cyclosporine A and

tacrolimus in peripheral-blood mononuclear cells of renal transplant recipients by the LIST with MTT assay procedure.

EXPERIMENTAL

Reagents

Cyclosporine A was kindly provided by Novartis Pharma Co. (Basel, Swizerland). Tacrolimus was kindly provided by Fujisawa Co. (Osaka, Japan). 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was obtained from Sigma Chemical Co. (St. Louis, MO). Ficoll-Paque was obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK). RPMI 1640 medium, fetal bovine serum, and Hank's balanced salt solution were obtained from Gibco Laboratories (Rockville, NY). Concanavalin A was obtained from Seikagaku Kogyo Co. (Tokyo, Japan). All other reagents were of the highest grade available.

Subjects

After informed consent was obtained, heparinized venous blood (20 ml) was taken from 41 renal transplant recipients (30 males and 11 females) just before operation. The mean (SD) age of these recipients was 36.9 (14.7) years. Mean HLA-AB missmatch number was 1.8. Mean HLA-DR missmatch number was 0.9 (Table 1). All of the transplant recipients received renal allografts from living donors after blood-sampling for analysis of their peripheral-blood mononuclear cells-response to immunosuppressive agents in vitro. All of the patients underwent renal transplantation from May 2002 to October 2004 at Niigata University Medical and Dental Hospital. The study was approved by the ethics review board of the Medical Faculty of Niigata University.

These patients were treated with maintenance immunosuppressive therapy after renal transplantation, which consisted of a combination of

Table 1. Characteristics of renal transplant recipients

	Patients (n = 41)
Mean age ± SD	36.9 ± 14.7
Male (%)	30/41 (73.2%)
Female (%)	11/41 (26.8%)
Mean HLA-AB missmatch number	1.8
Mean HLA-DR missmatch number	0.9

methylprednisolone, basiliximab (6 patients did not receive it), either cyclosporine A (Neoral cap., Novartis Pharma Co., Swizerland) or tacrolimus (Prograf cap., Fujisawa Co., Japan), and Mycophenolate mofetil (Celcept 250 mg Cap., Chugai Co., Japan). The starting doses of these agents were 125 mg/day for methylprednisolone, 2–3 mg/kg/day for cyclosporine A intravenously (or 8 mg/kg/day orally), 0.05 mg/kg/day for tacrolimus intravenously (or 0.2 mg/kg/day orally), and 1,000 mg b.i.d. for MMF. To measure the response of PBMCs to the drugs in vitro, a drug sensitivity test[1,5] was carried out in renal transplant recipients just before operation.

Isolation of Peripheral Blood Mononuclear Cells

Venous blood was taken and heparinized at just before immunosuppressive agents were administered for renal transplant recipients on a day on which hemodialysis was not performed. Isolation and culturing of peripheral-blood mononuclear cells were carried out according to the method we described previously.[1,5] In brief, 5 mL of heparinized blood was loaded onto 4 mL of Ficoll-Paque and centrifuged at 900 × g for 20 min at room temperature. The buffy coat was taken and rinsed 3 times with Hank's balanced salt solution. peripheral-blood mononuclear cells, including lymphocytes, were suspended in RPMI1640 medium containing 10% fetal bovine serum to a cell density of 1×10^6 cells/mL.

Peripheral Blood Mononuclear Cell Culture and Evaluation of Drug Potency

The cell suspension prepared as described above was placed into each well of microplates with 96 flat-bottomed wells. Saline containing concanavalin A was added to each well at a final mitogen concentration of 5.0 μg/mL. Subsequently, ethanol solution containing cyclosporine A was added to give a final drug concentration of 0.01, 0.1, 1, 10, 100, 1,000, 10,000 or 100,000 ng/mL. Similarly, ethanol solution containing tacrolimus was added to give a final drug concentration of 0.0001, 0.001, 0.01, 0.1, 1, 10, 100, or 1,000 ng/mL. The same volume of each vehicle solution was added to control wells. The plates were incubated for 4 days in an atmosphere of 5% CO_2 at 37°C.

MTT Assay

After 4 days of culture, 10 μL of 5 mg/mL MTT solution dissolved in saline was added to each well and then the cultures were re-incubated under 5% CO_2 at 37°C for 4–5 hours.[6–9] The plates were centrifuged at 375 × g for 5 min to precipitate cells and formazan produced by growing cells. Aliquots of the

supernatant were removed from each well and dimethylsulfoxide was added followed by shaking of the plate on a microshaker for 10 min to dissolve the formazan crystals. The absorbance was read with a microplate reader at 550 nm. Dose-response curves were plotted, and the concentrations of drugs giving 50% inhibition of PBMC-blastogenesis (IC_{50}s) were calculated.

Statistical Analysis

The correlation coefficiency between cyclosporine A IC_{50} and tacrolimus IC_{50} was analyzed with the Kendall test and Spearman test. The medians for calcineurin inhibitor IC_{50}s were compared between transplant recipients with and without acute rejection episodes by the Mann-Whitney tests. The incidence of acute rejection episodes was compared between cyclosporine A-treated recipients and tacrolimus-treated recipients by the χ^2 test.

The medians for calcineurin inhibitor IC_{50}s were also compared between the recipients with and without cytomegalovirus antigenemia(+) by the Mann-Whitney test. The incidence of cytomegalovirus antigenemia(+) was compared between cyclosporine A-treated recipients and tacrolimus-treated recipients by the χ^2 test.

Statistical analysis software SPSS 11.0J was used for the analysis (SPSS Japan Inc.).

RESULTS AND DISCUSSION

The pharmacological efficacy of cyclosporine A and tacrolimus on mitogen-induced growth of peripheral-blood mononuclear cells were evaluated in 41 renal transplant recipients by the LIST with MTT assay procedure. Typical dose-response curves for cyclosporine A and tacrolimus against the blastogenesis of peripheral-blood mononuclear cells obtained from one renal transplant recipient are shown in Fig. 1. The mean (SD) of cyclosporine A IC_{50} values was 1,488.0 (3,998.5) ng/mL ($n = 41$), and the median was 158.5 ng/mL. The IC_{50} range showed a huge variation from 0.07 to 18,765.3 ng/mL. The mean (SD) of tacrolimus IC_{50} values was 14.8 (69.7) ng/mL, and the median was 0.293 ng/mL. The IC_{50} ranged from 0.000128 to 442.0 ng/mL. The mean (SD) of the cyclosporine A IC_{50}/tacrolimus IC_{50} ratio was 27,700.9 (80,247.9), and its median was 555.6 ng/mL. The range of the ratio was 0.639 to 370,059.5 (Table 2) (Figure 2). The relationship between cyclosporine A IC_{50} and tacrolimus IC_{50} in transplant recipients as analyzed by sparing plots is shown in Fig. 3. Cyclosporine A IC_{50}s and tacrolimus IC_{50}s gave a significant Kendall coefficient correlation in these 41 renal transplant recipients by the LIST with MTT assay procedure ($r_k = 0.418$, $P < 0.01$). Cyclosporine A IC_{50}s and tacrolimus IC_{50}s also gave a significant Spearman coefficient correlation ($r_s = 0.586$, $P < 0.01$). We have reported

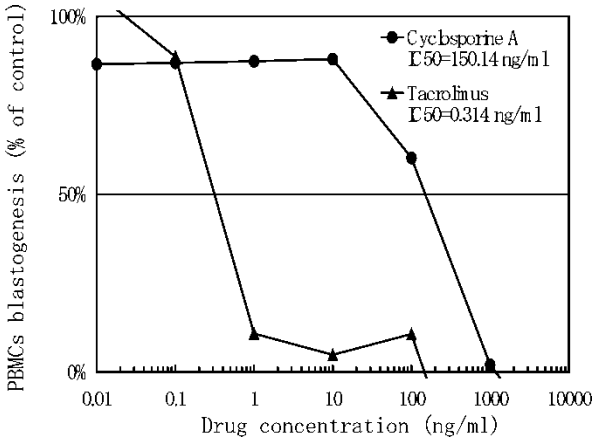

Figure 1. Typical dose response curves for calcineurin inhibitors on concanavalin A stimulated blastogenesis of PBMCs from one transplant recipient by the MTT assay procedure.

that the median of cyclosporine A IC_{50} as estimated by the LIST with ^{3}H-thymidine assay procedure was 4.8 ng/mL.[3] The median of tacrolimus IC_{50} was estimated to be 0.22 ng/mL by the same assay procedure.[3] The ratio of the cyclosporine A median IC_{50} determined by the LIST with MTT assay procedure/cyclosporine A median IC_{50} determined by the LIST with ^{3}H-thymidine assay procedure was 33.0. The ratio of the tacrolimus median IC_{50} determined by the LIST with MTT assay procedure/tacrolimus median IC_{50} determined by the LIST with ^{3}H-thymidine assay procedure was 1.3.

The number (%) of rejection episodes in these 41 renal transplant recipients was 9/41 (22.0%) during the 6 months after transplantation. The number of rejection episodes in the recipients with cyclosporine A-based immunosuppressant treatment was 8/32 (25.0%), whereas the number of rejection episodes in the recipients with tacrolimus-based immunosuppressant treatment was 0/9 (0%). Conversion of calcineurin inhibitor from

Table 2. Comparison of immunosuppressive potency between cyclosporine A and tacrolimus in renal transplant recipients (n = 41)

	IC_{50} (ng/ml) Cyclosporine A	IC_{50} (ng/ml) Tacrolimus	Ratio of IC_{50}s (cyclosporine A/ tacrolimus)
Mean ± SD	1488.0 ± 3998.5	14.8 ± 69.7	27700.9 ± 80247.9
Median	158.5	0.293	555.6
Minimum	0.07	0.000128	0.639
Maximum	18765.3	442.0	370059.5

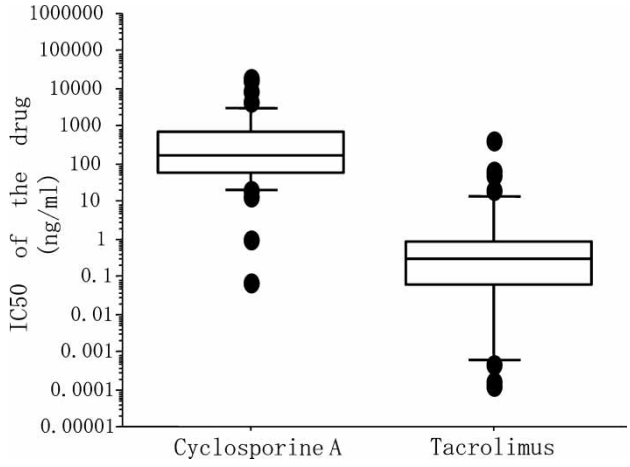

Figure 2. IC_{50} values of cyclosporine A and tacrolimus against blastogenesis of PBMCs from renal transplant recipients. Median IC_{50} values (158.5 ng/mL and 0.293 ng/mL for cyclosporin A and tacrolimus, respectively) are shown by horizontal bars in the boxes. The horizontal boundaries of the boxes represent the first and third quartiles. The vertical bars indicate the range from the 10th to the 90th percentile.

cyclosporine A to tacrolimus was carried out in 6/32 cases (18.8%), while the conversion from tacrolimus to cyclosporine A was carried out in 2/9 cases (22.2%). The number (%) of cytomegalovirus antigenemia(+) renal transplant recipients (n = 41) was 19/41 cases (46.3%) during the 6 months after transplantation. The number of cytomegalovirus antigenemia(+) renal transplant recipients under cyclosporine A-based immunosuppressant treatment was 14/32 cases (43.8%). The number of cytomegalovirus anti-genemia(+) recipients under tacrolimus-based immunosuppressant treatment was 5/9 cases (55.6%) (Table 3).

The number of cases that exhibited adverse events which were considered to have arisen from immunosuppressive drugs was 10 cases (24.4%). The incidence of adenovirus infection was 2 cases (4.9%). The number of ABO-incompatible donors was 3 cases (7.3%). Graft survival at 6 months was achieved in 39 cases (95.1%). Patient survival at 6 months after renal transplantation was 41 cases (100%).

We compared, in the present study, the pharmacological efficacy against PBMC-blastogenesis between cyclosporine A and tacrolimus in renal transplant recipients by the LIST with MTT assay procedure. Furthermore, we compared the data of IC_{50}s obtained here with those obtained in our previous study using the LIST with ^{3}H-thymidine incorporation procedure.[3,4] The data from the MTT assay procedure have been reported to correlate with those from the ^{3}H-thymidine assay procedure.[2] Therefore, the LIST based on either the ^{3}H-thymidine assay procedure or the MTT assay procedure would

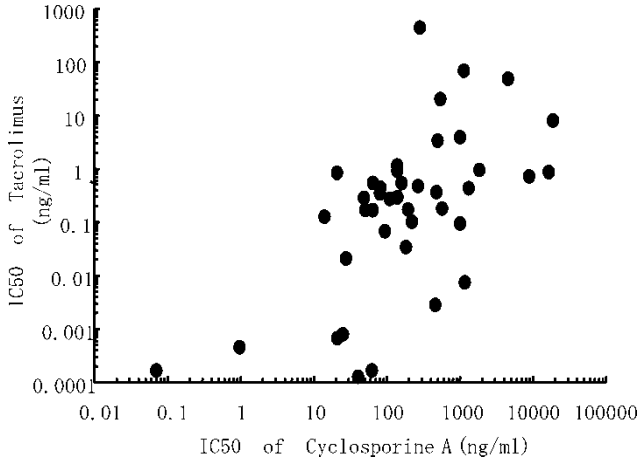

Figure 3. Relationship between cyclosporine A IC_{50}s and tacrolimus IC_{50} against blastogenesis of PBMCs of transplant recipients ($n = 41$). A significant correlation was observed between these values ($r_k = 0.418$ $P < 0.01$) by the Kendall coefficient correlation test. A significant correlation was also observed by the Spearman coefficient correlation test ($r_s = 0.586$, $P < 0.01$).

be useful for the evaluation of individual sensitivity to immunosuppressive drugs. The median of tacrolimus IC_{50} evaluated by the LIST with MTT assay procedure was almost equal to the median of tacrolimus IC_{50} estimated by the ^{3}H-thymidine assay procedure. However, the median of cyclosporine A IC_{50} evaluated by the MTT assay procedure was 33.0 times greater than that evaluated by the ^{3}H-thymidine assay procedure.

The MTT assay procedure is a rapid colorimetric assay. The MTT reagent is cleaved in active mitochondria and, therefore, the reaction occurs only in living cells. Thus, the MTT assay procedure can quantify the growing and proliferating cells.[2] Stephan et al. reported that cyclosporine A prevented mitochondrial permeability transition and cytochrome c release in the rat liver. However,

Table 3. Comparison of clinical events the occurred during 6 months after transplantation between cyclosporine A-treated recipients and tacrolimus-treated recipients

	Cyclosporine A treated (n = 32)	Tacrolimus treated (n = 9)
Acute rejection episode: Number of cases (%)	8 (25.0)	0 (0)
CNI[a] conversion: Number of cases (%)	6 (18.8)	2 (22.2)
Cytomegalovirus antigenemia(+): Number of cases (%)	14 (43.8)	5 (55.6)

[a]CNI: (calcineurin inhibitor).

tacrolimus is suggested to have no effect on mitochondrial permeability transition.[10] Hans et al. reported that calcium-induced swelling in isolated mitochondria from the hippocampus is depressed by cyclosporine A, but not by tacrolimus.[11] Both cyclosporine A and tacrolimus prevented the increase in DNA synthesis, lactase production, and ATP levels seen in response to mitogen stimulation.[11] The increase in mitochondrial uptake of Rhodamine 123 during blastoid transformation was significantly reduced by cyclosporine A, but not by tacrolimus.[12] Furthermore, nitric oxide-induced thymocyte apoptosis is related to the mitochondrial membrane potential. Cyclosporine A partially inhibited nitric oxide-induced thymocyte apoptosis, as well as reduction of the mitochondrial membrane potential.[13] In contrast, tacrolimus did not affect nitric oxide-induced thymocyte apoptosis.[13] The larger cyclosporine A IC_{50} values estimated by the MTT assay procedure, compared to those estimated by the ^{3}H-thymidine assay procedure, might have resulted from these specific effects of cyclosporine A on the mitochondrial membrane potential. Therefore, when measuring immunosuppressant-pharmacological efficacy, it should be kept in mind that the correlation coefficients of the data obtained by the MTT assay procedure and those obtained by the ^{3}H-thymidine assay procedure are different, especially in the case of cyclosporine A.

The MTT assay procedure for carrying out LIST has several merits as compared to the ^{3}H-thymidine assay procedure. The MTT assay procedure, for instance, does not require radioisotope metabolites. Furthermore, the assay is simple, can be carried out rapidly and easily, and is not expensive in comparison with the ^{3}H-thymidine assay procedure. Therefore, the LIST with MTT assay procedure is convenient for the estimation of immunosuppressant pharmacological efficacy in general hospitals.

In the present study, renal transplant recipients treated with basiliximab and tacrolimus had a lower rate of acute rejection episodes than the recipients treated with basiliximab and cyclosporine A. On the other hand tacrolimus treated recipients had a higher rate of cytomegalovirus antigenemia(+) episodes than cyclosporine A treated recipients. Therefore, tacrolimus-based immunosuppressant therapy is superior to cyclosporine A based therapy for controlling acute rejection, while tacrolimus-based therapy may have a tendency to cause excess immunosuppression as compared with cyclosporine A-based therapy.

In our previous study, we compared the immunosuppressive pharmacological efficacy of prednisolone and prednisolone sodium succinate in vitro using human peripheral blood mononuclear cells with the MTT assay. We found that the immunosuppressive potency of prednisolone sodium succinate was several-fold lower than that of prednisolone in vitro.[7] On the other hand, we have also shown that the pharmacological efficacies of azathioprine and its pro-drug 6-mercaputopurine against blastogenesis of human PBMCs were almost equal. Azathioprine has been suggested to be effective after conversion to 6-mercaputopurine in peripheral blood mononuclear cells in vitro.[8] Furthermore, we compared the pharmacological efficacy of mycophenolic acid, 6-mercaputopurine, and mizoribine by the

MTT assay procedure. The individual variation of the effect of mycophenolic acid in both renal transplant recipient's peripheral blood mononuclear cells and healthy peripheral blood mononuclear cells was smallest among the effect of the purine-synthesis inhibitors examined.[9]

CONCLUSION

These observations, together with our present findings concerning the cellular pharmacodynamics of calcineurin inhibitors, support the conclusion that the LIST with MTT assay procedure is useful for estimation of the pharmacological efficacy of immunosuppressive drugs in renal transplant recipients. We conclude, from the present observations, that the LIST with MTT assay procedure is useful for evaluating individual sensitivity to the therapeutic effects of immunosuppressive drugs in renal transplant recipients. When evaluating the sensitivity to cyclosporine A by the MTT assay procedure, underestimation of the drug potency by this method, compared to that of the ^{3}H-thymidine assay procedure, should be kept in mind. Establishment of the normal range of cyclosporine A IC_{50} is, thus, required for individualized monitoring of cyclosporine A pharmacodynamics by the LIST with MTT assay procedure.

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