Virology **304,** 211–221 (2002)
doi:10.1006/viro.2002.1685

Mycophenolic Acid Inhibits Dengue Virus Infection by Preventing Replication of Viral RNA

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Received May 7, 2002; returned to author for revision June 7, 2002; accepted July 22, 2002

Dengue fever is a mosquito-borne viral disease of global importance with no available antiviral therapy. We assessed the ability of mycophenolic acid (MPA), a drug currently used as an immunosuppressive agent, to inhibit dengue virus (DV) antigen expression, RNA replication, and virus production. Pharmacological concentrations of MPA effectively blocked DV infection, decreasing the percentage of infected cells by 99% and the levels of secreted virus by up to a millionfold. Results were reproduced with four hepatoma cell lines and different flaviviruses, including a recent West Nile virus isolate. Experiments were performed to define the stage in the viral lifecycle at which MPA abrogates infection. Early steps in viral infection, such as viral entry and nucleocapsid uncoating, were not the primary targets of MPA action since its inhibitory effect was retained when naked DV RNA was transfected directly into cells. Biosynthetic labeling experiments showed that MPA did not block the initial phase of viral translation but did interfere with viral protein synthesis in the amplification phase. Quantitative RT-PCR demonstrated that MPA prevented the accumulation of viral positive- and negative-strand RNA as the infection proceeded. We conclude that MPA inhibits flavivirus infection by preventing synthesis and accumulation of viral RNA.

Key Words: flavivirus; antiviral; dengue virus; mycophenolic acid; ribavirin.

INTRODUCTION

Dengue fever (DF), the most prevalent arthropod-borne viral disease in humans, is caused by dengue virus (DV). DV is a single-stranded, positive-polarity, enveloped RNA virus with a 10.7-kb genome that is translated in the cytoplasm as a single polyprotein and cleaved into three structural and seven nonstructural proteins. The four serotypes of DV are transmitted to humans by two species of mosquitoes, *Aedes aegypti* and *Aedes albopictus*. DV is a member of the *Flaviviridae* family and is related to the viruses that cause yellow fever; the Japanese, St. Louis, and West Nile encephalitides, and hepatitis C. Infection causes a spectrum of disease ranging from a debilitating, self-limited illness (DF) to a life-threatening syndrome (dengue hemorrhagic fever (DHF)). DV causes disease globally with an estimated 100 million new cases per year (Monath, 1994). At present, no vaccine exists and treatment is supportive. Despite its morbidity and mortality, few antiviral therapies have been tested.

Previous studies in our laboratory were directed at characterizing the efficacy and mechanism of agents with antiviral activity against DV such as interferons (Diamond *et al.,* 2000a). We then initiated a search for other molecules that could attenuate DV production. Here, we describe the antiviral activity of mycophenolic acid (MPA) against DV infection of human cells. MPA is a nonnucleoside inhibitor of inosine monophosphate (IMP) dehydrogenase; by blocking the synthesis of xanthosine monophosphate, it depletes intracellular guanosine pools. MPA is used clinically in the prevention of rejection of transplanted organs (Lipsky, 1996). MPA inhibits to varying degrees the infection of cells *in vitro* with several viruses by an unknown mechanism (Gong *et al.,* 1999; Ichimura and Levy, 1995; Neyts and De Clercq, 1998). A prior study reported that MPA blocked the cytopathic effect of YFV in monkey cells (Neyts *et al.,* 1996), although it had no inhibitory effect on hepatitis GB infection (Lanford *et al.,* 2001). In this article, we describe in detail the antiviral properties of MPA against DV infection of human cells, using as a comparison ribavirin (RBV), a competitive inhibitor of IMP dehydrogenase that was shown previously to have a modest antiviral effect against DV *in vitro* (Koff *et al.,* 1982). At concentrations well below its use as an immunosuppressive agent, MPA potently inhibited DV replication and protein production. An analysis of the mechanism suggested that MPA inhibited DV infection by preventing replication of both positive- and negative-strand viral RNA.

RESULTS

In an effort to characterize antiviral agents that could attenuate DV infection, MPA, a molecule that inhibits the cytopathic effect caused by infection with other viruses

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0042-6822/02 $35.00

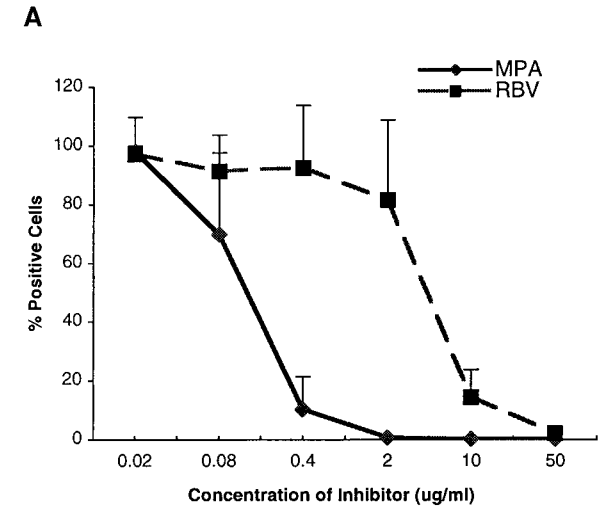
(Leyssen *et al.,* 2001; Neyts *et al.,* 1996), was assessed for its ability to inhibit DV type 2 (DV2) infection of human hepatoma cells. Clinical studies demonstrate DV antigen, DV nucleic acid, and cell death in the liver of patients infected with DV (Couvelard *et al.,* 1999; Marianneau *et al.,* 1998; Rosen *et al.,* 1999); thus, the liver is an important target of DV infection *in vivo.* Cell lines of hepatic origin were used as a suitable and relevant system for studying DV infection. For the purpose of comparison, cells were also treated with RBV, an antiviral agent that modestly inhibits DV infection (Koff *et al.,* 1982). Treatment of cells with MPA reduced the percentage of cells that expressed DV2 antigens in a dose-dependent manner, as measured by a flow cytometry assay that detects cells in which DV envelope (E) protein is expressed intracellularly (Diamond *et al.,* 2000a). MPA showed an approximately 65-fold greater potency than RBV (Fig. 1A), with an IC_{50} (concentration of inhibitor that reduced the viral antigen expression by 50%) of 0.1 μg/ml (0.31 μM). At the clinically therapeutic drug level, MPA (10 μg/ml, 30 μM) (Gong *et al.,* 1999; Lipsky, 1996) reduced virus production by greater than 6 log, whereas RBV (25 μg/ml, 100 μM) (Conner, 1984; Huggins *et al.,* 1991) decreased it by 3 log. The inhibition was not due to a toxic effect, as there was no difference in cell viability by trypan blue exclusion after treatment with MPA at the doses used (data not shown). At high concentrations, both MPA ($\geq$10 μg/ml) and RBV ($\geq$50 μg/ml) had a mild cytostatic effect, as they inhibited cell growth by 50%. In agreement with previous studies (Koff *et al.,* 1982; Neyts *et al.,* 1996), the inhibitory effects were completely reversed by the addition of 50 μg/ml of exogenous guanosine (data not shown).

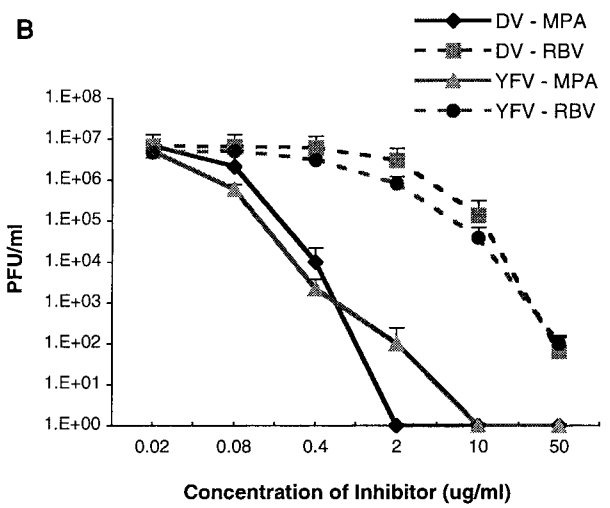
To confirm these effects, other cell lines were exposed to DV2, treated, and analyzed. Three other hepatoma cell lines (Huh-7, CRL-8024, and HepG2) exhibited a similar pattern of inhibition, with MPA showing a more potent antiviral effect than RBV (Table 1). In each cell type, MPA also showed a greater ability to inhibit the production of infectious virus (data not shown). Comparable results were observed with the SW13 human adrenal carcinoma cell line and BHK21 hamster fibroblasts (data not shown). To confirm that the inhibitory effects were not limited to the prototype DV2 strain (16681), we examined the ability of MPA to inhibit infection with a low-passage DV2 isolate, N1047 (Diamond *et al.,* 2000b). When Hep3B cells were infected with N1047 and then treated with MPA, expression of viral antigen and production of infectious virus were significantly blocked (97% reduction in antigen positive cells, >3 log decrease in virus production, data not shown). Similar results were observed with a low-passage recent isolate of the Southeast Asian genotype, K0049, in both Hep3B and HepG2 cells (data not shown). Thus, similar inhibitory effects were seen in several cell types and with both recent viral isolates and a prototype strain.

We assessed the antiviral effect of MPA against two other flaviviruses, yellow fever (YFV) and West Nile (WNV) viruses. Treatment of Hep3B cells with MPA and RBV immediately after infection with YFV resulted in a dose-dependent inhibition of viral antigen and infectious virus production (Fig. 1B, and data not shown). Again, MPA was more potent than RBV, as MPA (2 μg/ml) completely blocked YFV production, whereas the highest concentration of RBV tested was unable to eliminate virus production. Finally, treatment of murine primary glial cells with MPA decreased production of WNV by greater than 100-fold (Fig. 1C).

To assess the kinetics of the effect of MPA and RBV on DV2 infection, we performed time-course studies. Hep3B cells were exposed to RBV or MPA either before (24 or 4 h) or after (4 or 24 h) infection with the prototype DV2 strain (Fig. 2A). Pretreatment of Hep3B cells prior to infection dramatically reduced the percentage of cells that express viral antigen (MPA, >99% inhibition; RBV, 76–81%) and the production of infectious virions (MPA, >6 log reduction; RBV, >2 log). However, if MPA was washed out after the cells were exposed to virus, the inhibitory effect was completely lost. Consistent with this, when MPA was first added to Hep3B cells 4 or 24 h after exposure to DV2, viral antigen expression (99 and 94% inhibition, respectively) and infectious virus production (6 and 3 log reduction, respectively) were markedly reduced. Because MPA inhibited the production of infectious virus in Hep3B cells when administered 24 h after infection, we assessed its activity at later times after infection (Fig. 2B). Hep3B cells were exposed to DV2 and allowed to propagate virus for between 24 and 72 h. At each time point after infection, cells were extensively washed to remove newly generated virus and exposed to medium with or without MPA for 48 additional hours, and then supernatants were harvested for quantification of infectious virus. Without MPA treatment, viral titers peaked ($36 + 48 = 84$ h, 3.1×10^7 PFU/ml) and waned ($72 + 48 = 120$ h, 8.0×10^6 PFU/ml). In contrast, in cells treated with MPA, the inhibition was greatest at the earlier time points (5800-fold reduction at 72 h and 800-fold at 84 h), whereas later in infection a smaller effect was observed (15-fold reduction at 108 h and 3.6-fold difference at 120 h).

We next investigated the stage in the viral lifecycle at which MPA blocked infection. Although the antiviral action of MPA presumably occurred because of depleted intracellular guanosine, it was not clear which guanosine- or GTP-dependent step in the viral lifecycle (e.g., trafficking, translation, or replication) was most susceptible. To confirm that MPA did not inhibit the early steps (attachment, entry, nucleocapsid escape, and uncoating) of DV infection, an indirect assay was utilized in which naked viral RNA was transfected into cells (Diamond and Harris, 2001). If MPA inhibited an early step, it should not significantly inhibit DV replication in cells





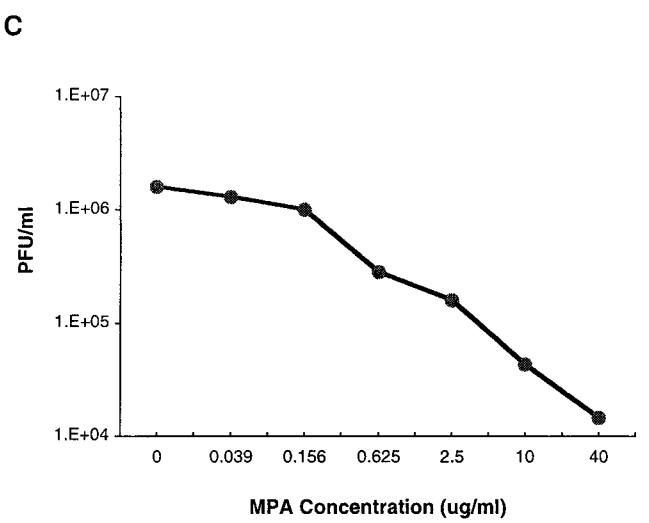


FIG. 1. (A) Effect of MPA and RBV on generation of DV2 antigen-positive cells. Hep3B cells were exposed to DV2 and incubated with increasing concentrations of MPA or RBV. Seventy-two hours later, cells were subjected to indirect immunofluorescent flow cytometry. The results are expressed as the percentage of cells that express the E protein of DV2 relative to the untreated (medium) control. The data are the average of three experiments ($n = 3$) and the error bars represent the standard deviations. (B) Effect of RBV and MPA on yields of DV2 and YFV from Hep3B cells. Cells were infected as described above with the exception that YFV was also used. Seventy-two hours after infection supernatants were harvested for viral plaque assays. The plaque assay data are expressed as the number of plaque forming units per milliliter (PFU/ml) based on cytopathic effect in BHK21 cells ($n = 3$). (C) Effect of MPA on WNV infection in primary glial cells. After infection, cells were incubated with increasing concentrations of MPA. Seventy-two hours later, supernatants were harvested for viral plaque assays in Vero cells. The data are expressed as above and represents one of two independent experiments.

TABLE 1

Effect of RBV and MPA on DV2 Infection in Hepatoma Cell Lines

Cell line[a]	RBV IC_{50} (μM)[b]	MPA IC_{50} (μM)
Hep3B	20	0.3
HepG2	60	3
CRL-8024	100	1.2
Huh-7	40	1.9

[a] Human hepatoma cells were exposed to DV2 (strain 16681), incubated with increasing concentrations of RBV or MPA, cultured for 72 h, harvested, and subjected to flow cytometry with mAbs against the E protein.

[b] The IC_{50} was defined as the concentration of RBV or MPA that inhibited expression of DV antigen in 50% of the infected cells relative to a medium-treated control. The data reflect the average of at least two independent experiments per cell line, and the standard deviations for the dose-response curves ranged from 5 to 25%.

transfected with purified viral RNA. DV2 RNA was isolated under denaturing conditions and transfected into Huh-7 cells with cationic liposomes. In the presence of medium alone, significant levels of viral proteins accumulated within the cells, and infectious virus was produced in cell supernatants (Figs. 3A and 3B). In contrast, treatment of Huh-7 cells with MPA still virtually abolished production of viral antigen and infectious progeny after transfection with DV RNA. Notably, significant levels of positive-strand viral RNA were detected in the MPA-treated cells at 4 h posttransfection, confirming that viral RNA entered these cells but was not able to replicate efficiently (data not shown). Since the inhibitory effect of MPA was still observed after transfection of naked viral RNA, its antiviral action occurred at a step after the release of the viral genome into the cytoplasm.

Because low intracellular guanosine concentrations may interfere with the fidelity and processivity of DNA and RNA polymerases (Crotty *et al.,* 2000, 2001; Huggins, 1989), the effect of MPA on viral RNA accumulation was assessed. Hep3B cells were exposed to DV2, treated with medium, RBV, or MPA, and harvested for RNA determination by an asymmetric, quantitative, competitive

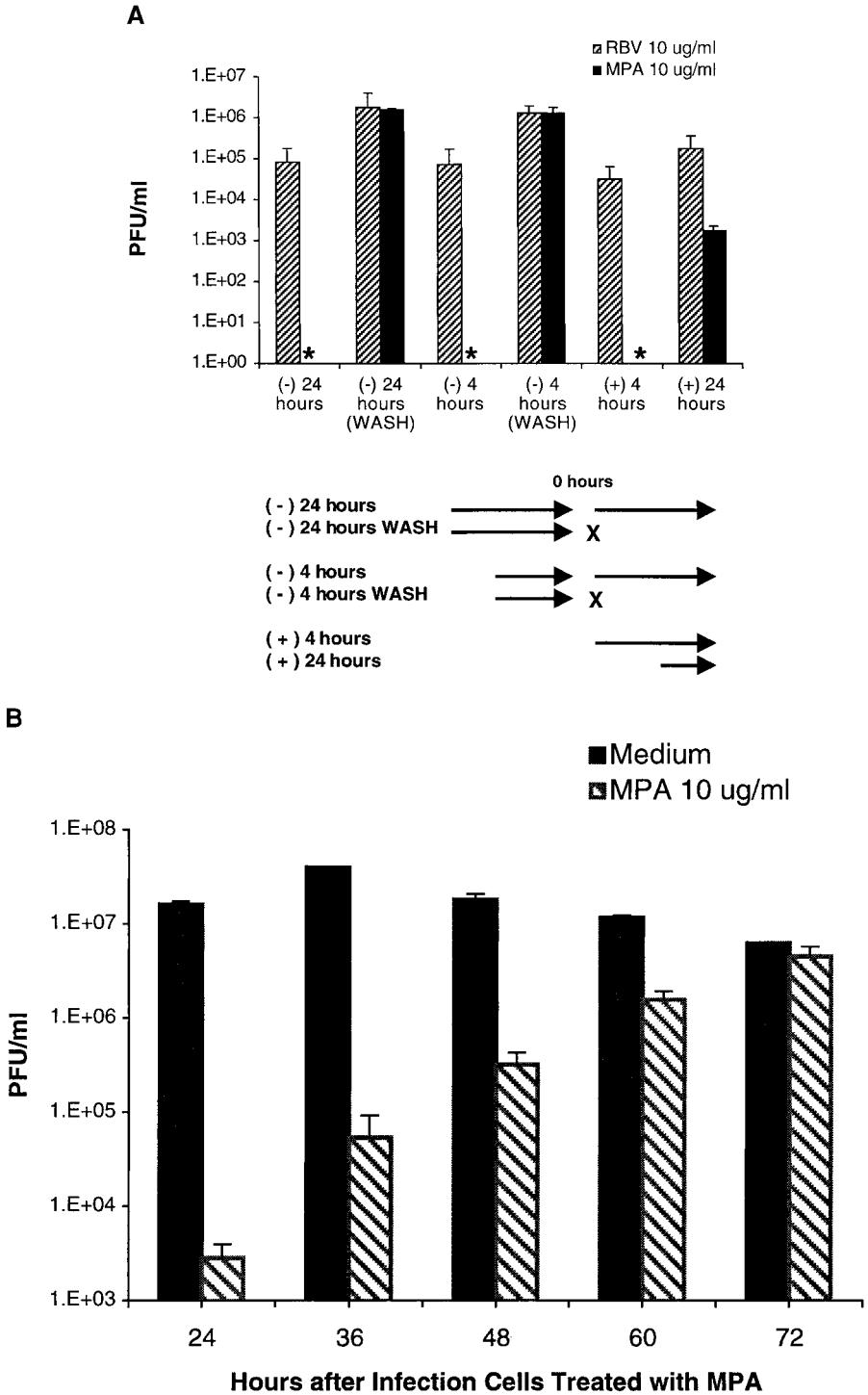


FIG. 2. (A) Time course of the effect of RBV and MPA treatment on DV2 infection. Hep3B cells were exposed to DV2 and incubated for 72 h. Cells were exposed to RBV (10 μg/ml) or MPA (10 μg/ml) either prior to ("(−) 24 or (−) 4 h") or after ("(+) 4 or (+) 24 h") incubation with virus. WASH indicates that the inhibitor was removed by the washing protocol after infection but was not added back. Cells that were treated with medium alone yielded $1.9 \times 10^6 \pm 0.9 \times 10^5$ PFU/ml. After infection, cell supernatants were harvested and assayed by viral plaque assay as described in Fig. 1 ($n = 2$). Asterisks are indicated when no viral plaques were detected (limit of detection = 10 PFU/ml). A diagram of the addition, washing, and readdition of RBV and MPA is also depicted. The X indicates that inhibitor was not added back after infection. (B) Effect of MPA on DV2 production at later stages of infection. Hep3B cells were exposed to DV2. After washing, cells were incubated for the time period indicated (24, 36, 48, 60, or 72 h) and then treated with either medium or MPA (10 μg/ml). Forty-eight hours later, cell supernatants were harvested for viral plaque assays as described in Fig. 1 ($n = 2$).

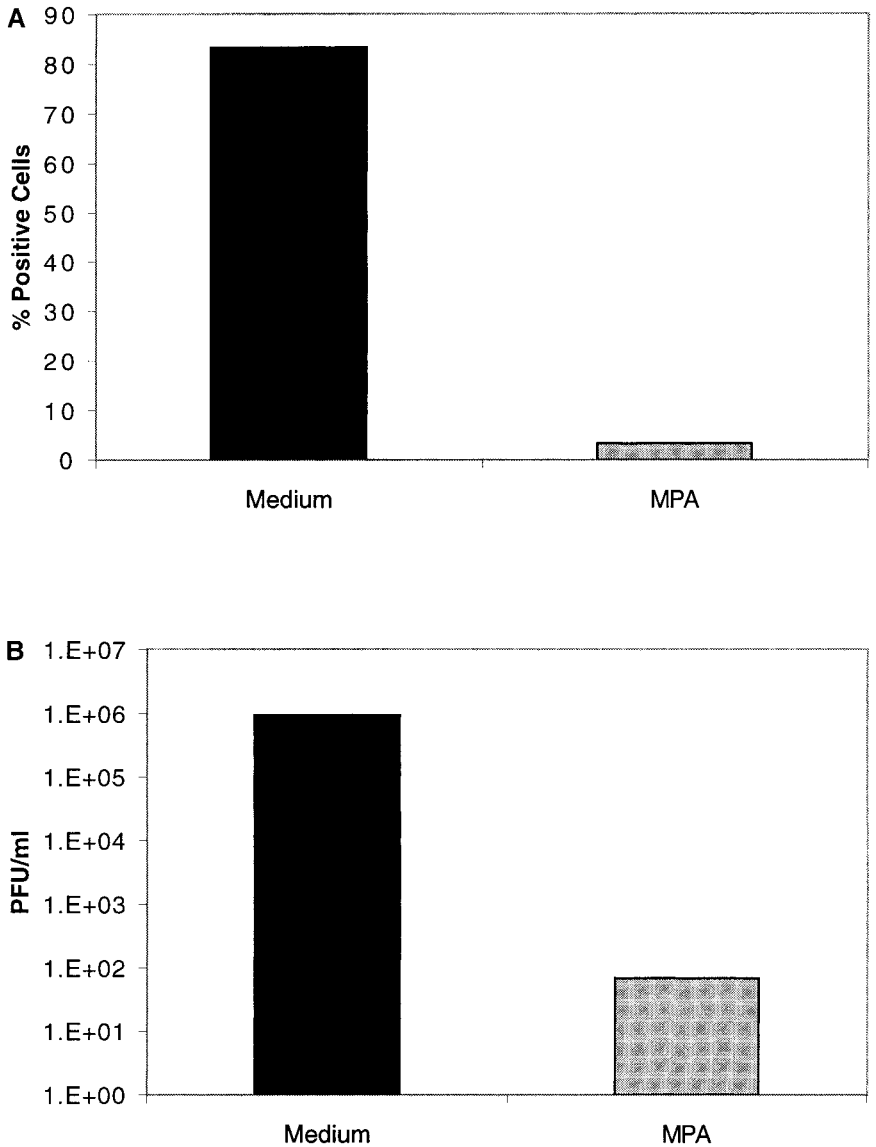


FIG. 3. Transfection of Huh-7 cells with DV2 RNA. Cells were transfected with equivalent amounts of purified DV RNA and incubated in the presence or absence of MPA. Forty-eight hours after transfection, cells were harvested and processed for antigen expression (A) or virus production (B) as described in Fig. 1. The data are representative of two independent experiments.

RT-PCR assay (Diamond *et al.,* 2000a,b). At 48 h postinfection, treatment of cells with RBV or MPA reduced positive-strand viral RNA 10- to 200-fold and negative-strand viral RNA 10- to 1000-fold reduction (Fig. 4A). Kinetic analysis revealed that at early time points (2, 6, and 12 h postinfection), MPA did not appear to affect the steady-state level of the positive or negative strands (Fig. 4B). The low level of viral RNA at these time points likely reflected that from the input virus. After 12 h, the medium-treated cells manifested an abrupt logarithmic increase in positive- and negative-strand viral RNA. In contrast, no dramatic increase or rapid decrease was observed in the steady-state levels of positive- or negative-strand viral RNA in the MPA-treated cells over time.

Because MPA blocked the accumulation but did not accelerate the degradation of viral RNA in cells infected with virus or transfected with viral RNA, two possible mechanisms were hypothesized: MPA inhibited the translation of the infectious viral RNA (by interfering with GTP-dependent steps in translation) or MPA impeded the synthesis of viral RNA. To investigate whether it affected input-strand translation, Hep3B cells were infected with DV, treated with medium or medium plus MPA for 6, 9, 12, 15, or 18 h, and then biosynthetically labeled for 20 min. Synthesis of viral proteins was detected in lysates after 9 h in cells that were treated with medium but not in uninfected cells or cells treated with MPA (Fig. 5A). The identity of DV NS5 and NS3 was confirmed by either Western blot (Fig. 5B) or by comparison with gels containing lysates of virally infected cells at later times (>24

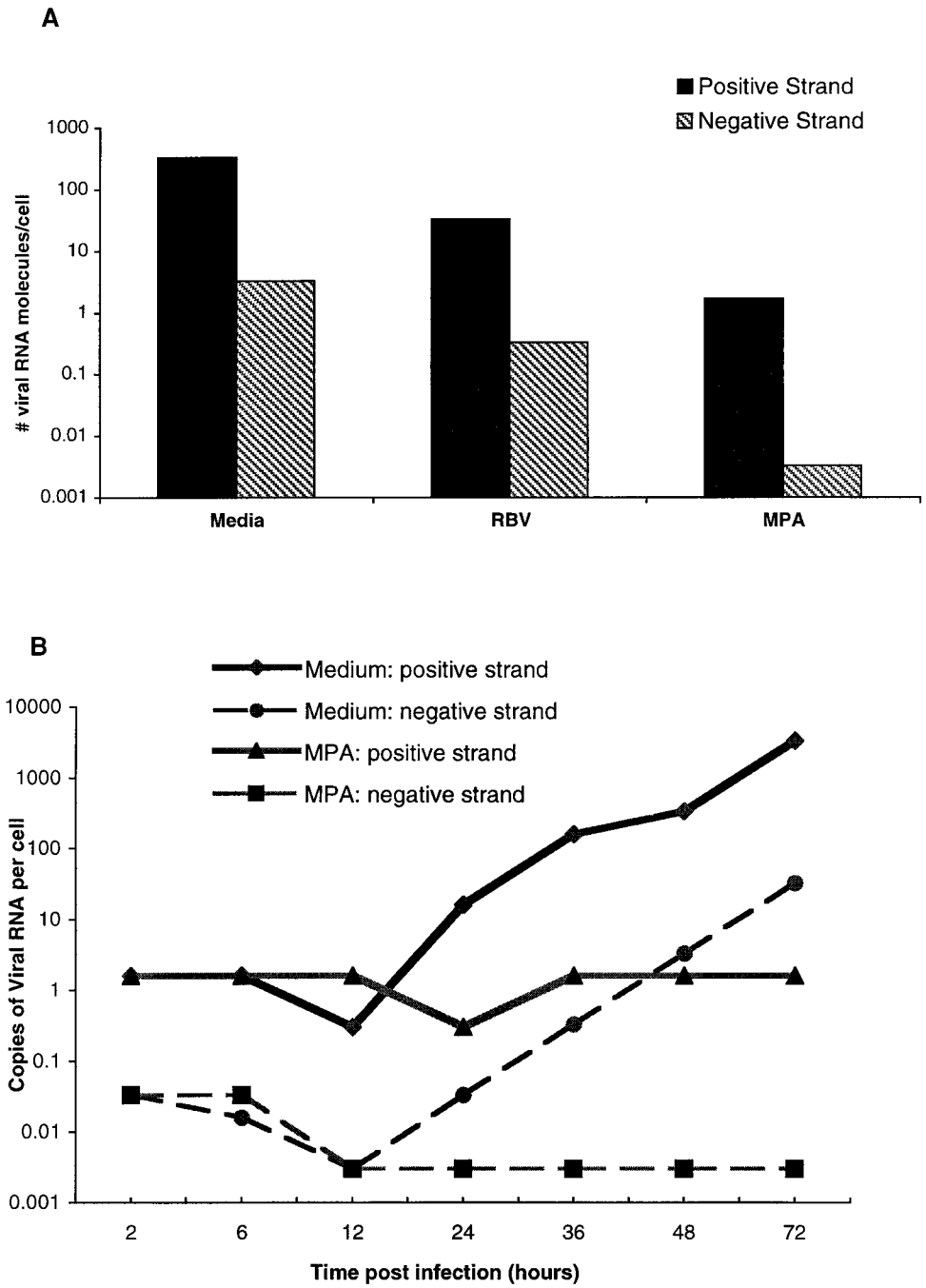


FIG. 4. (A) Effect of RBV and MPA on the levels of positive and negative strand of DV2 RNA. Hep3B cells were exposed to DV2 (strain 16681) and then incubated with medium, RBV (10 μg/ml), or MPA (10 μg/ml). Seventy-two hours later, cells were harvested, RNA was isolated, and quantitative asymmetric RT-PCR was performed. The amount of viral RNA was determined from the competitor concentration that produced competitor and DV bands of equal intensity. One representative experiment of two is shown. (B) Prospective time course of the effect of MPA on DV2 RNA production. Hep3B cells were infected with DV2 (strain 16681) at an m.o.i. of 3. After incubating with MPA or medium for the indicated times (2, 6, 12, 24, 26, 48, or 72 h), cells were harvested for viral RNA determination by asymmetric, competitive RT-PCR.

h) in the course of infection. One interpretation of these data was that MPA blocked DV infection by preventing translation of the input strand of DV RNA.

To further investigate the stage at which inhibition occurred, experiments were designed so that translation of input-strand viral RNA could be observed at very early time points after entry yet prior to replication of viral RNA. BHK21 cells were infected with wild-type (16681) DV at a high m.o.i. to generate a large pool of translatable viral RNA at early time points. Immediately prior to biosynthetic labeling, excess (175 mM) NaCl was added; this treatment reduces the rate of synthesis of cellular proteins more than viral proteins (Erdei, 1981; Schrader and Westaway, 1990; Svitkin *et al.,* 1978). In contrast to the 6-

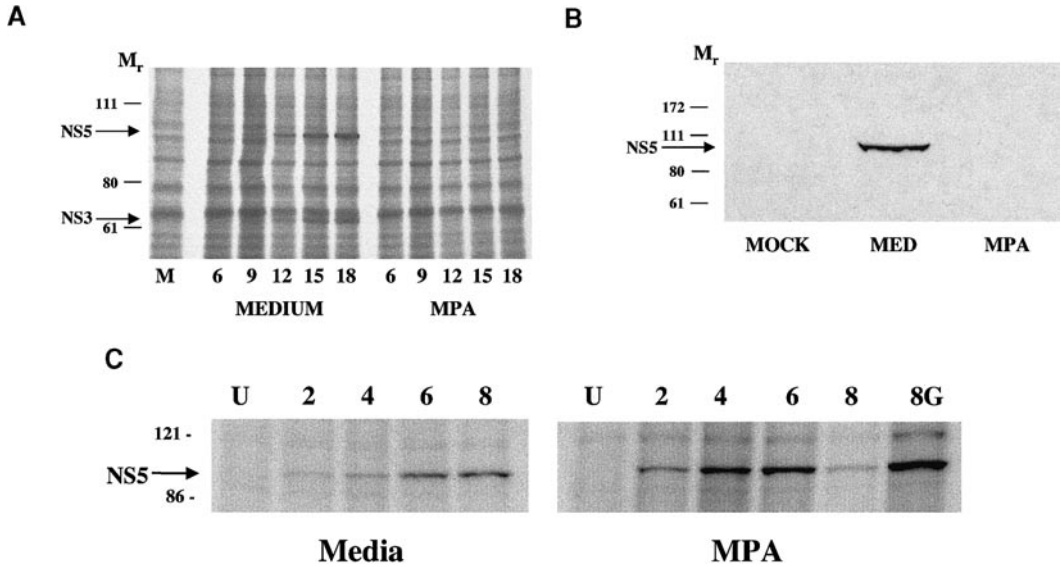


FIG. 5. (A) Effect of MPA on viral protein translation in Hep3B cells. Hep3B cells were either not infected (U) or infected with DV2 in the presence of medium or MPA. After the indicated times postinfection (6, 9, 12, 15, and 18 h), cells were labeled biosynthetically with ^{35}S for 20 min. After washing, cells were lysed, and the proteins resolved by 8% SDS–PAGE and autoradiography. The migration of viral proteins (arrows) was confirmed by Western blot (B) and with labeled markers. (B) Immunoblot analysis of Hep3B cells infected with DV2. Hep3B cells were infected with DV2 in the presence of medium or medium plus MPA. After 24 h, cells were harvested and protein extracts were analyzed by SDS–PAGE. Proteins were transferred to a nitrocellulose membrane and probed with polyclonal anti-NS5 antibodies. (C) Effect of MPA on translation of NS5 at early time points after infection in BHK21 cells. BHK21 cells were pretreated for 6 h with media or MPA (2.5 μg/ml). Subsequently, cells were mock-infected (lanes marked with U) or infected with DV2 in the presence or absence of MPA. At the indicated time points after infection, cells were labeled for 30 min with ^{35}S in the presence of 175 mM excess NaCl as described under Materials and Methods. Cells were detached and lysed in SDS–sample buffer, and the proteins were resolved by 7% SDS–PAGE and autoradiography. The migration of NS5 and labeled markers is indicated.

and 9-h time points in Hep3B cells (Fig. 5A), in BHK21 cells in the absence of significant cellular protein synthesis, translation of NS5 was detected in both medium- and MPA-treated cells at 2, 4, and 6 h after infection (Fig. 5C). However, by 8 h after infection, whereas there was a noticeable increase in synthesis of NS5 in medium-treated cells, a marked decrease was observed in the MPA-treated cells. This decrease was reversed by the addition of excess guanosine (Fig. 5C). These early time-course translation studies suggested that MPA did not prevent translation of the infectious DV RNA.

Because MPA did not inhibit the early phase of viral translation, we hypothesized that it must directly inhibit viral replication, by blocking the synthesis of negative- and/or positive-strand viral RNA. Studies were repeated at early time points after infection with a more sensitive and quantitative real-time RT-PCR (Houng *et al.,* 2001) to distinguish small increases in positive- and negative-strand viral RNA replication. At the earliest time points, a higher m.o.i. was used to enhance sensitivity of detection. No increases in viral RNA were observed at 2 and 4 h after infection (m.o.i. = 100) in the medium and MPA-treated cells. However, reproducible increases in negative and positive viral RNA were observed at 6 h after infection only in the medium-treated cells (Figs. 6A and 6B). To distinguish whether MPA blocked negative- and/or positive-strand viral RNA synthesis, the effect of MPA was measured at a point during the course of infection when both strands would be replicating. BHK cells were infected with DV (m.o.i. = 3), incubated for 12 h to generate a pool of actively replicating viral RNA, and then treated with medium or MPA. At several time points after treatment, the levels of positive- and negative-strand viral RNA were determined by real-time RT-PCR (Figs. 6C and 6D). Significantly, treatment with MPA resulted in an abrupt cessation in the accumulation of both positive- and negative-strand viral RNA.

DISCUSSION

This article establishes that infection of human cells *in vitro* by DV is completely prevented by pharmacologically relevant concentrations of MPA, a nonnucleoside inhibitor of IMP dehydrogenase. Using quantitative RT-PCR, biosynthetic labeling, RNA transfection, flow cytometry, and viral plaque assays, we demonstrated that MPA potently inhibited DV infection by significantly reducing the levels of viral RNA, intracellular viral antigen, and infectious progeny virus. The inhibitory effect of MPA was reversed by the addition of exogenous guanosine and was not due to cellular toxicity. Time-course and washout studies revealed that the antiviral effect required the continued presence of MPA. When the mechanism of inhibition was analyzed, we found that although the later phases of viral protein synthesis were inhibited,

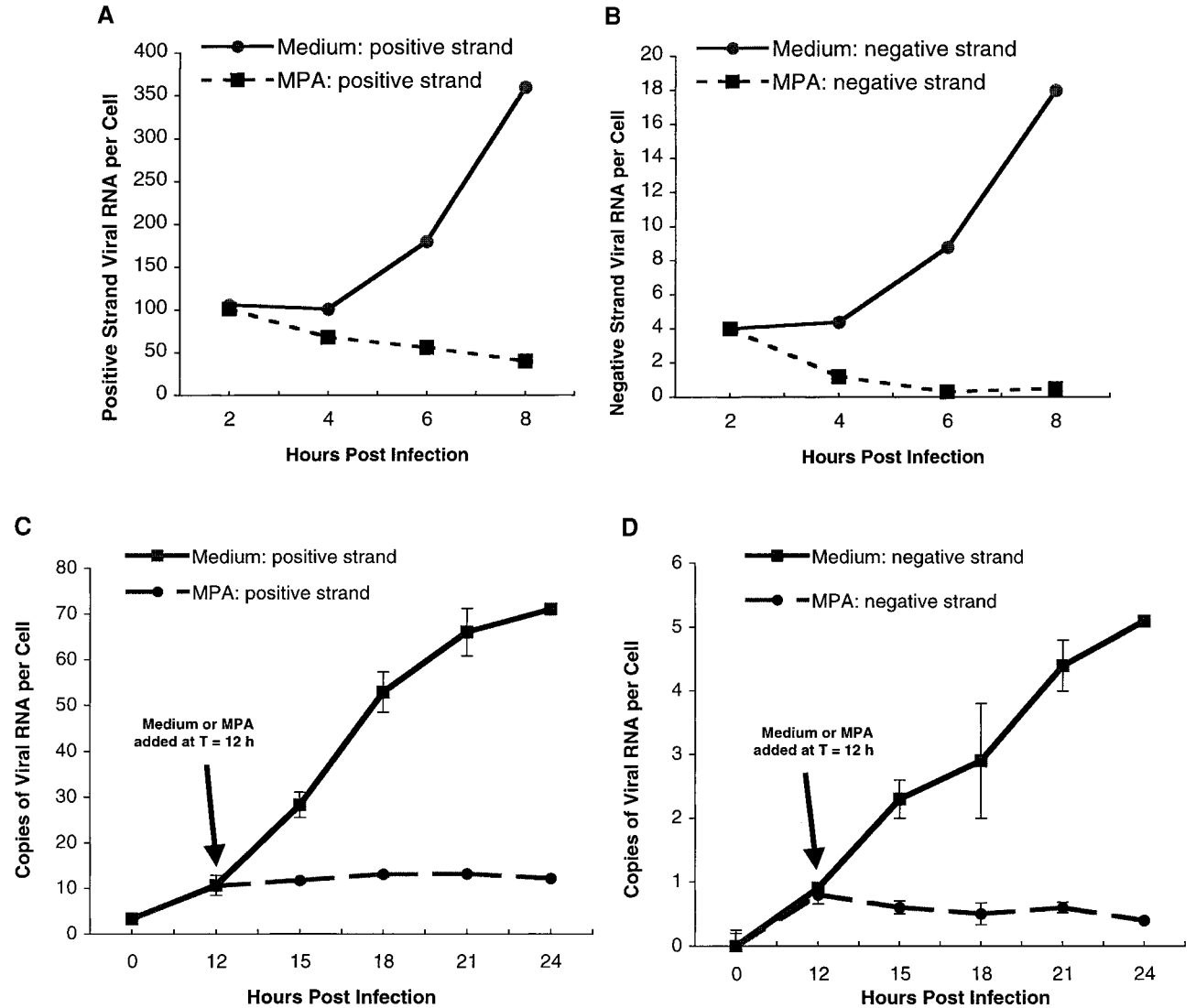


FIG. 6. Effect of MPA on the accumulation of positive- and negative-strand DV RNA at early times postinfection. (A and B) Early time course (0 to 8 h) of positive- (A) and negative- (B) strand DV RNA accumulation in BHK21 cells using Taq-Man fluorogenic quantitative RT-PCR. Cells were treated with medium or MPA 18 h prior to infection (m.o.i. = 100) and harvested at the indicated time after infection. Samples were run in triplicate and reflect the average of two different experiments. The absolute amount of viral RNA was determined using standards that were measured by spectrophotometer. (C and D) Effect of MPA on positive- (C) and negative- (D) strand DV RNA accumulation in DV-infected BHK21 cells. Cells were infected (m.o.i. = 3) for 12 h and then exposed to medium or MPA (arrow). After the indicated times, cells were harvested, and RNA was measured by real-time fluorogenic RT-PCR. Samples were run in duplicate and reflect the average of two different experiments.

MPA did not block translation of the input strand of viral RNA but instead prevented synthesis of viral RNA.

MPA is currently used as an immunosuppressive agent to prevent organ rejection in transplantation; in this capacity, it is believed to act by blocking T cell proliferation (Allison and Eugui, 1993). Previous studies have reported that MPA inhibited *in vitro* infection by several viruses (Gong *et al.,* 1999; Ichimura and Levy, 1995; Neyts and De Clercq, 1998; Neyts *et al.,* 1996) in a guanosine-dependent manner. However, there were no prior studies with MPA and DV infection. Our data show convincingly that MPA is a potent anti-DV agent that can abrogate a new infection or attenuate an existing infection. Moreover, preliminary studies demonstrated a significant inhibitory effect of MPA on infection in primary glial cells by low-passage WNV isolates. Although additional studies must be performed, these investigations suggest that MPA may have broad antiviral properties against flaviviruses. Experiments are planned to test the efficacy of MPA against WNV and DV infection in a mouse model of disease.

Because the inhibitory effects of MPA are reversed by guanosine, its antiviral activity depends on its capacity to deplete intracellular guanosine levels. MPA likely does not act as a viral mutagen; it is not a nucleoside analog and thus cannot incorporate into the nascent RNA strand. Here, the stage at which MPA inhibited DV infection was examined; no prior study has described which

guanosine-dependent step in the viral lifecycle is limited by MPA. In DV-infected BHK21 cells, translation of input-strand viral RNA was observed at very early time points in both medium- and MPA-treated cells using a high dose of infectious virus and conditions that reduced host cell protein synthesis. A sensitive real-time RT-PCR assay demonstrated that translation of NS5 clearly preceded replication of viral RNA in medium-treated cells, and that neither negative- nor positive-strand viral RNA accumulated in MPA-treated cells. Collectively, these experiments suggest that MPA blocks DV infection by preventing replication of viral RNA. Studies are underway to determine the exact mechanism, but at this time we hypothesize that low intracellular guanosine levels adversely affect the processivity of the viral replicase.

RBV has broad-spectrum antiviral activity *in vitro* and *in vivo* against a number of viruses. However, as an antiviral agent against flaviviruses, RBV has modest inhibitory activity. High doses of RBV were required to inhibit WNV replication in human neural cells (Jordan *et al.,* 2000) and YFV infection in monkey kidney cells (Neyts *et al.,* 1996). In prior experiments with DV *in vitro,* high concentrations of RBV (100 μM) were required to reduce viral titer in monkey kidney cells (Koff *et al.,* 1982). Our data in human hepatoma cells are consistent with this, as we observed a significant inhibitory effect only at the highest (100 μM) concentrations used. The limited *in vivo* experience with DV has not been auspicious, as RBV failed to attenuate DV infection in a murine intracerebral inoculation model (Koff *et al.,* 1983) and a rhesus monkey model (Malinoski *et al.,* 1990).

Recent studies suggest that the pathogenesis of DHF may be explained by an enhanced viremia (Vaughn *et al.,* 2000) coupled with an unrestrained immune system response with extensive T lymphocyte activation and production of inflammatory cytokines (Rothman and Ennis, 1999). The data we present in this article suggest that at clinically relevant concentrations MPA potently inhibits DV infection by blocking replication of viral RNA. Given the immunopathogenesis of DHF, it is intriguing to consider therapeutic trials with MPA, a drug that efficiently prevents and attenuates viral translation but also, as an immunosuppressive, blunts some of the proliferative and cytokine responses of the immune system. Independent of its clinical utility as an antiviral agent, by specifically disrupting flavivirus replication, MPA may become a useful tool to disrupt steps in the viral lifecycle that are coupled to replication.

MATERIALS AND METHODS

Reagents, cell culture, antibodies, and viruses

MPA, RBV, and guanosine were purchased commercially (Sigma Chemical Co., St. Louis, MO). Human cells were cultured in RPMI 1640 as described previously (Diamond *et al.,* 2000a) and obtained from the following sources: HepG2 hepatoma cells (the American Type Culture Collection (ATCC), Rockville, MD), Hep3B hepatoma cells (a gift of J. Boothroyd, Palo Alto, CA), CRL-8024 hepatoma cells (ATCC), and Huh-7 hepatoma cells (a gift of A. Dasgupta, Los Angeles, CA). BHK21-15 hamster kidney cells were maintained in α-modified Eagle's media, and C6/36 mosquito cells were cultured in Leibovitz's L15 media as described (Diamond *et al.,* 2000a). Glial cells were isolated from the cerebral cortex of neonatal C57BL/6 mice according to a published protocol (Goslin and Banker, 1992). These cells were maintained in Eagle's media supplemented with 20% fetal calf serum and used after one passage. Hybridoma cells that produce monoclonal antibodies against the envelope proteins of DV (3H5-1, anti-DV2; 5D4-11, anti-DV3; 4G2, antiflavivirus) were obtained (ATCC) and cultured in DMEM (Diamond *et al.,* 2000b). DV2 strains used included a prototype DHF strain (16681) from Thailand (Russell and Nisalak, 1967) and a recent DF isolate (N1047, passage 2) from Nicaragua (Balmaseda *et al.,* 1999; Diamond *et al.,* 2000b). The YF virus was derived from the 17D vaccine strain. The WNV strain (3000.0259, passage 1) was isolated from a mosquito in New York in 2000 (Ebel *et al.,* 2001) and obtained from Dr. Laura Kramer (Albany, NY).

Plaque and flow cytometric assays

Virus production was titered by plaque assays as described previously (Diamond *et al.,* 2000a). For the experiments in which RBV and MPA were used, after a 2-h exposure to virus, BHK21 cells were washed four times with medium prior to the addition of the agarose overlay. To determine the percentage of cells that expressed viral antigen, flow cytometric analysis was performed as described (Diamond *et al.,* 2000a).

Cell infection with virus

Cells were infected with DV2, YF, or WNV; briefly, virus was added to monolayers at a given multiplicity of infection (m.o.i.) and incubated at 37°C for 2 h (Diamond *et al.,* 2000a). Subsequently, the cells were washed four times to remove residual unbound virus. In some experiments, cells were treated with a given concentration of RBV or MPA prior to, during, or after exposure to DV2. In the early time-course studies, RBV or MPA was added to cells before and/or after DV infection. For some experiments, guanosine (50 μg/ml) was added to the cells after infection with DV2. Supernatants and cells were harvested 72 h after infection for quantitation of viral antigen, positive- and negative-strand RNA, and infectious virion. In the late time-course experiments, cells were infected with DV2, incubated for a given time (24, 36, 48, 60, or 72 h), extensively washed, and then in some cases incubated with MPA (10 μg/ml). Forty-eight hours after

this addition, cell supernatants were harvested for virus titration by plaque assay.

Cell transfection with RNA

Huh-7 cells were transfected with purified RNA using cationic liposomes. RNA was purified from DV2 viral supernatants (3×10^8 PFU) using the RNEasy Mini Kit (Qiagen, Valencia, CA) and eluted in RNAse-free ddH_2O. The cationic lipid reagent DMRIE-C was used according to the manufacturers' instructions (Gibco-BRL). Briefly, Huh-7 cells were washed in Opti-MEM I medium (Gibco-BRL) and plated (2×10^5 cells/well) in a 12-well plate. Liposomes were prepared, and immediately after, purified DV2 RNA was added; the RNA-liposome suspension was added to Huh-7 cells in the presence or absence of MPA (10 μg/ml) for 6 h at 37°C. Subsequently, the liposome suspension was removed, and complete medium was added with or without MPA. Cells and supernatants were harvested 72 h later for plaque assay, flow cytometric analysis, and viral RNA determination.

Quantitative RT-PCR

For experiments with Huh-7 cells, positive- and negative-strand DV RNA was quantitated using a competitive RT-PCR assay (Diamond *et al.,* 2000a). We observe a small yet reproducible amount of negative-strand viral RNA in our input virus samples. This low level is present after virion purification by sucrose gradient sedimentation and may reflect viral particles that contain fragments of negative-strand viral RNA (Novak and Kirkegaard, 1991) or residual membrane fragments with associated negative-strand RNA that cosediment with virions. For experiments with BHK21 cells, positive- and negative-strand viral RNA was measured by real-time PCR using the Taq-Man RT-PCR buffer system and an ABI 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). To quantitate DV, two primers and a probe were generated using a sequence in the DV2 3′ noncoding region according to a previously published protocol (Houng *et al.,* 2001).

Biosynthetic labeling

For biosynthetic labeling in Huh-7 cells, published protocols were modified (Baxt and Bablanian, 1976; Baxt *et al.,* 1977). Hep3B cells were plated (0.7×10^5) in 48-well tissue culture plates, infected with DV2 (m.o.i. of 200), and treated with medium or medium plus MPA (10 μg/ml) for 5, 8, 11, 14, and 17 h. Cells were washed three times in RPMI cysteine$^-$ methionine$^-$ (cys^-met^-) medium supplemented with 5% dialyzed FBS in the presence or absence of MPA and incubated for 1 h. Subsequently, 0.1 mCi of [^{35}S]cys-met was added for 20 min. Cells were then washed three times in PBS at 4°C, detached, pelleted, and lysed in 0.2 ml lysis buffer (1% Triton X-100, 140 mM NaCl, 25 mM Tris–HCl pH 7.8, 0.025% NaN_3, 1 mM phenylmethylsulfonylfluoride, 2 mg/ml iodacetamide, 0.2 TIU/ml aprotinin, and 0.5% bovine hemoglobin) on ice. After 15 min, the lysate was clarified by centrifugation at 10,000 *g* (10 min at 4°C). SDS sample buffer with 5% β-mercaptoethanol was added to an aliquot (40 μl) of the clarified lysate and subjected to 7% SDS–PAGE and autoradiography. For biosynthetic labeling in BHK21 cells, a protocol was used that maximized the translation of viral proteins at early time points (Erdei, 1981; Schrader and Westaway, 1990; Svitkin *et al.,* 1978). Cells (1.5×10^5) were plated in 24-well plates and pretreated overnight in media or MPA (2.5 μg/ml) and infected at an m.o.i. of 100. One hour prior to labeling, cells were washed and incubated with RPMI cys^-met^- medium. Twenty minutes prior to labeling, 175 mM excess NaCl was added. Cells were labeled for 30 min with 0.075 mCi of [^{35}S]cys-met and then detached. After washing by centrifugation, cell pellets were lysed in 50 μl SDS sample buffer supplemented with 5% β-mercaptoethanol and 2 M urea, boiled, and electrophoresed.

Immunoblot analysis

Hep3B cells (3.0×10^5) were infected with DV2 (m.o.i. of 100) and treated with medium or medium plus MPA (10 μg/ml) for 24 h. Cells were then washed three times in PBS, detached, and pelleted. Cell lysates were prepared by freeze-thawing once and resuspended in 6× SDS sample buffer. One-half of the lysate was loaded per lane, subjected to SDS–PAGE and transferred to nitrocellulose (MSI, Westborough, MA). The membrane was blocked with 5% nonfat dry milk, probed with mouse polyclonal anti-NS5 antibodies (1:1000), washed five times with PBS-Tween 20 (0.1% v/v), incubated with goat anti-mouse secondary antibody conjugated to horseradish peroxidase (Sigma Chemical Co.) at a dilution of 1:2000, and washed five times with PBS–Tween 20. After incubation with the substrate Luminol (Sigma Chemical Co.), NS5 was visualized by autoradiography.

ACKNOWLEDGMENTS

The authors thank J. Boothroyd and D. Gupta for providing cell lines; L. Kramer for WNV isolates; R. Beatty for polyclonal anti-NS5 antibodies; J. Ernst, S. Schlesinger, R. Beatty, and S. Shresta for editorial comments; and M. Engle for technical assistance. The work was supported by a National Institutes of Health (NIH) grant to E. Harris (AI-42052), a Washington University-Pharmacia grant to M. Diamond, and by fellowships from the Infectious Diseases Society of America to M. Diamond and by fellowships from the University of California Berkeley, Howard Hughes Medical Institute, and the American Society for Microbiology to M. Zachariah.

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