Comparison of the in vitro antiproliferative effects of five immunosuppressive drugs on lymphocytes in whole blood from cats

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Objective—To compare the in vitro immunosuppressive effects of cyclosporine and 4 novel immunosuppressive drugs on lymphocytes in whole blood collected from healthy cats.

Sample Population—Whole blood samples collected from 10 healthy adult domestic shorthair cats.

Procedure—Mitogen-stimulated lymphocyte proliferation in whole blood incubated with and without various concentrations of cyclosporine, tacrolimus, sirolimus, mycophenolic acid (MPA), or A771726 was measured by use of [^{3}H]thymidine incorporation. Drug concentrations that resulted in a 50% inhibition of mitogen-induced proliferation (IC_{50}) were calculated. Lymphocyte viability was determined by use of the trypan blue dye exclusion method.

Results—An obvious dose-response relationship for the antiproliferative effects of each drug was detected. Mean IC_{50} determined with concanavalin A was 46 n*M* for cyclosporine, 9 n*M* for tacrolimus, 12 n*M* for sirolimus, 16 n*M* for MPA, and 30 m*M* for A771726, whereas with pokeweed mitogen, mean IC_{50} was 33 n*M* for cyclosporine, 5 n*M* for tacrolimus, 15 n*M* for sirolimus, 14 n*M* for mycophenolic acid, and 25 m*M* for A771726. Mitogen-stimulated and nonstimulated lymphocytes remained viable, regardless of drug evaluated.

Conclusions and Clinical Relevance—Tacrolimus, sirolimus, MPA, and A771726 inhibited in vitro mitogen-stimulated proliferation of feline lymphocytes in a dose-dependent manner. These novel immunosuppressive drugs may be useful for management of immune-mediated inflammatory diseases and prevention and treatment of rejection in cats that undergo organ transplantation. (*Am J Vet Res* 2000;61: 906–909)

Immunosuppressive drugs are commonly administered to human and veterinary patients to control immune-mediated inflammatory diseases and to prevent and treat allograft rejection after transplantation. New immunosuppressants that have been recently approved for clinical use in humans include tacrolimus (FK506), sirolimus (rapamycin), **mycophenolate mofetil (MMF)**, and leflunomide.[1] Administration of a combination of established and novel immunosuppressive drugs that have different mechanisms of action and affect different stages of the cell cycle should result in more effective immunosuppression with fewer drug-induced adverse effects.

Tacrolimus binds to the immunophilin **FK-binding protein (FKBP)** in the cytosol of lymphocytes. The tacrolimus-FKBP complex then binds to calcineurin and inhibits its phosphatase activity. This results in an inhibition of gene expression of lymphokines responsible for lymphocyte activation and expression of cell surface activation molecules. Tacrolimus thus blocks cell cycle progression in lymphocytes at the G_o to G_1 phase.

Sirolimus also binds FKBP. The sirolimus-FKBP complex inhibits the 70-kd S6 protein kinases and other proteins involved in cell proliferation, which results in inhibition of interleukin-2 and other growth factor-mediated signal transduction pathways. Additionally, the calcium-independent CD28-B7 costimulatory pathway is also inhibited. Sirolimus prevents lymphocytes from progressing from the G_1 to the S phase of the cell cycle.

Mycophenolate mofetil is converted by liver and plasma esterases to the active metabolite **mycophenolic acid (MPA)**. Mycophenolic acid inhibits the enzyme inosine monophosphate dehydrogenase, which is involved in de novo purine synthesis. Moreover, MPA is a selective inhibitor of B and T lymphocytes, as these cells depend on de novo purine nucleotide synthesis during activation, whereas most other cells can replicate, using only the salvage pathway. Mycophenolic acid blocks cell cycle progression of lymphocytes through the S phase.

Leflunomide is converted by hydrolysis in the plasma and intestinal tract mucosa to A771726, which inhibits the enzyme dihydro-orotate dehydrogenase. Dihydro-orotate dehydrogenase is involved in de novo pyrimidine synthesis. At high concentrations, A771726 inhibits protein tyrosine kinase, which is involved in T-cell receptor and growth factor signal transduction. Moreover, A771726 arrests cell cycle progression at the late G_1 phase in T and B lymphocytes and is particularly effective in inhibiting B-cell proliferation and antibody production.

The purpose of the study reported here was to compare the antiproliferative effects of the immunosuppressive drugs cyclosporine, tacrolimus, and sirolimus, and the active metabolites of MMF (ie, MPA) and leflunomide (ie, A771726) on mitogen-stimulated proliferation of lymphocytes in blood collected from healthy cats. The whole blood mitogen-stimulated lymphocyte proliferation assay has been described

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for the in vitro evaluation of lymphocyte proliferation in a number of species.[2,3] Because clonal expansion of lymphocytes is essential for antigen-induced immune responses, the ability of immunosuppressive drugs to inhibit mitogen-stimulated lymphocyte proliferation provides an in vitro assessment of the effect of these drugs on immune function. Potency of the immunosuppressive effects of the 5 drugs evaluated in this study varies considerably among species, and effects in cats cannot be inferred from results of studies in other species. We believe that the results of the study reported here will provide a rational basis for choosing immunosuppressive regimens for experimental and clinical use in cats.

Materials and Methods

Animals—Sixteen 2-year-old domestic shorthair cats with a mean (± SD) body weight of 4.7 ± 0.61 kg were used for this study. Cats were examined before the study to ensure that they were healthy. The experimental protocol was approved by the Institutional Animal Care and Use Committee at the University of California, Davis.

Media and mitogens—Culture media was prepared, using RPMI 1640[a] supplemented with penicillin (100,000 U/L), streptomycin (100,000 µg/L), and amphotericin B (250 µg/L). Stock solutions of the mitogens **concanavalin A**[b] (**ConA**; 10 µg/ml) and **pokeweed mitogen**[a] (**PWM**; 5 µg/ml) were prepared by dilution in culture media and stored at –20 C.

Preparation of immunosuppressive drugs—Stock solutions (1 m*M*) were prepared by diluting cyclosporine,[c] tacrolimus,[d] sirolimus,[e] and MPA[a] powder in ethanol. A stock solution (1 m*M*) of A771726[f] was prepared in sodium hydroxide solution (0.3 *M*). All drugs were stored at –70 C, warmed to room temperature (approx 20 C), and diluted in culture media to final concentrations before each experiment.

Experimental protocol—Three milliliters of fresh whole blood was obtained from the jugular vein of each cat. Preservative-free heparin (100 U/ml of blood) was used as an anticoagulant. Whole blood from 6 cats (cats 1 to 6) was used in the initial lymphocyte proliferation assays performed to evaluate cyclosporine (cyclosporine concentration range, 100 to 10,000 n*M*). Additional assays, performed to generate more points on the cyclosporine concentration versus [^{3}H]thymidine incorporation curve, used whole blood from an additional 4 cats (cats 7 to 10) and lower cyclosporine concentrations (10, 25, and 50 n*M*). Lymphocyte proliferation assays performed to evaluate effects of tacrolimus, sirolimus, and MPA used whole blood from 8 cats (cats 1 to 8). Whole blood from 10 cats (cats 1 to 10) was used to evaluate A771726 because of the greater intercat variability we observed in results obtained with this drug. The lymphocyte proliferation assay was performed for all drugs evaluated with a single blood sample from each cat to avoid differences in lymphocyte proliferation between blood samples collected at different times from the same cat.

Whole blood mitogen-stimulated lymphocyte proliferation assay—The assay was performed as described.[2,4] Briefly, 80 µl of well-mixed heparinized whole blood was combined with 775 µl of culture media and 45 µl of the appropriate dilution of immunosuppressive drug or 45 µl of culture media (stimulated and unstimulated controls) in a 5-ml polypropylene culture tube. Final drug concentrations evaluated were based on results of previous studies[2] and preliminary investigations. Final concentrations of cyclosporine were 10, 25, 50, 100, 500, 1,000, 5,000, and 10,000 n*M*; concentrations of tacrolimus and sirolimus were 1, 10, 25, 50, and 100 n*M*; concentrations of MPA were 10, 25, 50, 100, and 1,000 n*M*; and concentrations of A771726 were 5, 12.5, 25, 50, and 200 µ*M*.

Tubes were vortexed and allowed to stand at room temperature for at least 15 minutes to ensure homogenous drug distribution within all blood constituents. Then 150 µl of each tube's contents was pipetted into each of 5 wells of a 96-well microtiter plate. For wells prepared from blood to which drug had been added and to wells prepared for the stimulated control, 50 µl of mitogen (either ConA or PWM) was pipetted into each well, resulting in a final blood dilution of 1:15. For wells prepared for the unstimulated control, 50 µl of culture media was substituted for the mitogen.

Tissue culture plates were incubated for 4 days at 37 C in an atmosphere with 99% relative humidity, 5% CO_2, and 95% air. Lymphocyte proliferation was assessed by measuring incorporation of [^{3}H]thymidine[g] (1 µCi in 10 µl of culture media; specific activity; 6.7 Ci/mmol) added during the last 18 hours of culture to 4 of each set of 5 wells. [^{3}H]Thymidine was not added to the fifth well; this well was used to determine cell viability. Cells were harvested from wells with [^{3}H]thymidine onto glass fiber filters, washed with PBS solution, and decolorized with methanol, using a cell harvester.[h] Beta emissions from each filter disc were quantitated as **counts per minute (CPM)**, using a liquid scintillation analyzer.[i]

Determination of inhibition of proliferation—Mean cpm determined for each set of 4 wells and mean percentage of inhibition of mitogen-induced proliferation determined for each concentration of the 5 drugs were calculated. Percentage inhibition was calculated from the following equation:

$$\text{Percentage inhibition} = (1 - [\mathbf{CPM}_{exp}/\mathbf{CPM}_{stim}]) \times 100$$

where CPM_{exp} = mean CPM determined from 4 replicate wells of whole blood incubated with mitogen and drug, and CPM_{stim} = mean **CPM** of 4 replicate wells of whole blood incubated with mitogen alone (ie, stimulated controls). For each of the 5 drugs with each mitogen, **drug concentration that induced 50% inhibition of [^{3}H]thymidine incorporation (IC_{50})** was calculated after fitting the drug concentration versus percentage of inhibition curve into a pharmacodynamic model.[j]

Determination of lymphocyte viability—Lymphocyte viability was determined after 4 days of culture by use of the trypan blue dye exclusion method.[5] Trypan blue (0.4%) was diluted 1:1 with isotonically buffered saline (0.9% NaCl) solution and filtered immediately before use. Two drops of dilute stain were added to the cell suspension in the fifth of each set of 5 wells (ie, the well without [^{3}H]thymidine) and allowed to stand at room temperature for 2 to 3 minutes. Well contents were microscopically examined, using a hemacytometer, and categorized as viable (nonstained lymphocytes) or nonviable (stained lymphocytes).

Blood was collected from 6 additional cats (cats 11 to 16) and incubated with each mitogen alone (stimulated control) and with the IC_{50} of each immunosuppressive drug determined for that mitogen. The trypan blue dye exclusion method was repeated, 100 cells were viewed with the hemacytometer, and the number of viable cells recorded.

Results

For each drug, there was an obvious dose-response relationship regarding the percentage of inhibition of

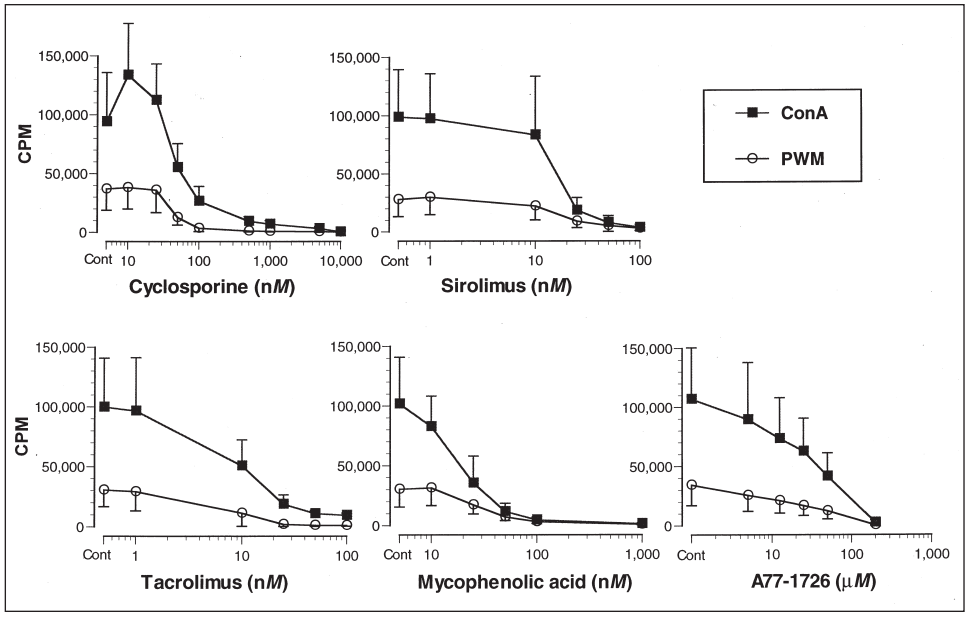

Figure 1—Mean (± SD) incorporation of [^{3}H]thymidine (counts per minute [CPM]) by lymphocytes in whole blood collected from cats and cultured in vitro with concanavalin A or pokeweed mitogen alone (cont) and with various concentrations of cyclosporine (n = 10), tacrolimus (8), sirolimus (8), mycophenolic acid (8), or A771726 (10).

mitogen-induced lymphocyte proliferation (**Fig 1**). Mean IC_{50} determined with ConA was 46 n*M* for cyclosporine, 9 n*M* for tacrolimus, 12 n*M* for sirolimus, 16 n*M* for MPA, and 30 µ*M* for A771726. Mean IC_{50} determined with PWM was 33 n*M* for cyclosporine, 5 n*M* for tacrolimus, 15 n*M* for sirolimus, 14 n*M* for MPA, and 25 µ*M* for A771726.

Results of trypan blue exclusion revealed that most of the lymphocytes were viable in all mitogen-stimulated wells, regardless of drug or concentration of drug evaluated. The mean (± SD) percentage cell viability with ConA stimulation at the IC_{50} of each drug was 94 ± 4% in stimulated controls, 91 ± 4% for cyclosporine, 91 ± 4% for tacrolimus, 90 ± 3% for sirolimus, 90 ± 3% for mycophenolic acid, and 88 ± 5% for A771726, whereas with PWM, the percentage cell viability was 83 ± 11% in stimulated controls, 83 ± 8% for cyclosporine, 80 ± 13% for tacrolimus, 82 ± 8% for sirolimus, 82 ± 11% for mycophenolic acid, and 80 ± 10% for A771726.

Discussion

Several pharmacodynamic assays that measure the in vitro effects of immunosuppressive drugs have been developed. The whole blood mitogen-stimulated lymphocyte proliferation assay is easier, faster, and more economical than assays that use isolated lymphocytes. The former assay also requires only a small amount of blood, which is important when performing multiple assays using blood from small animals such as cats.[5,6] In addition, this assay has better reproducibility and results in better cell viability and may better reflect in vivo immunologic status, compared with assays using isolated lymphocytes.[7,8] Results of preliminary studies in our laboratory indicated that, of the available mitogens for lymphocyte stimulation, ConA, which stimulates T cells, and PWM, which stimulates T and B cells, induce the greatest and most consistent degree of proliferation of feline lymphocytes.

The in vitro effects of cyclosporine on mitogen-stimulated proliferation of lymphocytes in whole blood collected from cats have been described, although the IC_{50} was not reported.[4] Cyclosporine and tacrolimus share a similar mechanism of action; however, tacrolimus is reported to inhibit in vitro proliferation of human and murine lymphocytes 70 to 100 times more potently than cyclosporine.[9] In the present study, tacrolimus was only 5 and 6.8 times more potent than cyclosporine in inhibiting ConA- and PWM-stimulated lymphocyte proliferation, respectively. This difference in relative potency may be important when estimating the optimal dosing regimen and potential therapeutic plasma concentration of tacrolimus for experimental and clinical use in cats.

The results of studies evaluating the immunosuppressive effects of sirolimus, using the whole blood mitogen-stimulated lymphocyte proliferation assay, have been described, using blood from humans, rats, and rabbits.[3] Human lymphocytes were the least sensitive, with an IC_{50} of 38 n*M*, and rabbit lymphocytes were the most sensitive, with an IC_{50} of 6 n*M*. Interestingly, there was a sex difference noticed in rats; lymphocytes from male rats were less sensitive to the inhibitory effects of sirolimus than lymphocytes from female rats (29 vs 3 n*M*).

To our knowledge, the effects of MPA on results of the whole blood mitogen-stimulated lymphocyte proliferation assay have not been described. The IC_{50} of MPA determined using mitogen-stimulated isolated peripheral blood mononuclear cells from humans ranged from 20 to 100 n*M*,[10] and in rats, MPA inhibited lymphocyte proliferation, measured by use of flow cytometry, in whole blood in a dose-dependent manner.[11]

The effects of **malononitriloamide (MNA)** analogs, including A771726, on results of the whole blood mitogen-stimulated lymphocyte proliferation assay have been described, using blood from a number of species including cats.[2] The IC_{50} of A771726 determined for lymphocytes stimulated with ConA differed significantly among species (rats, 7 μ*M*; dogs, 12 μ*M*; humans, 98 μ*M*; rhesus macaques, 141 μ*M*). The IC_{50} that we determined in the present study for lymphocytes stimulated with ConA (30 μ*M*) or PWM (25 μ*M*) were similar to those reported previously for feline lymphocytes (ConA, 48 μ*M*; PWM, 57 μ*M*). In addition, the IC_{50} of the MNA analogs MNA 279 and MNA 715 have been reported in cats; IC_{50} were 67 and 49 μ*M* for ConA-stimulated lymphocytes, respectively, and 134 and 64 μ*M* for PWM-stimulated lymphocytes, respectively.

To our knowledge, the clinical or experimental use of the novel immunosuppressants tacrolimus, sirolimus, MMF, and leflunomide in cats has not been reported. These immunosuppressive drugs may improve efficacy of treatment of immune-mediated inflammatory diseases such as hemolytic anemia, thrombocytopenia, autoimmune and allergic skin disease, immune-mediated arthritis, allergic bronchitis, lymphocytic-plasmocytic enteritis, myasthenia gravis, and systemic lupus erythematosus.

Immunosuppressive treatment regimens combining cyclosporine and prednisolone have allowed for successful renal transplantation in non-major histocompatibility complex-matched cats.[12] Novel immunosuppressive agents may also reduce the incidence of acute and chronic kidney rejection, improve the treatment of ongoing rejection, reduce drug-mediated adverse effects, and improve allograft survival. There are currently no alternative immunosuppressive protocols available for cats that cannot tolerate cyclosporine (eg, cats that develop cyclosporine-mediated hemolytic uremic syndrome).[k] The 4 novel immunosuppressive agents evaluated in our study are candidates for further pharmacokinetic, toxicologic, experimental, and clinical studies.

The whole blood mitogen-stimulated lymphocyte proliferation assay has other potential experimental and clinical applications. The assay can serve as an in vitro assessment of possible in vivo effects of various combinations of immunosuppressive drugs.[3] The assay may also be useful for therapeutic drug monitoring of immunosuppressive drugs. The trough plasma cyclosporine concentration has traditionally been used to adjust the dosing regimen and maintain therapeutic plasma drug concentrations. However, trough plasma cyclosporine concentration does not correlate well with drug exposure, allograft rejection, or nephrotoxicity in human renal transplant recipients.[13] Use of the whole blood mitogen-stimulated lymphocyte proliferation assay for therapeutic drug monitoring may correlate better with therapeutic and toxicologic effects of immunosuppressive drugs and, thus, improve the efficacy and safety of immunosuppressive therapy.

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[a]Sigma Chemical Co, St Louis, Mo.
[b]Vector Laboratories Inc, Burlingame, Calif.
[c]Sandoz, East Hanover, NJ.
[d]Fujisawa USA, Deerfield, Ill.
[e]Wyeth-Ayerst, Princeton, NJ.
[f]Hoechst-Marion-Roussel, Weisbaden, Germany.
[g][^{3}H]thymidine, New England Nuclear, Boston, Mass.
[h]PHD Cell Harvester, Cambridge Technology, Watertown, Mass.
[i]Tricarb 2000 liquid scintillation analyzer, Packard Instrument Co, Downers Grove, Ill.
[j]WinNonlin, Scientific Consulting Inc, Cary, NC.
[k]Aronson LR, Gregory CR. Hemolytic uremic syndrome in two cats following renal transplantation (abstr). *Vet Surg* 1997;26:436.

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