JMB Papers in Press. First Published online Sep 20, 2018
DOI: 10.4014/jmb.1807.06054

Manuscript Number: JMB18-06054

Title: Influence of pretreatment with immunosuppressive drugs on viral proliferation

Article Type: Research article

Keywords: Foamy virus, immunosuppressive drug, viral replication
Influence of pretreatment with immunosuppressive drugs on viral proliferation

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Brief running title: Immunosuppressive drugs affect viral proliferation
Abstract

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 the immune-compromised persons who are using immune-suppressive drugs may be especially vulnerable to viral infection originated from pets.

Keywords: Foamy virus, Immunosuppressive drug, 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 a 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 Rafii [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
immunosuppressant result in increased susceptibility to pathogens such as bacteria, fungi, and 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 sub-family Spumaretrovirinae, one of 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, we are focusing on investigate on the drug-induced viral infection. Recently cats are companion animals for more people. There is a possibility for the immune-compromised persons, who are 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 the 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 with drug-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 (St. Louis, MO, 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, Seoul, 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 × 10⁴ 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 for 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; kind gift from Dr. Martin Löchelt, Heidelberg, 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 (gift from Dr. Martin Löchelt, Heidelberg, Germany) derived from the CRFK carrying FFV LTR-β-galactosidase reporter gene was maintained with 100 μg/mL G418 (Invitrogen, Carlsbad, CA, USA). Approximately 2 × 10⁵ 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 antiserum was prepared by immunizing rabbits with a synthetic peptide; 15 amino acids (GPPGPNPYRRFGDGG) 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., Buckinghamshire, England) at 40 V for 1.5 h using semi-dry transfer (Hoefer, Inc., Holliston, MA, 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, Waltham, MA, 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 to 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 concentration for each drug at concentrations 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 concentration were marked with a gray box in the table of Figure 2.

Next, in order to study 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 to compare immunosuppressive drug effects (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. After 48 h post-infection, the culture supernatants were collected, and then the viral titers were measured using the
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 pictures of the infected cells showed differences in the blue-cell numbers that depended on the immunosuppressive drug type and concentration used. DEX pretreatment 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 to be more susceptible to FFV infection (Fig. 4B-F 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 concentration 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 (Fig. 4B-F). 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 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 drugs concentration 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, 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 µM 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 [18]. Both drugs are widely used to treat many conditions including arthritis, asthma, colitis, bronchitis, allergies, and skin problems. We expected similar effects of 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 drugs. Glucocorticoids shift the cytokine response form T helper 1 immunity to T helper 2 immunity to suppress inflammation [21]. T helper 1 response support 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 level 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 research indicate 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 dose of immunosuppressive drugs affects host immunity in different ways.

In summary, our research shows that the different influences of pretreatment with immunosuppressive drugs on viral proliferation. Nowadays, more people than ever are raising cats as companion animals. In the most of the cases we do not have feeling of wariness about the potential diseases which may be infected from pets. However there are many diseases or infection originated from pets. Especially the infection derived from pets may cause serious disease in the immune-compromised human or the persons who are taking immunosuppressive drugs. We recommend careful monitoring of immunosuppressive therapy patients with a viral serological test. Further investigations on doses of drugs and times of viral infection after drug pretreatment is needed to better understand the effects of immunosuppressive drugs on the host immune system and viral infections.
Acknowledgement This work was supported by a grant from the National Research Foundation of Korea (NRF) funded by the Korean government (NRF-2015R1D1A1A01059592) to Cha-Gyun Shin, and by the Chung-Ang University Graduate Research Scholarship in 2016 to Ga-Eun Lee.

References


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**Figure legends**

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

**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, which are shown on Fig 1.

**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.

**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.
Dexamethasone
IC$_{50}$ = 4.8 mM

Prednisolone
IC$_{50}$ = 0.59 mM

Cyclosporin A
IC$_{50}$ = 5.67 µM

Aspirin
IC$_{50}$ = 1.02 mM

Methotrexate
IC$_{50}$ = 0.98 mM

Fig. Fig 1.
Fig. 2.

![Graph showing drug concentration vs. cell viability (% of control).]

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Fig. 3.
Fig. 4.