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Mycophenolic acid inhibits avian reovirus replication

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Abstract

Avian reoviruses (ARV) are economically important pathogens, especially in the poultry industry, where they cause viral arthritis and tenosynovitis. Mycophenolic acid (MPA) is an inhibitor of inosine monophosphate dehydrogenase (mainly used clinically for immunosuppression) that inhibits the replication of several viruses. We demonstrate in this study that MPA also is capable of inhibiting ARV replication in QM5 quail fibrosarcoma cells. The selectivity index of MPA in QM5 cells was determined as approximately 41. Concentrations of $\geq$3 μg/ml MPA inhibited infectious ARV progeny production in QM5 cells by more than 100-fold. Inhibition of ARV replication also was seen in other cell lines, including HD-11 and Vero. Addition of exogenous guanosine to MPA-treated ARV-infected QM5 cells restored viral replicative capacity to nearly normal levels.

Keywords: Avian reovirus; Mycophenolic acid; Ribavirin

1. Introduction

Viruses have evolved as remarkably successful intracellular pathogens. Indeed, most viruses employ so much of their hosts' intracellular machinery that it has historically proven difficult to separate virus functions from cellular functions. Thus, many viral diseases are difficult to treat, and, as little as a decade ago, only five drugs were licensed for the treatment of viral infections (De Clercq, 2001a). Presently, because of a greater understanding of several viral life cycles and the elucidation of virus-specific events as targets for antiviral agents, there are more than 30 antiviral drugs approved for treatment of viral infections today and several others are in advanced phase III clinical trials (De Clercq, 2001c). Half of these agents are for the treatment of human immunodeficiency virus, while the remainder are primarily for hepatitis B virus, influenza viruses and herpesviruses (De Clercq, 2002). Unfortunately, we still lack effective therapies for several important viral infections and current treatments are not always well tolerated. These deficiencies highlight the need for further refinement of antiviral drug design and development.

Mycophenolic acid (MPA) is a non-nucleoside non-competitive, reversible inhibitor of eukaryotic inosine monophosphate dehydrogenase (IMPDH). IMPDH catalyzes a rate-limiting step in the de novo biosynthesis of purine mononucleotides and is involved in the early steps of GMP synthesis. It is responsible for the conversion of IMP to XMP which is further converted to GMP, GDP, dGDP, GTP and dGTP (for review, see Allison and Eugui, 2000). IMPDH inhibitors are believed to primarily affect viral RNA and/or DNA synthesis when there is an increased need for synthesis as in the case of virus-infected cells. IMPDH inhibition decreases levels of intracellular guanosine nucleotide pools required for adequate RNA and DNA synthesis. Thus, inhibition of IMPDH with MPA has antiviral (Williams et al., 1968; Sidwell et al., 1972), antiproliferative (Carter et al., 1969), immunosuppressive (Mitsui and Suzuki, 1969), antimicrobial (Mizuno et al., 1974), and antiparasitic (Berman and Webster, 1982; Hupe et al., 1986) effects. Currently, MPA is used clinically in combination with steroids and cyclosporine A to prevent heart and kidney transplant rejection (Sollinger, 1995; Behrend et al., 1997; Kobashigawa et al., 1998). MPA can inhibit the replication of several viruses in vitro (Malinoski and Stollar, 1981; Ichimura and Levy, 1995; Neyts et al., 1998; Gong et al., 1999; Margolis et al., 1999; Diamond et al., 2002; Morrey et al., 2002) and potentiates the inhibitory effects of acyclic guanosine analogs (such as acyclovir, penciclovir and ganciclovir) against herpesviruses (Neyts and De Clercq, 1998; Neyts et al., 1998). MPA also potentiates the activity of nucleoside analogs against human immunodeficiency virus (Heredia et al., 1999; Margolis et al., 1999; Hossain et al., 2002), which is thought to occur by an enhancement of antiviral activity caused by depletion of normal

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dGTP substrate pools. This dGTP depletion decreases the competition that nucleoside analogs experience from the normal substrate during the DNA polymerization reaction. Thus, incorporation of the analog and chain termination are increased (De Clercq, 2001b; De Clercq et al., 2001). Despite some progress made in the understanding of antiviral properties of MPA, the potential application of its use as a broad-spectrum antiviral agent against both positive- and negative-stranded RNA viruses has not been fully realized.

In an effort to further delineate the antiviral mechanism and role of MPA, we examined the effects of MPA on avian reovirus (ARV). ARV are members of the genus *Orthoreovirus* in the family *Reoviridae*. These viruses possess double-stranded (ds) segmented RNA genomes encased in a non-enveloped icosahedral shell consisting of two or more concentric protein capsid layers (for review, see Nibert and Schiff, 2001). The ARV differ from the better studied mammalian reoviruses (MRV; the prototypic *Orthoreoviruses*) because the ARV lack hemagglutinin, but are able to induce cell fusion. Although the prototypic MRV is not pathogenic in humans, many of the viruses in the *Reoviridae* family cause significant disease in animals. These include rotavirus (Keating, 1999; Estes, 2001), Bluetongue virus (Roy, 2001), and ARV, the last of which causes viral arthritis/tenosynovitis (leg weakness) and other disease conditions in chickens, making ARV a major cause of economic loss in the poultry industry.

Few studies have examined the effects of antiviral compounds on these double-stranded RNA viruses. Four classes of antiviral agents shown to be effective against MRV are the *S*-adenosylhomocysteine hydrolase inhibitors (e.g. Neplanocin A) (De Clercq et al., 1989; Robins et al., 1998), the orotidylic acid carboxylase inhibitors (e.g. pyrazofurin) (De Clercq, 1993), the CTP synthetase inhibitors (e.g. cyclopentylcytosine) (De Clercq et al., 1990), and the IMPDH inhibitors (e.g. Ribavirin, mycophenolic acid) (Rankin et al., 1989; Gong et al., 1999). In addition, foscarnet, which binds to the pyrophosphate cleavage site of the polymerase, has been shown to have an antiviral effect on the related rotaviruses (Rios et al., 1995). For this study, we confirmed that ribavirin inhibits ARV replication. We then performed a more in-depth analysis on mycophenolic acid and report here that MPA inhibits replication of ARV in several cell lines when used at dosages less than those used clinically for immunosuppression (Sollinger, 1995; Behrend et al., 1997; Kobashigawa et al., 1998; Gong et al., 1999; McEvoy, 2003a), and that addition of exogenous guanosine nearly completely reverses this inhibition.

2. Materials and methods

2.1. Reagents, cells and viruses

Ribavirin, MPA and guanosine were purchased commercially (Sigma Chemical Co.). The continuous quail fibrosacroma cell line QM5 was cultured in Media 199 (1× M199) (Gibco-Invitrogen) supplemented to contain 10% fetal calf serum (Gibco), 10% tryptose phosphate broth (2% tryptone, 0.2% dextrose, 8.6 mM NaCl, 1.8 mM Na_2HPO_4) and 2 mM L-glutamine at 5% CO_2 and 37 °C. HD11 cells (kindly provided by Dr. Roy Duncan, Dalhousie University) were maintained as described (O'Hara et al., 2001) and Vero (African Green monkey kidney) cells were cultured in Dulbecco modified MEM (Gibco) supplemented to contain 10% FCS, 2 mM L-glutamine, and 3.5 g/l D-glucose as described (Keirstead and Coombs, 1998). Confluent cell monolayers were passaged by trypsinization and subculturing at 1:6–1:8 ratios twice weekly.

ARV strain 138 (ARV 138) and ARV strain 176 (ARV 176) were amplified in QM5 cell monolayers supplemented to contain 100 U of penicillin per ml, 100 μg/ml of streptomycin sulfate and 1 μg/ml of amphotericin B as described (O'Hara et al., 2001).

2.2. Virus infections and drug treatments

Except where differences are noted, cells were routinely treated with various concentrations of ribavirin or mycophenolic acid (MPA) 1 h prior to infection. Media was removed and treated cells then were infected with stocks of ARV 138 or ARV 176 at various multiplicities of infection (MOI). A mixture of fresh media and pre-adapted media (3:1; supplemented to contain the same amount of ribavirin or MPA as used during pre-treatment) was then added to infected cells which were incubated at 37 °C. Supernatants and cells were harvested at various times post-infection (hpi) for virus titration by plaque assay as described (O'Hara et al., 2001). In time-course studies, cells were treated with drug at various times prior to, during, or post-ARV exposure and virus was harvested at 70 hpi. In some experiments, infected cells were incubated with 50 μg/ml guanosine.

3. Results

3.1. Ribavirin inhibits replication of avian reovirus

Ribavirin has been used to inhibit replication of several viruses, including mammalian reovirus (Rankin et al., 1989; Connolly and Dermody, 2002). A dosage of 200 μM (50 μg/ml) ribavirin has been used to inhibit MRV replication, so initial studies of the effect of ribavirin on ARV replication used this same dose (Fig. 1). In the absence of ribavirin (filled symbols), QM5 cells infected with either ARV 138 or ARV 176 at an MOI of 1 PFU/cell replicated and produced up to 100-fold the amount of input virus by 30-h post-infection (hpi). However, treatment of QM5 cells with 200 μM ribavirin (open symbols) prior to and during infection led to no observable increase in infectious progeny above input virus. Thus, this dose of ribavirin inhibited the growth of ARV by between 1 and 2 orders of magnitude,

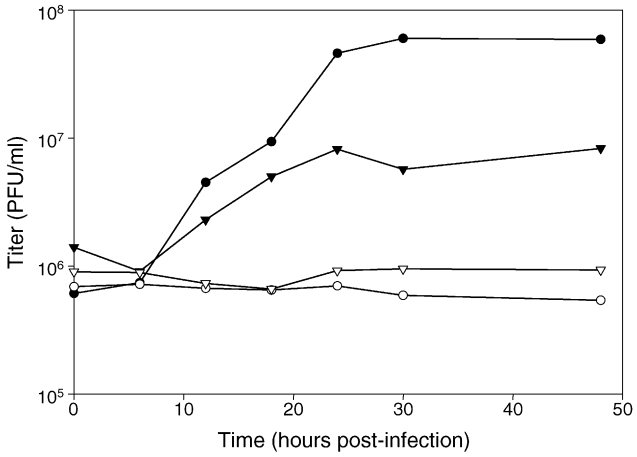

Fig. 1. Avian reovirus production in the presence of ribavirin. QM5 cells were infected with ARV 138 and ARV 176 at an MOI of 1 PFU/cell. After virus adsorption, cells were overlaid with 1× M199 that contained 0 or 200 μM ribavirin and incubated at 37 °C. Virus was harvested at indicated times post-infection and viral titer was determined. (●) ARV 138 0 μM ribavirin; (○) ARV 138 200 μM ribavirin; (▼) ARV 176 0 μM ribavirin; (▽) ARV 176 200 μM ribavirin. Results represent a single experiment.

consistent with recent reports that examined viral and cellular RNA and protein synthesis (Bodelon et al., 2002; Labrada et al., 2002).

3.2. Mycophenolic acid (MPA) also inhibits replication of avian reovirus

As indicated above, previous work has demonstrated anti-ARV properties of ribavirin. However, ribavirin appears to distribute slowly into some tissues (McEvoy, 2003b). Thus, we sought additional antiviral compounds that might be useful in treating this economically important virus. We focused our subsequent work on mycophenolic acid, which like ribavirin, is an IMPDH inhibitor, and which may have better tissue distribution. MPA has been reported to affect mammalian reovirus strain T1L replication (Gong et al., 1999; Diamond et al., 2002), although the data and dosages used are not readily available. The first step in characterizing MPA was to determine an effective dose capable of inhibiting ARV replication. In addition, preliminary studies (subsequently completed and described below) suggested that antiviral effects were greatest at MOIs less than 1 PFU/cell.

Concentrations of MPA ≤0.03 μg/ml had no significant effect upon the replication of either ARV 138 or ARV 176 when QM5 cells were infected at a low MOI of 0.14 PFU/cell (Fig. 2A). Virus replication was affected by higher doses of MPA. A dose of 0.3 μg/ml MPA led to an approximately two to fivefold decrease in the amount of detectable infectious progeny. Higher concentrations of MPA (>0.3 μg/ml) led to decreased virus production for both strains. These plaque reduction assays demonstrated decreased production of infectious viral progeny was maximal at MPA concentrations above 3 μg/ml, where inhibition ranged from about 100- to almost 1000-fold compared to untreated controls. MPA was capable of inhibiting the replication of both tested strains of avian reovirus, ARV 138 and ARV 176 (Fig. 2A). Concomitant with decreased viral replication, addition of 0.1 μg/ml or more MPA led to reduction in cytopathic effect (CPE) in viral-infected QM5 cells (Fig. 2A). The dose of MPA required to reduce CPE by 50% (CPE_{50}; as determined by direct microscopic observation of infected monolayers) was determined as approximately 0.125 μg/ml (Fig. 2A). The toxicity of MPA on QM5 cells also was determined, using the WST-1 assay, and the dose of MPA that induced 50% cell toxicity (TD_{50}) was determined as approximately 5.1 μg/ml (Fig. 2B). Thus, the selectivity index of MPA in QM5 cells (ratio of TD_{50} to CPE_{50}) was calculated as 40.8. For most subsequent experiments, we routinely used a dosage of 3 μg/ml MPA, which showed near-maximal viral inhibition, is below the TD_{50}, and which is lower than the typically used dose of ≥10 μg/ml for clinical immunosuppresion (Allison and Eugui, 1993; Gong et al., 1999; McEvoy, 2003a).

3.3. Inhibition of ARV by MPA is dependent upon MOI and time of treatment

In a natural setting, organisms usually are initially exposed to a relatively small inoculum of infectious agent. If the infectious agent is capable of replicating in the organism, other cells may then be exposed to significantly larger quantity of infectious agent. Thus, to more fully characterize conditions that may affect the antiviral activity of MPA, we determined the amount of infectious progeny virus produced when cells were treated with 3 μg/ml MPA at various times pre- and post-infection and infected with various amounts of virus. Maximal inhibition was seen when MPA was added prior to infection and cells were infected with low MOIs of either ARV 138 (Fig. 3A) or ARV 176 (Fig. 3B). There was very little inhibition if cells were infected with higher MOIs of virus or if the drug was added later than 12-h post-infection. Thus, for subsequent experiments we routinely pre-treated cells for 1-h with MPA, infected at MOIs less than 0.2 PFU/cell, and maintained the cells in media supplemented with MPA for the duration of incubation.

3.4. Addition of exogenous guanosine rescues viral growth

MPA's antiviral activity probably occurs through intracellular guanosine nucleotide pool depletion (Allison and Eugui, 2000; Sintchak and Nimmesgern, 2000). Therefore, addition of excess exogenous guanosine to virus-infected cells treated with MPA should yield viral titers comparable to untreated controls. QM5 cells were mock-treated or pre-treated for 1-h with 3 μg/ml MPA, infected with ARV 138 or ARV 176, and guanosine added at a concentration of 0 or 50 μg/ml. Infections were harvested at 70 hpi and titered. Addition of guanosine at a concentration of 50 μg/ml to infections in the presence of MPA restored viral replication to near-normal levels (Fig. 4). These results are in agreement

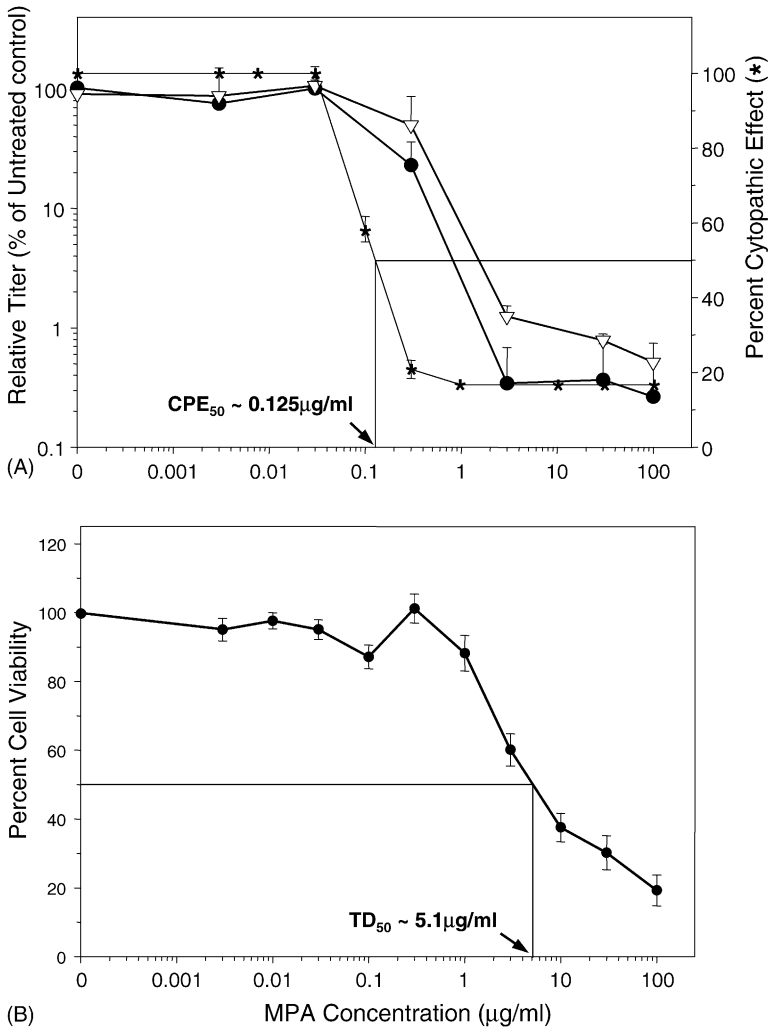

Fig. 2. Effect of MPA on production of infectious avian reovirus progeny in QM5 cells. QM5 cells were pre-treated with the indicated concentrations of MPA for 1-h before infection with ARV 138 (●) and ARV 176 (▽) at an MOI of 0.14 PFU/cell. After virus adsorption, cells were overlaid with 1× M199 that contained the indicated concentrations of MPA and incubated at 37 °C. Virus was harvested at 70-h post-infection and viral titer was determined. Results are displayed as a relative titer, with infectious progeny virus produced at each MPA concentration expressed as a proportion of virus produced in the untreated control (0 μg/ml MPA). The data represents the average of a minimum of two experiments and the error bars represent one standard deviation.

with some previous studies (Neyts and De Clercq, 1998; Diamond et al., 2002).

The role of MPA as an IMPDH inhibitor, as well as rescue of progeny infectious production by addition of guanosine, strongly suggests that viral RNA production is inhibited, as seen with ribavirin (Bodelon et al., 2002; Labrada et al., 2002). To directly test this possibility, ARV 138 and ARV 176 infections that had been pre-treated with 0 or 3 μg/ml MPA were incubated for various periods of time, then viral RNA was extracted and resolved by gel electrophoresis. Addition of 3 μg/ml MPA reduced the amount of viral RNA to undetectable levels (Fig. 5).

3.5. Addition of MPA abolishes ARV replication

Viral growth curves in the presence and absence of MPA were performed to better characterize the effects of antiviral compound has on ARV replication (Fig. 6). Untreated samples showed a decline in virus titer at 6 hpi (corresponding to the eclipse phase) and rise in titer by 12 hpi, which peaked by 24 hpi. In contrast, MPA-treated infections showed moderate declines in virus titer during the first 12 hpi with no increase in titer over input virus throughout the course of the experiment, indicating the drug completely inhibited replication of both strains of ARV.

3.6. MPA inhibits production of infectious reovirus progeny in other cell lines

Since the sensitivity of virus/cell culture systems to ribavirin, another IMPDH inhibitor, is often dependent on the host cell line being used (Richman et al., 1987; Rankin et al., 1989), and because, in a natural setting, viruses often may replicate in a variety of tissues, we examined the effects of MPA on the production of infectious ARV progeny in two other cell lines, HD-11 and Vero. Similar to what was

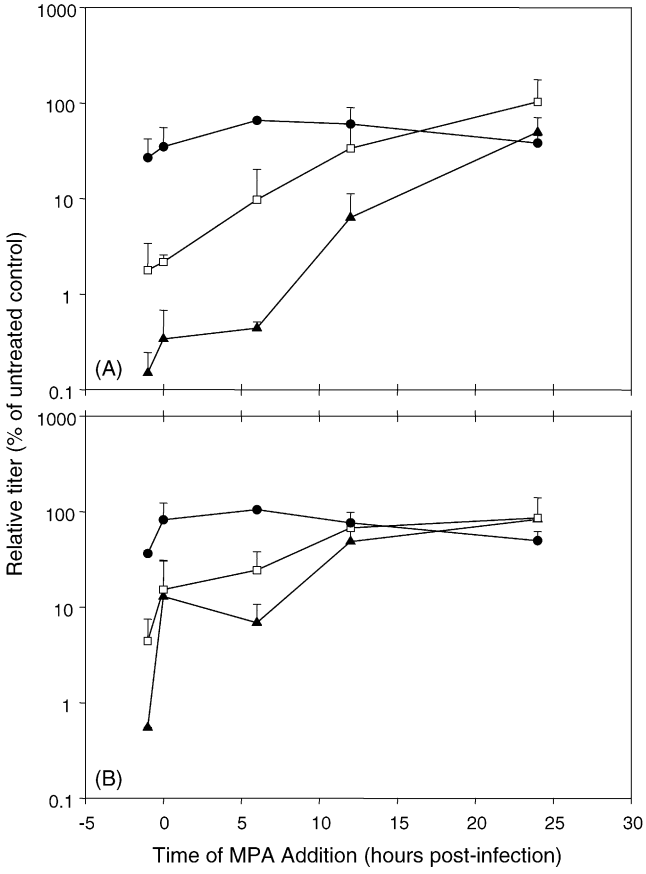

Fig. 3. Effects of multiplicity of infection and time of addition of MPA on inhibition of infectious progeny. QM5 cells were treated with 3 μg/ml MPA at the indicated times prior to, or during, infection with ARV 138 (A) or ARV 176 (B) at multiplicities of infection of 0.14 (▲), 1.4 (□), or 14 (●) PFU per cell. Virus was harvested at 70-h post-infection and viral titer was determined. Results are displayed as a relative titer compared to progeny virus production in untreated control. The data represents the average of a minimum of two experiments and the error bars represent one standard deviation.

found in QM5 cells, concentrations of ≤0.03 μg/ml MPA had no significant effect on either ARV 138 or ARV 176 virus production, MPA concentration of 0.3 μg/ml resulted in slight decreases in progeny virus production, and higher MPA doses inhibited viral growth slightly less than 100-fold in both HD-11 and Vero cells (data not shown).

4. Discussion

Mycophenolic acid has been shown to interfere with replication of a number of viruses, including Sindbis virus (Malinoski and Stollar, 1981), HIV (Ichimura and Levy, 1995), herpesvirus (Neyts et al., 1998), hepatitis B virus (Gong et al., 1999), orthopoxviruses (Smee et al., 2001), dengue virus (Diamond et al., 2002), and West Nile virus (Morrey et al., 2002). This study demonstrates that MPA is also capable of inhibiting replication of the double-stranded RNA avian reoviruses. Inhibition of ARV replication by

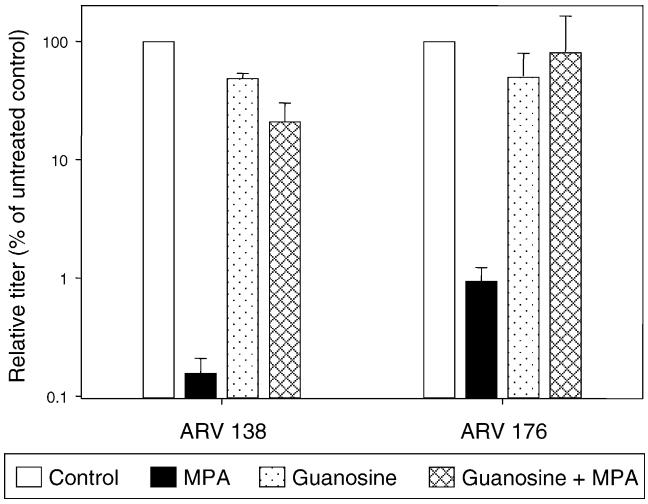

Fig. 4. Effect of exogenous guanosine on infectious virus progeny production in the presence of MPA. QM5 cells were pre-treated with MPA for 1-h before infection with ARV 138 or ARV 176 at an MOI of 0.14 PFU/cell. After virus adsorption cells were overlaid with 1× M199 that contained either no MPA and no guanosine, 3 μg/ml MPA only, 50 μg/ml guanosine only, or a combination of MPA plus guanosine. Virus was harvested 70-h post-infection and viral titer was determined. The data represents the average of a minimum of two experiments and the error bars represent one standard deviation.

MPA appears to be virtually complete, as seen by lack of detectable rise in titer in the presence of 3 μg/ml MPA during a 70-h incubation period (Fig. 6) and as seen by inability to detect progeny RNA (Fig. 5).

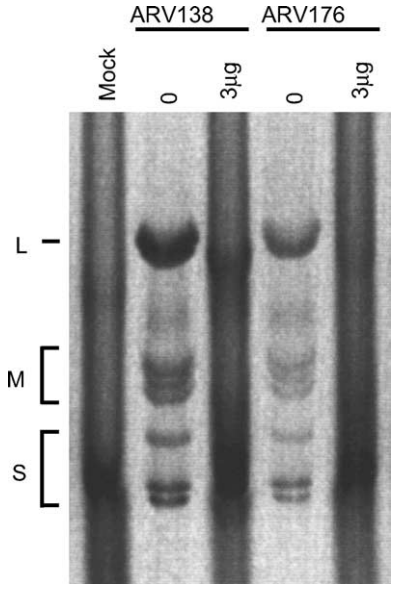

Fig. 5. Effect of MPA on viral RNA production. Agarose gel analysis of viral RNA. Pre-treated QM5 cells were either mock-infected (Mock) or infected with ARV 138 or ARV 176 in the presence of 0 or 3 μg/ml MPA and incubated at 37 °C as detailed in Section 2. Viral dsRNA was purified from infected cells at 24-h post-infection and agarose gels were run at 125 V for 2-h. The location of the L, M and S gene segments are indicated. Results from 12 or 48-h incubation were similar to the 24-h results (data not shown).

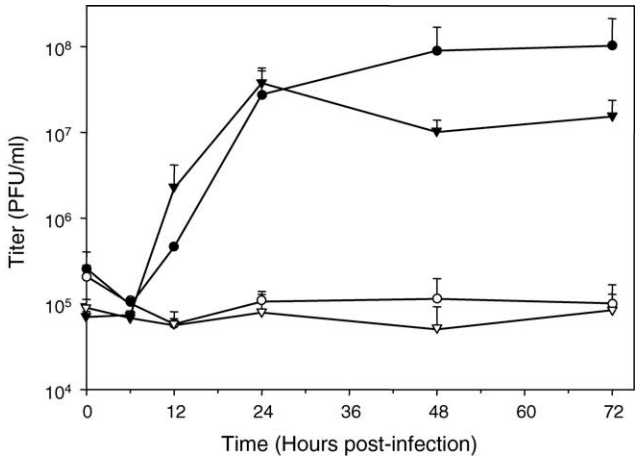

Fig. 6. Avian reovirus production in the presence of MPA. QM5 cells were infected with ARV 138 and ARV 176 at an MOI of 0.14 PFU/cell. After virus adsorption, cells were overlaid with 1× M199 that contained 0 or 3 μg/ml MPA and incubated at 37 °C. Virus was harvested at indicated times post-infection and viral titer was determined. The data represents the average of a minimum of two experiments and the error bars represent one standard deviation. (●) ARV 138 0 μg/ml MPA; (○) ARV 138 3 μg/ml MPA; (▼) ARV 176 0 μg/ml MPA; (▽) ARV 176 3 μg/ml MPA.

MPA appears to exert its antiviral effects through intracellular guanosine nucleotide pool depletion, consistent with its known role as a non-nucleoside non-competitive, reversible inhibitor of eukaryotic IMP dehydrogenase, an enzyme that catalyzes the rate-limiting step in the de novo biosynthesis of purine mononucleotides (for review, see Allison and Eugui, 2000). Thus, depletion of guanosine nucleotides would be expected to seriously impair viral RNA synthesis and addition of excess exogenous guanosine would be expected to restore capacity for ARV to replicate in MPA-treated cells (Fig. 4). Similar modes of MPA's antiviral effects have been reported for some other viruses (Williams et al., 1968; Ichimura and Levy, 1995; Gong et al., 1999; Smee et al., 2001; Diamond et al., 2002; Morrey et al., 2002).

Ribavirin is another IMPDH inhibitor, which, in its monophosphate form, has been shown to effectively inhibit growth of a number of viruses, including mammalian (Rankin et al., 1989; Connolly and Dermody, 2002) and avian reovirus (Bodelon et al., 2002; Labrada et al., 2002; see also Fig. 1). Since both ribavirin and MPA are proposed to exert their antiviral effects through the depletion of guanosine nucleotide pools, it is interesting to compare the effects of the two drugs. MPA and ribavirin had similar effects on avian reovirus infectious viral progeny production (compare Figs. 1 and 6). The production of infectious viral progeny was virtually halted in the presence of both agents. However, a much greater concentration of ribavirin (200 μM; =49 μg/ml) was needed in comparison to MPA (3 μg/ml; =9.3 μM) to bring about this virtually complete antiviral activity. Similarly, results obtained with dengue virus showed higher levels of ribavirin than MPA are needed to abrogate virus replication (Diamond et al., 2002).

In conclusion, we have demonstrated that concentrations of ~3 μg/ml MPA appear to completely inhibit replication of avian reoviruses, and that inhibition is not cell type specific. Since MPA is used therapeutically as an immunosuppressive agent at higher doses of ≥10 μg/ml (Allison and Eugui, 1993; Gong et al., 1999; McEvoy, 2003a), and because MPA exerts antiviral activity against a wide range of RNA and DNA viruses (see beginning of this section), this suggests it may be useful as a broad-spectrum antiviral agent in patients and some economically important animals to attenuate or prevent viral disease.

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