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Influence of Pretreatment with Immunosuppressive Drugs on Viral Proliferation

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Immunosuppressive drugs are used to make the body less likely to reject transplanted organs or to treat autoimmune diseases. In this study, five immunosuppressive drugs including two glucocorticoids (dexamethasone and prednisolone), one calcineurin inhibitor (cyclosporin A), one non-steroid anti-inflammatory drug (aspirin), and one antimetabolite (methotrexate) were tested for their effects on viral proliferation using feline foamy virus (FFV). The five drugs had different cytotoxic effects on the Crandell-Ress feline kidney (CRFK) cells, the natural host cell of FFV. Dexamethasone-pretreated CRFK cells were susceptible to FFV infection, but pretreatment with prednisolone, cyclosporin A, aspirin, and methotrexate showed obvious inhibitory effects on FFV proliferation, by reducing viral production to 29.8−83.8% of that of an untreated control. These results were supported by western blot, which detected viral Gag structural protein in the infected cell lysate. As our results showed a correlation between immunosuppressive drugs and susceptibility to viral infections, it is proposed that immune-compromised individuals who are using immune-suppressive drugs may be especially vulnerable to viral infection originated from pets.

Keywords: Foamy virus, immunosuppressive drugs, viral replication

Introduction

Immunosuppressive drugs inhibit inflammatory activity of the host immune system. Understanding immune mechanisms and discovery of novel and safer immunosuppressive drugs have been of major interest in clinical and immunobiological research, largely because of transplant rejection and autoimmune disorders. Immunosuppressive drugs can be classified into glucocorticoids, cytostatics, antibodies, drugs acting on immunophilins, and other drugs.

Dexamethasone (DEX) is a prominent member of the glucocorticoid family known to induce cell-mediated immunosuppression and lower resistance to bacterial and viral infection in various animal species [1−3]. Prednisolone (PRED) is a synthetic adrenal steroid with appropriately potentiated glucocorticoid activity. PRED has many effects on cytokines, but it downregulates pro-inflammatory cytokines, mainly by inhibition of nuclear factor kappa B (NF-κB) induced transcription of cytokine mRNA. Cyclosporin A (CsA) is a metabolite of the soil fungi *Cylindrocarpon lucidum* and *Polysporium Rafti* [4] and works as a calcineurin inhibitor. CsA strongly inhibits Ca^{2+}-dependent T-cell receptor-mediated signal transduction, leading to IL-2 production. CsA has mainly been used to maintain immunosuppressive therapy in renal transplantation. Aspirin (ASP) is the most commonly used analgesic and anti-inflammatory agent. Many studies suggest that regular use of ASP, long-term or short-term, could reduce cancer incidence and death, as well as decrease the risk of distant metastasis among cancer survivors [5−7]. Methotrexate (MTX), the cytostatic antimetabolite for cancer chemotherapy, has become an important therapeutic alternative in the treatment of severe psoriasis [8] and in the suppression of graft-versus-host rejection after bone marrow transplantation [9] (Fig. 1).

Until quite recently, many studies and clinical cases have reported about the efficacy and adverse effects of anti-inflammatory agents on immunosuppressive therapy. The majority of immunosuppressants result in increased susceptibility to pathogens such as bacteria, fungi, and

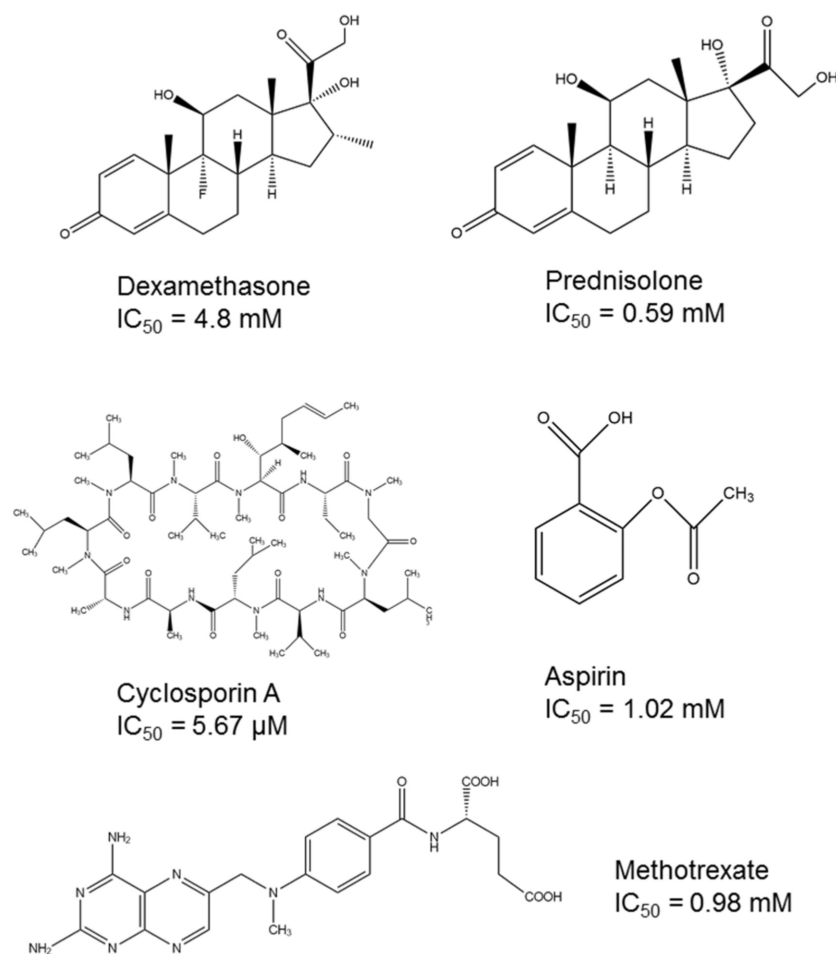


Fig. 1. Chemical structures of five immunosuppressive drugs and their 50% inhibitory concentration (IC_{50}) for in vitro cell growth.

viruses. There are also some studies about inhibition of viral replication and reactivation with immunosuppressive drugs, but they deal only with such virus types as hepatitis C virus, hepatitis B virus and human immunodeficiency virus (HIV) [10−12].

Feline foamy virus (FFV) belongs to the subfamily *Spumaretrovirinae*, within the *Retroviridae* family. FFV replicates well in feline kidney cells as other retroviruses do, by inserting viral DNA into the host chromosome [13]. There has been controversy on whether FFV is nonpathogenic as the virus is generally asymptomatic in infected cats and does not cause severe disease.

In this study however, we are focusing on investigation drug-induced viral infection. Recently, more and more people are keeping cats as companion animals. There is an increasing possibility for immune-compromised patients taking immune-suppressive drugs to be infected by foamy viruses through opportunistic infection. Therefore it is very important to understand viral infectivity in the cells treated with immune-suppressive drugs. Here, FFV proliferation and infectivity were studied in Crandell-Ress feline kidney (CRFK) cells pretreated with five different immune-suppressive drugs, respectively. FFV viral replication and viral protein synthesis are shown to be affected in a dose- and pretreatment time-dependent manner in cell systems in vitro.

Materials and Methods

Immunosuppressive Drugs and Cell Culture

The immunosuppressive drugs used for the study were purchased from Sigma-Aldrich (USA). Dexamethasone and methotrexate were dissolved in distilled water. Prednisolone, cyclosporin A, and aspirin were dissolved in 20% dimethyl sulfoxide (DMSO). The CRFK cell (Korean Cell Line Bank, Korea) was cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal

bovine serum (Gibco Life Technologies, USA), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Cytotoxicity Test

The in vitro cytotoxic effects of immunosuppressive drugs on the cultured cells were measured by MTT colorimetric dye reduction, as described previously [14]. 4×10^4 cells/well were cultured on 96-well plates in the presence of immunosuppressive drugs from 1 nM to 10 mM, as a final concentration. After 48 h, 50 μl of 0.1 mg/ml MTT was supplemented and incubated for 4 h. After discarding the whole solution, cells were digested by DMSO and incubated at 37°C for 10 min. The absorbance was measured at 595 nm using a microplate reader. Three independent experiments were conducted in duplicate at different time points.

Viral Production and FeFAB Assay for Viral Titer Determination

FFV was produced in CRFK cells transfected with pCF7 DNA (FFV molecular clone; a kind gift from Dr. Martin Löchelt, Germany). Then, we used the FFV for infection in the viral proliferation experiments, where immunosuppressive drugs were pretreated for 2 h. Viral titer was evaluated by FeFAB assay. The FeFAB cell line (also by gift of Dr. Martin Löchelt, Germany) derived from the CRFK-carrying FFV LTR-β-galactosidase reporter gene was maintained with 100 μg/ml G418 (Invitrogen, USA). Approximately 2×10^5 FeFAB cells were infected with FFV in 48-well culture plates. At 48 h post infection, cells were fixed with 1% formaldehyde and 0.2% glutaraldehyde in PBS. The fixed FeFAB cells were incubated for 4 h with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) staining solution, and the blue cells were counted using an inverted microscope.

Production of Specific Antibody Against FFV-Gag Protein

Specific anti-FFV-Gag antisera was prepared by immunizing rabbits with a synthetic peptide; 15 amino acids (GPPGPNPYR RFGDGG) represent residue from 431 to residue 445 in the FFV-Gag polypeptide.

Western Blot Analysis

Non-infected and infected CRFK cells were lysed in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% SDS, and 0.5% sodium deoxycholate, and protein concentration was measured using the Bradford assay. The collected protein of 10 μg was loaded onto SDS-PAGE and separated at 110 V for 1.5 h. Proteins were then transferred to nitrocellulose membranes (GE Healthcare UK Ltd., England) at 40 V for 1.5 h using semi-dry transfer (Hoefer, Inc., USA). The membranes were blocked for 16 h at 4°C with blocking buffer PBST [5% (w/v) non-fat dry milk, 0.1% (w/v) Tween 20 in PBS]. The membranes were then probed with the in-house rabbit polyclonal antibody against FFV-Gag protein (1:1,000 dilution) in PBST solution for 1 h. After washing with PBST, the membranes were incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (1:10,000 dilution, Sigma-Aldrich, USA) in PBST for 1 h at room temperature. The membranes were washed three times with PBST and developed with a chemiluminescence detection kit (Bionote, Korea). As an internal control, β-actin was probed with a mouse monoclonal antibody against β-actin (1:5,000 dilution, ThermoScientific, USA) and then with goat anti-mouse IgG conjugated to horseradish peroxidase.

Statistical Analysis

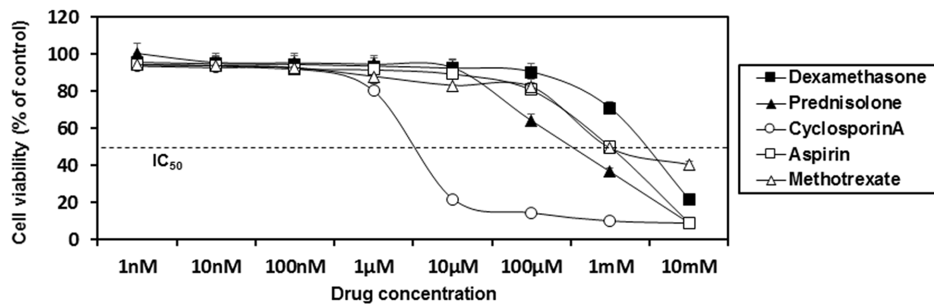
All data are expressed as mean ± SEM. Statistical significance was analyzed with a two-paired Student's t test. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Results and Discussion

First, we investigated the cytotoxicity of the five immunosuppressive drugs; DEX, PRED, CsA, ASP, and MTX on CRFK cells. Cells were incubated with drugs at final concentrations from 1 nM to 10 mM with 10-fold dilution for 48 h. Cell growth was measured by MTT assay. Compared to untreated control cells, all drug-treated cells showed more than 90% cell growth compared to the control from 1 nM to 100 nM final concentration. But cell viability was reduced in a dose-dependent manner. DEX was less toxic to CRFK cells but PRED and CsA reduced cell viability to 64.3% and 14.3% of the control in the 100 μM final concentration, respectively. We selected three drug concentrations for each drug at amounts not toxic to cell culture for 48 h. Therefore 1, 10, and 100 μM for DEX; 100 nM, 1 μM, and 10 μM for PRED; 1, 10, and 100 nM for CsA and MTX; and 10 nM, 100 nM and 1 μM for ASP were selected. The selected concentrations were marked with a gray box in the table of Fig. 2.

Next, in order to study the correlation between drug treatment and viral proliferation, we investigated the optimal time of drug pretreatment. The CRFK cells cultured in the 60-mm culture plate were treated with 5 μM DEX for 0, 2, 4, 6, and 24 h, and then infected with FFV of 1 multiplicity of infection (MOI) at the same time. Based on infection time, 2 h pretreatment was the optimal condition for comparing the effects of the immunosuppressive drugs (Fig. 3).

In further experiments, the CRFK cells were treated with each of three different drug concentrations for 2 h, and then were infected with FFV of 1 MOI. At 48 h post-infection, the culture supernatants were collected, and then the viral titers were measured using the FeFAB assay. FeFAB assay indicated that the culture supernatants collected from the infected and drug-pretreated cells contained infectious FFV virions, which induce expression of the β-galactosidase in the indicator cells (Fig. 4A). Mock infected CRFK cells were used as a negative control for FeFAB assay, and untreated cells were used as a control for the virus titer. Microscopic



	1nM	10nM	100nM	1µM	10µM	100µM	1mM	10mM
Dexamethasone	95.4	94.7	94.3	93.4	92.3	90.3	70.8	21.9
Prednisolone	100.5	95.7	95.4	94.7	92.9	64.3	36.7	9.1
Cyclosporin A	93.8	92.9	92.4	80.3	21.9	14.3	9.9	8.7
Aspirin	94.5	93.9	92.1	91.7	89.4	80.7	50.1	9.2
Methotrexate	94.1	93.7	92.6	87.8	83.0	82.3	49.3	40.5

Fig. 2. Effects of five immunosuppressive drugs on cell growth.
CRFK cells were grown on 96-well culture plates in the presence of the five immunosuppressive drugs from 1 nM to 10 mM as a final concentration. After 48 h, the cell growth was detected using MTT and absorbance is presented as percentages of control, with no drug treatment. Closed squares; dexamethasone, closed triangles; prednisolone, open circles; cyclosporin A, open squares; aspirin, open triangles; methotrexate. Based on dose-response curves, the IC_{50} for DEX, PRED, CsA, ASP, and MTX was approximately 4.8 mM, 0.59 mM, 5.67 μM, 1.02 mM, and 0.98 mM respectively, as shown in Fig. 1.

pictures of the infected cells showed differences in the blue-cell numbers that depended on the immunosuppressive drug type and concentration used. DEX pretreatment

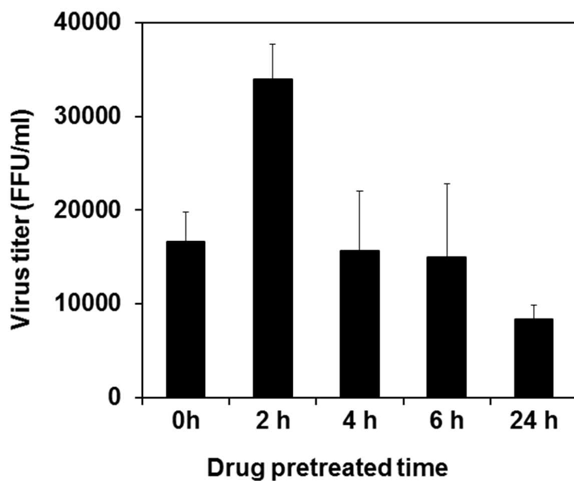


Fig. 3. Virus titer of time-dependent DEX pretreatment.
CRFK cells were pretreated with DEX at a final concentration of 5 μM for 0, 2, 4, 6, and 24 h, and then infected with FFV of 1 MOI. Culture supernatants at 48 h post-infection were used to infect FeFAB cells to study virus titers. Data represent the mean ± SEM from the triplicates of the three independent experiments. Statistical significance was analyzed with a two-paired Student's t test. * : $p < 0.05$, ** : $p < 0.01$, *** : $p < 0.001$.

increased viral proliferation but the other four drugs reduced viral proliferation in a dose-dependent manner. The untreated control showed $(1.24 \pm 0.06) \times 10^6$ blue cells, and pretreatment with 100 μM DEX, 10 μM PRED, 100 nM CsA, 1 μM ASP, and 100 nM MTX resulted in $(1.88 \pm 0.02) \times 10^6$, $(1.04 \pm 0.06) \times 10^6$, $(6.33 \pm 0.30) \times 10^5$, $(3.70 \pm 0.06) \times 10^5$, $(4.06 \pm 0.10) \times 10^5$ blue cells per mL of culture supernatant, respectively, indicating that only DEX pretreatment made CRFK more susceptible to FFV infection (Figs. 4B−4F bar graph).

We investigated whether the level of viral production was correlated with the production of the viral proteins, especially with the viral structural Gag protein, in the cytoplasm of the infected cells. We collected cell lysate from the FFV-infected CRFK cells pretreated with the three different concentrations of each immunosuppressive drug. We analyzed the expression of FFV-Gag protein in the cell lysates using specific anti-FFV-Gag antisera made by immunizing rabbits with 15 synthetic amino acids (Figs. 4B−4F). The FFV Gag proteins were detected with two different-sized bands at 52 kDa (a full length) and at 48 kDa (a cleaved product) [15−17]. The levels of FFV-Gag viral structural protein detected in the cell lysates showed the same disposition with the virus titer, which suggests that the immunosuppressive drugs affect the viral protein synthesis and eventually viral production. Viral production

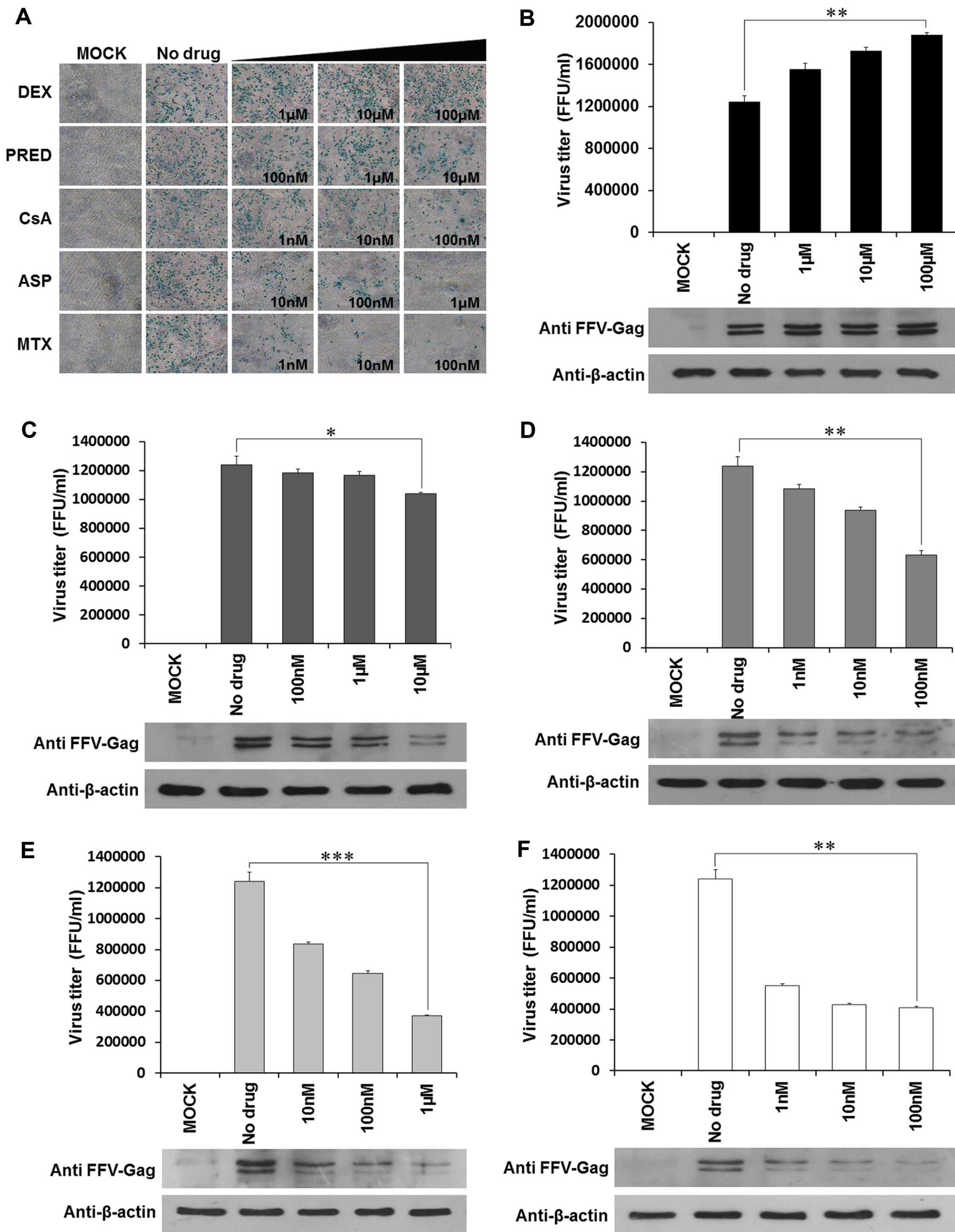


Fig. 4. Effects of five immunosuppressive drugs on FFV viral proliferation and viral protein synthesis.
After pretreatment with the immunosuppressive drugs for 2 h, CRFK cells were infected with FFV of 1 MOI. At 48 h post-infection, culture supernatant was used to infect FeFAB cells for virus titer determination and cell lysates were used for western blot analysis. (**A**) Microscopic observation of FeFAB assay. (**B–F**) Virus titer and western blot analysis of FFV-infected cell lysates. FFV-Gag protein was detected by polyclonal anti-FFV-Gag antisera. β-actin was also detected as a loading control. (**B**) Dexamethasone (**C**) Prednisolone (**D**) Cyclosporin A (**E**) Aspirin (**F**) Methotrexate. MOCK, mock infection; No drug, untreated. Data represent the mean ± SEM from the triplicates of the three independent experiments. Statistical significance was analyzed with a two-paired Student's t test. * : $p < 0.05$, ** : $p < 0.01$, *** : $p < 0.001$.

from DEX-pretreated CRFK cells for 2 h showed a growing trend, but PRED-, CsA-, ASP- and MTX-pretreated CRFK cells showed a decline when the drug concentrations increased 10-fold.

Immunosuppressive drugs have been tested for transplantation medicine in nephrology, ophthalmology, dermatology, gastroenterology, and rheumatology, but their therapeutic potential and toxicity profiles hold a few surprises. In this study, the cytotoxicity of the five immunosuppressive drugs on CRFK cells was evaluated with final concentrations from 1 nM to 10 mM (Fig. 2). DEX had the least cytotoxic effect, having over 90% cell viability to 100 μm. CsA and MTX inhibited cell proliferation at higher concentration and especially, PRED and CsA showed a rapid decline between 10 μ M and 100 μM, and between 1 μM and 10 μM, respectively. Besides drug-specific toxicity, the main risk of immunosuppressive therapy using anti-inflammatory drugs is infection, including viral infection. DEX and PRED are synthetic glucocorticoids used for suppressing the immune system and inflammation. DEX and PRED are shown on the WHO model list of essential medicines as anti-allergy and anti-anaphylaxis drugs [18]. Both are widely used to treat many conditions including arthritis, asthma, colitis, bronchitis, allergies, and skin problems. We expected similar effects from both DEX and PRED on viral infection with glucocorticoid common mechanisms that suppress inflammation by down-regulating the expression of pro-inflammatory cytokines such as IL-6 and TNF-α, or by up-regulating cytokines such as IL-10, which in turn suppress the production of pro-inflammatory mediators [19, 20]. However, DEX induced FFV proliferation in CRFK, in a dose-dependent manner, but PRED inhibited viral proliferation like the other drugs, CsA, ASP, and MTX (Fig. 4). This controversial observation might result from the properties of the drugs. Glucocorticoids shift the cytokine response from T helper 1 immunity to T helper 2 immunity to suppress inflammation [21]. T helper 1 response supports the activities of macrophages and cytotoxic T-cells of the cellular immune system, whereas the T helper 2 response promotes the actions of the B-cells of the humoral immune system, and they are mutually inhibitory [22−24]. Although the mechanism of glucocorticoid-induced viral proliferation is unclear, anti-inflammatory cytokine expression levels could be different in DEX and PRED pretreated cells, depending on time and drug doses.

Combination therapy with CsA and MTX in severe rheumatoid and psoriatic arthritis might increase the risk of reactivating past infections or acquiring new infections. Delia et al showed that the patients with psoriatic arthritis are exposed to multiple viruses with increased prevalence seropositive compared to the normal population, but they did not develop any virus-related clinical symptoms [25, 26]. Some researches indicates that CsA inhibits the replication of influenza A virus [27]. High-dose CsA inhibited the replication of murine cytomegalovirus (CMV) [28]; post-infection treatment with CsA inhibited the replication of murine CMV and vesicular stomatitis virus. However, in a guinea pig model of CMV infection [29], orally administered CsA (lower doses) prolonged and exacerbated CMV infection more than in controls and corticosteroid-treated animals. Zhang *et al*. [30] found that treatment with CsA increased murine CMV infection and appeared to foster chronic infection. These controversial results suggest that immunosuppressive drugs affect host immunity in different ways.

In summary, our research shows the different influences of pretreatment with immunosuppressive drugs on viral proliferation. Nowadays, more people than ever are raising cats as companion animals. In most cases there is no need for concern about the potential for diseases transmitted by pets. However, there still exists the possibility of infection originating from pets, and such infections may cause serious disease particularly in the immune-compromised individuals who are taking immunosuppressive drugs. We recommend careful monitoring of such immunosuppressive therapy patients with viral serological testing. Further investigations on doses of drugs and times of viral infection after drug pretreatment are needed to better understand the effects of immunosuppressive drugs on the host immune system and viral infections.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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