Mycophenolic acid inhibits replication of Type 2 Winnipeg, a cerebrospinal fluid-derived reovirus isolate

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BACKGROUND: The role of reoviruses in human disease is uncertain. Most identified cases are sporadic and asymptomatic or produce minor upper respiratory or gastrointestinal symptoms. In November 1997, a reovirus was isolated from the cerebrospinal fluid of a severe combined immune deficient infant in Winnipeg, Manitoba. RNA characterization and sequencing studies demonstrated this reovirus isolate to be unique. Thus, the virus was named Type 2 Winnipeg (T2W).
OBJECTIVES: Mycophenolic acid (MPA), a drug primarily used as an immunosuppressive agent, was assessed in the capacity to inhibit T2W viral growth.
METHODS: The effects of MPA on viral growth were determined by plaque reduction assays. Cells were treated with different MPA concentrations, infected with T2W and incubated at 37°C for 0 h to 72 h. Virus titres were determined and compared with untreated controls.
RESULTS: Production of infectious T2W progeny decreased more than 99% at 3 µg/mL MPA compared with untreated controls. Inhibition was not caused by cell toxicity because there was no difference in cell viability. The 50% cell toxic dose was 30 µg/mL MPA.
CONCLUSIONS: MPA was able to inhibit viral growth of the novel reovirus T2W. Although MPA is usually used as an immunosuppressive agent, and despite the fact that T2W was isolated from an immunocompromised patient, these results suggest that MPA could have been used as a possible treatment at subimmunosuppressive doses. Animal studies to better define the antiviral and immunosuppressive activities of MPA (and its prodrug mycophenolate mofetil) appear warranted.

Key Words: *Mycophenolic acid; Reovirus; Severe combined immune deficiency*

L'acide mycophénolique inhibe la réplication du Winnipeg de type 2, un isolat de réovirus dérivé du liquide céphalorachidien

HISTORIQUE : Le rôle des réovirus dans la maladie humaine demeure incertain. La plupart des cas dépistés sont sporadiques et asymptomatiques ou produisent des symptômes mineurs des voies respiratoires supérieures ou de l'intestin. En novembre 1997, un réovirus a été isolé dans le liquide céphalorachidien d'un nourrisson atteint d'un déficit immunitaire combiné sévère de Winnipeg, au Manitoba. La caractérisation de l'ARN et des études de séquençage ont démontré que cet isolat de réovirus est unique. C'est pourquoi le virus a été nommé Winnipeg de type 2 (WT2).
OBJECTIFS : La capacité de l'acide mycophénolique (AMP), un médicament surtout utilisé comme immunosuppresseur, à inhiber la croissance virale du WT2 a été évaluée.
MÉTHODOLOGIE : Les effets de l'AMP sur la croissance virale ont été déterminés par titrages de la réduction des plaques. Les cellules ont été traitées à l'aide de diverses concentrations d'AMP, infectées par le WT2 et incubées à 37 °C pendant 0 heure à 72 heures. Les titrages viraux ont été déterminés et comparés à ceux de sujets témoins non traités.
RÉSULTATS : La production d'une descendance de WT2 infectieuse chutait de plus de 99 %, à 3 µg/mL d'AMP, par rapport à celle des sujets témoins non traités. L'inhibition n'était pas imputable à une toxicité cellulaire parce qu'on ne décelait aucune différence dans la viabilité des cellules. La dose cellulaire toxique à 50 % était de 30 µg/mL d'AMP.
CONCLUSIONS : L'AMP a pu inhiber la croissance virale du nouveau réovirus WT2. Bien que l'AMP soit généralement utilisé comme immunosuppresseur, et même si le WT2 a été isolé chez un patient immunocompromis, ces résultats laissent supposer que l'AMP aurait pu être utilisé comme traitement éventuel à des doses sous-immunosuppressives. Des études sur des animaux pour mieux définir les activités antivirales et immunosuppressive de l'AMP (et de sa prodrogue, le myfatil de mycophénolate) semblent justifiées.

Reoviruses are members of the genus *Orthoreovirus* in the family *Reoviridae*. They are nonenveloped viruses with a double-layered protein capsid that surrounds a genome composed of 10 segments of double-stranded RNA (1). Mammalian reoviruses are ubiquitous in nature. They are easily found in the environment – in rivers, stagnant water and sewage (2,3) – and they infect insects, fish, reptiles and a variety of mammalian species, including humans, monkeys, rats and mice (4). The name 'reovirus' (*r*espiratory *e*nteric *o*rphan) was proposed by Sabin (5) to describe a group of viruses typically isolated from human respiratory and gastrointestinal tracts, but not associated with serious disease (hence the orphan designation). Despite the presence of antireovirus antibodies in the majority of the population (6), symptomatic disease appears only rarely. Most identified cases are sporadic in nature, with no seasonal predilection. In fact, most infections appear to be asymptomatic or produce minor upper respiratory or gastrointestinal symptoms indistinguishable from other illnesses seen in infancy or early childhood (4).

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One of the hallmarks of reovirus infection in neonatal mice is central nervous system (CNS) disease; thus, it is not unexpected that there have been isolated reports describing an association between reovirus and human CNS disease. The most persuasive case is a report of a previously healthy three-month-old girl who developed aseptic meningitis (7). The child seroconverted, and serotype 1 reovirus was isolated from the cerebrospinal fluid (CSF) after inoculation onto green monkey kidney cells. There are other less well-documented cases that associate reoviruses with human CNS disease. In some cases, reovirus was isolated only from stool (and not CSF); in these instances, seroconversion was not documented (8,9). This raises the possibility that reovirus infection may have been coincidental rather than causal. In some other cases, reovirus has been isolated from brain tissue (8,10).

In November 1997, a novel reovirus was isolated from the CSF of an eight-week-old infant hospitalized at the Children's Hospital (Winnipeg, Manitoba) for the investigation of congenital immune deficiency. We recently described (11) partial characterization of this new reovirus, named Type 2 Winnipeg (T2W) after the city where it was isolated. Ongoing sequencing studies confirm that T2W is a novel reovirus isolate, and recovery of T2W from CSF suggests that T2W is capable of infecting cells derived from the human CNS.

The study of antireoviral agents is limited because reovirus is not considered to be a significant human pathogen. However, inhibition of in vitro reovirus replication has been achieved with a variety of antiviral compounds, including the inosine monophosphate dehydrogenase (IMPDH) inhibitor, ribavirin (12-14). IMPDH catalyzes an important step in the synthesis of guanine nucleotides. GMP, an essential precursor to DNA, RNA and glycoprotein synthesis, is produced in cells by two pathways. The first pathway, the de novo pathway, involves the stepwise assembly of the purine ring to form inosine monophosphate (IMP). IMP is then converted to xanthosine monophosphate (XMP), a precursor to GMP. The enzyme IMPDH catalyzes the conversion of IMP to XMP. The second pathway, the salvage pathway, forms GMP from guanosine or guanine (15,16). Mycophenolic acid (MPA) is a nonnucleoside inhibitor of IMPDH. It binds to IMPDH and blocks the synthesis of XMP, thereby depleting intracellular guanosine triphosphate pools.

Unfortunately, ribavirin appears to distribute slowly into the CSF over four to seven weeks (17). Thus, although ribavirin was not used, it probably would have been ineffective in treating the infant for the reovirus infection. MPA is another known IMPDH inhibitor capable of penetrating the CNS (18). MPA is the active form of the prodrug mycophenolate mofetil (MMF). MMF is rapidly hydrolyzed by esterases in the intestines into MPA. MMF and MPA have been used primarily as immunosuppressive agents at dosages of 10 μg/mL or greater (19,20), especially in patients receiving organ transplantation, to prevent acute graft rejection (16,19). Previous in vitro studies have shown that MPA inhibits replication of other viruses (21). Also, it has recently been demonstrated that MPA inhibits replication of some laboratory-adapted reovirus strains (22).

Previous work has demonstrated that MPA can inhibit reovirus replication (22) and can inhibit different reovirus strains to different extents, ranging from about 100-fold to more than 1000-fold. In addition, replication of some reovirus strains was not completely abolished (22). Thus, the purpose of the present study was to determine whether MPA also exhibits antiviral activity against the clinical T2W isolate. The extent of any observed inhibition was also examined. The present study shows that MPA had an antiviral effect on T2W viral titres in vitro and the dose-response and kinetics of MPA inhibition. The relationship between MPA antiviral effects and the effective in vivo dosages of MPA and MMF are discussed.

METHODS

Reagents, cells and viruses

MPA and guanosine were purchased commercially (Sigma Chemical Co, USA). Mouse L929 fibroblast cells were cultured in Joklik modified minimum essential media (GIBCO-BRL, USA) supplemented to contain 2.5% fetal calf serum (Intergen, USA), 2.5% VSP neonate bovine serum (Biocell, USA) and 2 mM l-glutamine. T2W was grown in mouse L929 cell monolayers supplemented to contain 100 U/mL of penicillin, 100 μg/mL of streptomycin sulfate and 1 μg/mL of amphotericin B as described (23).

Virus infections and drug treatments

Except where differences are noted, cells were routinely treated with various concentrations of MPA 1 h before being infected. Most of the media was removed and some was saved (as 'preadapted media'). Treated cells were then infected with stocks of T2W at a multiplicity of infection (MOI) of 0.12 plaque forming units (PFU)/cell. A mixture of fresh media and preadapted media (3:1, supplemented to contain the same amount of MPA used during pretreatment) was then added to infected cells incubated at 37°C. The supernatant and the cells were harvested at various times postinfection for virus titration by plaque assay as previously described (23). In some experiments, infected cells were incubated with 50 μg/mL guanosine.

Cell toxicity

L929 cell monolayers were evaluated microscopically for the presence of cytopathic effect due to MPA. The cells were then harvested and counted in a hemocytometer. Cell viability was determined by cell doubling times and Trypan Blue exclusion. Cell viability in the presence of MPA was also examined with the Cell Proliferation Reagent WST-1 (Roche Applied Science, Canada). Briefly, 5×10^3 L929 cells were added to each well of a 96-well plate and incubated with various concentrations of MPA at 37°C. After 24 h, 10 μL of WST-1 reagent was added and the cells were incubated for an additional 4 h. After incubation, cells were thoroughly shaken for 1 min on a shaker and the absorbance of the sample was determined at 440 nm.

RESULTS

MPA inhibited production of infectious T2W progeny in L929 fibroblasts

MPA was assessed for its ability to inhibit T2W replication in mouse L929 fibroblast cells. The dose response of MPA on the production of infectious T2W reovirus progeny was determined for concentrations between 0 μg/mL and 100 μg/mL (Figure 1A). Viral replication was reduced at an MPA concentration greater than 30 ng/mL when cells were infected with T2W at an MOI of 0.12 PFU/cell. These plaque reduction assays demonstrated maximal inhibition at 3 μg/mL MPA with a 184-fold decrease in viral replication (P=0.017). Viral inhibition was not caused by cell toxicity

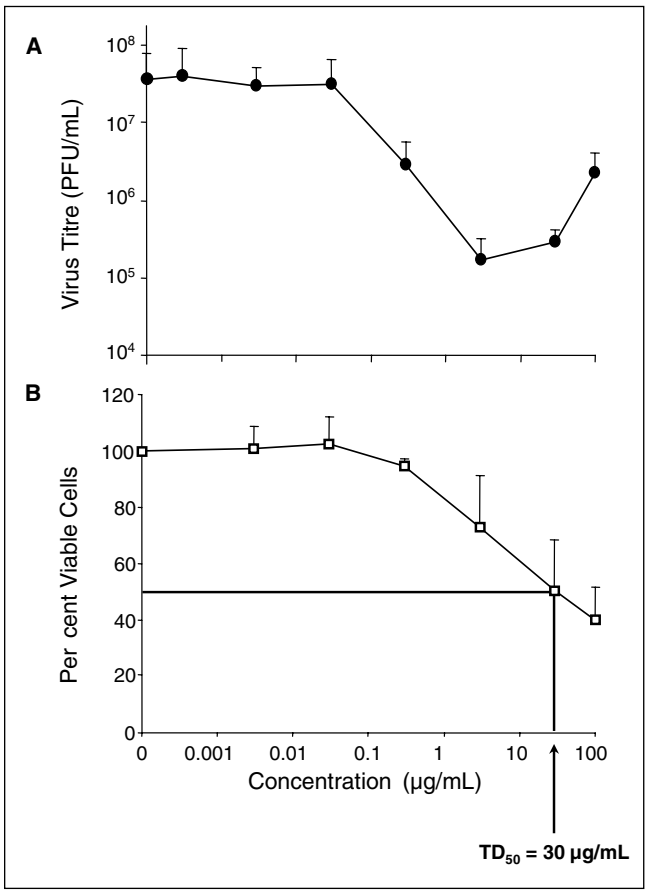

Figure 1) *Effect of mycophenolic acid (MPA) on Type 2 Winnipeg (T2W).* **A** *Effect of MPA on production of infectious T2W reovirus progeny. L929 cells were pretreated with concentrations of MPA ranging from 0 µg/mL to 100 µg/mL for 1 h before infection with T2W at a multiplicity of infection of 0.12 plaque forming units(PFU)/cell. After virus adsorption, cells were overlaid with fresh minimal essential media containing the appropriate concentrations of MPA and incubated at 37°C. Virus was harvested between 65 h and 72 h postinfection and viral titre was determined. The data represent the average of a minimum of two experiments (n=2) and the error bars represent 1 SD.* **B** *Measurement of the cytotoxic effects of MPA on L929 cells. L929 cells were added to a 96-well plate (5×10^3 cells/well) and incubated with various concentrations of MPA for 24 h. Cellular response to MPA was measured with the Roche Cell Proliferation Reagent WST-1 (Roche Applied Science, Canada) and plotted against MPA concentration. The data represent the average of a minimum of five experiments (n=5) and the error bars represent 1 SD. TD_{50} 50% toxic dose*

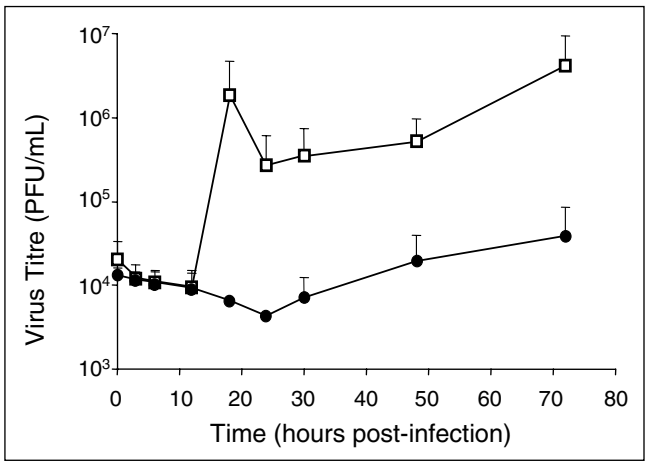

Figure 2) *Production of Type 2 Winnipeg virus progeny over time in the presence of mycophenolic acid (MPA). L929 cells were pretreated with 0 µg/mL or 3 µg/mL MPA for 1 h before infection with Type 2 Winnipeg at a multiplicity of infection of 0.12 plaque forming units (PFU)/cell. After virus adsorption, cells were overlaid with fresh minimal essential media that contained either 0 µg/mL (□)or 3 µg/mL (●) MPA and incubated at 37°C. Virus was harvested at indicated times between 0 h and 72 h postinfection and viral titre was determined. The data represent the average of a minimum of two experiments (n=2) and the error bars represent 1 SD*

because there were only small differences in cell viability, as determined by cell doubling times and Trypan Blue exclusion at MPA doses examined (data not shown); however, cell monolayers began to deteriorate within 24 h after exposure to 100 µg/mL MPA. Use of the more sensitive WST-cell viability assay determined the 50% toxic dose to be 30 µg/mL MPA (Figure 1B). A dose of 100 µg/mL MPA produced a paradoxical effect whereby production of infectious viral progeny was increased relative to lower MPA concentrations (eg, less viral inhibition at higher MPA concentrations). Concentrations of 3 µg/mL MPA were used for subsequent experiments to ensure lower cytotoxicity levels (relative to the levels seen at higher MPA concentrations). This lower concentration ensured that any observed inhibition would be due to the inhibitory effects of MPA on the virus rather than the cytotoxic effects on the cells.

T2W growth is inhibited by MPA for up to 48 h postinfection

A time course study was performed to help characterize the effects of MPA at various times throughout the reovirus infection. Subconfluent L929 cells, pretreated with MPA at 0 µg/mL or 3 µg/mL for 1 h, were infected with T2W at an MOI of 0.12 PFU/cell and harvested between 0 h and 72 h to generate a growth curve (Figure 2). In non-MPA-treated control samples, viral titre increased dramatically after the 12 h viral eclipse phase. In contrast, the titre of T2W in MPA-treated samples continued to decrease to a low at 24 h postinfection (hpi). At this point, viral titre started to increase to a level slightly higher than input titre at 72 hpi, suggesting limited progeny virus growth. The present study showed that MPA does not prevent T2W reovirus infection, though it dramatically reduces viral replication.

Addition of exogenous guanosine rescues T2W viral growth

The mechanism of MPA antiviral activity likely occurs through the depletion of intracellular guanine nucleotide pools (15,16). Therefore, the addition of excess exogenous guanosine to virus infected cells treated with MPA should yield viral titres comparable with untreated controls. L929 cells were

either mock treated or pretreated for 1 h with 3 μg/mL MPA, infected with T2W at an MOI of 0.12 PFU/cell and harvested at 72 hpi. The addition of 50 μg/mL guanosine to infections in the presence of MPA restored viral replication to levels similar to non-MPA-treated control cells with added guanosine (data not shown). These results are in agreement with previous studies (22,24,25).

DISCUSSION

The objective of the present study was to determine whether MPA could have been used for the treatment of T2W CSF infection. Reoviruses have not been definitively shown to produce human disease; thus, there has only been limited investigation of antireoviral therapy. The administration of antiviral antibodies, virus-specific immune cells and protease inhibitors has been tested in experimental animals (26). The activities of a number of antiviral agents against reovirus have been tested in cell culture systems and a number of agents have been reported to have in vitro activity (this has never been tested in vivo). These agents include neplanocin A and some of its derivatives (27-29), ribavirin (12-14), acivicin (30), cicloxolone sodium (31) and cyclopentenylcytosine (32,33). In addition, MPA was reported to inhibit reovirus replication in a previous study, although primary data are not available (34). Some of our prior results also indicate strain-dependent differences in reovirus inhibition (22).

Until recently, the most comprehensive study of antireoviral agents was with done with ribavirin. Rankin et al (14) found that a ribavirin concentration of 3 μg/mL inhibited viral multiplication, single strand RNA formation, double strand RNA formation and protein synthesis by approximately 90%. They proposed a model in which ribavirin triphosphate binds to a site close to the catalytic site of the transcriptase and postulated that the binding inhibited the helicase function of the transcriptase, lowered its affinity for template RNA and increased premature termination (14). Since this early study, ribavirin has been used at higher concentrations (up to 48 μg/mL) in both mammalian and avian reovirus studies to inhibit the production of reovirus RNA and protein (12,13). Despite the known antiviral effects of ribavirin on reovirus, the ability of ribavirin to inhibit T2W replication was not examined in the present study because, as indicated earlier, it appears to distribute slowly into the CSF (17). MPA, another known IMPDH inhibitor capable of penetrating the CNS (18,35), is also capable of inhibiting replication of various viruses (21,24,34,36,37). Therefore, MPA was examined for its utility as an antiviral agent against T2W.

The present study demonstrated that MPA inhibits the production of T2W infectious viral progeny. Maximum viral inhibition was 184-fold, which represents a greater than 99% decrease at a concentration of 3 μg/mL. The inhibitory effect of MPA was not due to cell toxicity and was reversed by the addition of exogenous guanosine. These observations are in agreement with reports of in vitro antiviral activity in other viral systems and consistent with a model where the antiviral effects of MPA are due to depletion of intracellular guanine nucleotide pools (21,22,24,34,36,38,39).

MMF, the morpholinoethyl ester of MPA, has increased bioavailability in humans compared with MPA. After oral administration, MMF is rapidly hydrolyzed by esterases in the intestine and blood to release MPA (40). Both MMF and MPA are typically used as immunosuppressive agents at dosages of 10 μg/mL or greater (17,19,20,34). Thus, we found that MPA inhibited T2W replication at dosages threefold to 30-fold lower than those typically used in the clinical setting. Although the inhibitory effect of MMF on reovirus replication was not examined in the present study, work with herpesvirus has shown a comparable reduction in virus yield between MPA and MMF in Vero cells (41). Work in the Coombs laboratory has also shown that equivalent molar concentrations of MMF and MPA leads to comparable inhibition of other strains of reovirus (22). It is likely that MMF would inhibit T2W reovirus replication to the same extent as MPA.

MPA is able to penetrate the CNS (18,35) and inhibit viral replication of T2W at concentrations below therapeutic immunosuppressive levels; therefore, MMF may have been effective for the treatment of CSF T2W infection. There have been some studies that investigated viral infections (particularly cytomegalovirus infections) in transplant patients receiving MMF. Generally, results indicated that MMF reduces the severity and complications from cytomegalovirus disease (which leads to increased graft survival) but does not decrease the incidence of the disease after transplantation (42-44). However, a study (45) in a pediatric population found that renal transplant recipients had increased susceptibility to varicella infection. This increase in varicella infection was attributed to the enhanced immunosuppression achieved with MMF over other immunosuppressive regimens. It is possible that the use of MPA (or MMF) in the immunocompromised patient from which T2W was originally isolated may have made the infant more susceptible to other infectious agents. However, the concentration of MPA required to inhibit production of T2W infectious viral progeny is significantly less than that required in organ transplant patients; this makes infection at low concentrations of MPA less likely. As such, it is possible that MPA could have been used as a treatment at subimmunosuppressive doses for the CSF reovirus infection seen in the immunocompromised infant. These results also suggest that MMF and/or MPA may be a more effective antiviral than ribavirin (approximately 180-fold inhibition with MPA [the present study] compared with approximately 10-fold inhibition with ribavirin [14] at 3 μg/mL). Also, Diamond et al (24) demonstrated that significantly higher doses of ribavirin than MPA were required to abrogate dengue virus replication. Thus, MPA shows promise as an antiviral agent. To better determine the potential usage of MMF as an in vivo broad-spectrum antiviral agent, additional animal studies – including those that incorporate pharmacodynamics and the assessment of antiviral and immunosuppressive activities – appear warranted.

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