THE EFFECT OF AMANTADINE ON MOUSE HEPATITIS VIRUS REPLICATION

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INTRODUCTION

Amantadine has been known to be a potent inhibitor of influenza A virus infection for many years (1). Although amantadine's antiviral effect on many other viruses is much less potent that observed for influenza A, it does have a fairly broad antiviral spectrum (2,3). The extreme sensitivity of influenza A virus to the drug has been shown by genetic and molecular biologic studies to reside with the M2 protein. Amantadine interacts with M2 and inhibits its ability to function as a pH-gated ion channel, and thus appears to interfere with a step in influenza virus uncoating and assembly (4-6). Amantadine exerts its more broad-spectrum antiviral effects by virtue of its lysomotropic properties. The drug accumulates in endocytic vesicles, raises their pH, and thus interferes with the ability of viruses requiring low pH to complete uncoating and penetration from entering the cytosol (2,3).

The effect of amantadine on coronavirus replication has not been fully investigated. It has been noted that the bovine coronavirus (BCV) is sensitive to amantadine, probably at a post-uncoating stage in the viral replicative cycle (7). In this work, we report that, like BCV, mouse hepatitis virus (MHV) is sensitive to amantadine at doses comparable to those reported to inhibit Semliki forest virus and vesicular stomatitis virus (VSV). However, unlike these viruses, amantadine exerts its antiviral effects on MHV at a late stage in viral replication.

MATERIALS AND METHODS

Cells and Virus

The origin and maintenance of the cell lines used in these studies has been reported previously (8). The origin, and growth of the MHV-A59, MHV-JHM, and

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VSV stocks used in these studies has been described (8). Plaque reduction, assays were performed by diluting viral stocks to approximately 100 PFU/ml in medium containing various concentrations of amantadine hydrochloride (Sigma Chemicals), letting the virus adsorb to monolayers of L2 cells for 60 minutes, and then overlaying the cells with media containing 0.8% agarose and the same concentration of amantadine as the virus inoculum. Plaques were stained and enumerated at 2 days post infection.

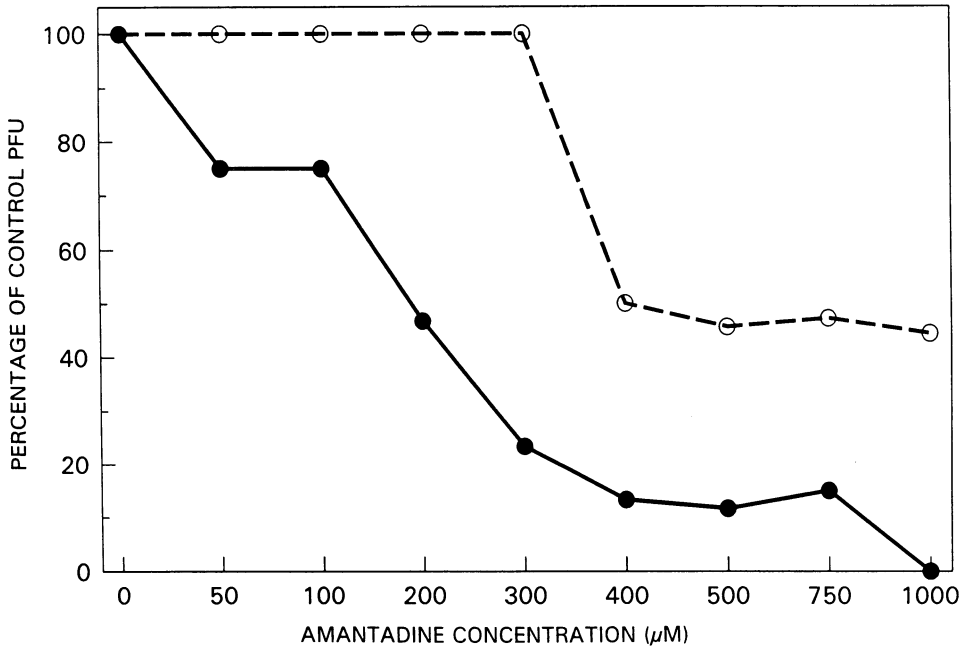

Figure 1. The effect of amantadine addition on plaque formation by MHV and VSV. Approximately 100 PFU of MHV (●) and VSV (o) were assayed in the presence of various concentrations of amantadine and in the absence of the drug. Results are expressed as a percentage of the plaques observed in the absence of amantadine.

RNA preparation and gel electrophoresis

Cells were incubated with ^{32}P-orthophosphate from 7-8 hours post infection in the presence of 5 μg/ml actinomycin D. RNA was extracted with guanidium-thiocyanate as described (9) and electrophoresed on 0.8% agarose gels containing formaldehyde as a denaturant.

Antibodies, immunoprecipitation and immunofluorescence

A monospecific polyclonal goat antisera directed against the MHV-A59 S protein was graciously provided by Dr. K.V. Holmes. The monoclonal antibodies J2.7 (anti-M, obtained from Dr. John Fleming) and 1.16.1 (anti-N) have been described previously (10,11). All other antibodies used in this work were purchased from Jackson Research.

The conditions of metabolic labeling of cells with ^{35}S-methionine, immunoprecipitation, and immunofluorescence microscopy have been described previously (12).

RESULTS

The effect of amantadine on MHV replication

To determine if amantadine inhibited MHV replication we performed a plaque reduction assay incorporating various amounts of amantadine in the virus inoculum and overlay medium. As shown in Figure 1, amantadine inhibited plaque formation with MHV-A59. Fifty percent inhibition was achieved at about 200 μM amantadine and complete inhibition was reached at 1 mM concentrations. Similar results were obtained with MHV-JHM (not shown), although slightly higher doses of amantadine were required to reach the same degree of inhibition. VSV, although reported in the literature to be sensitive to amantadine was considerably less so than MHV.

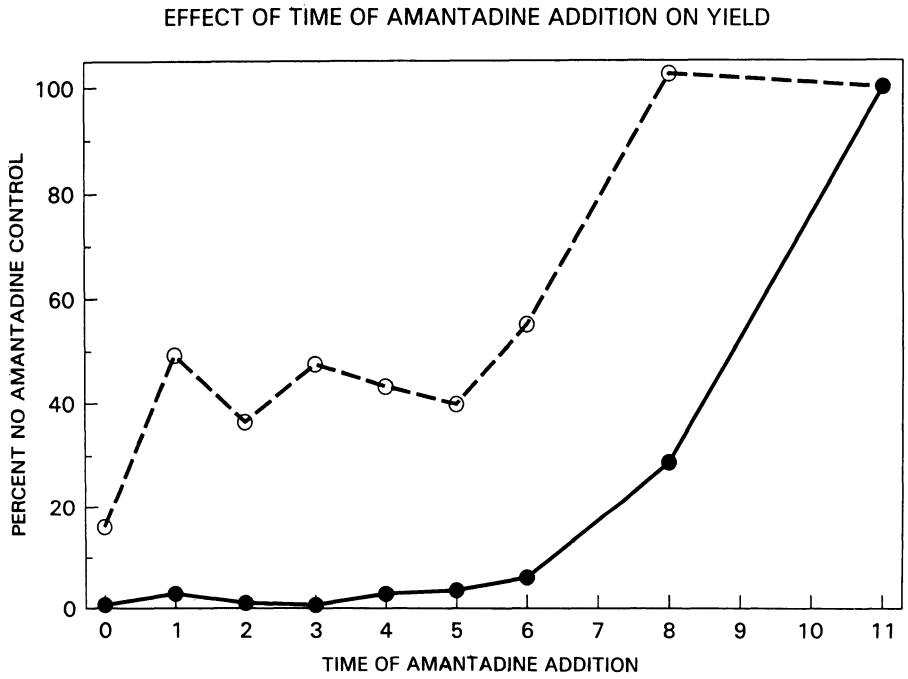

Figure 2. The effect of time of addition of amantadine on MHV replication. Cells were infected with either MHV (●) or VSV (o) at a M.O.I. of 3 and amantadine added to 500 μM at the times indicated. Cultures were harvested at 11 hour post infection and virus yield determined by plaque assay in the absence of amantadine.

To investigate the step in viral replication which is sensitive to amantadine we added the drug at various times post infection and assessed the effect on viral yield. Replicate cultures were infected with either MHV-A59 or VSV in the presence or

absence of amantadine. At various times post infection amantadine was added to cultures which had not contained the drug. All cultures were harvested at 11 hours post infection and the amount of virus determined by plaque assay in the absence of amantadine. As shown in Figure 2, amantadine inhibited MHV replication when added as late as six hours post infection. This did not to be due to general toxicity of the drug since the effect on VSV (Figure 2) replication was much less pronounced, the

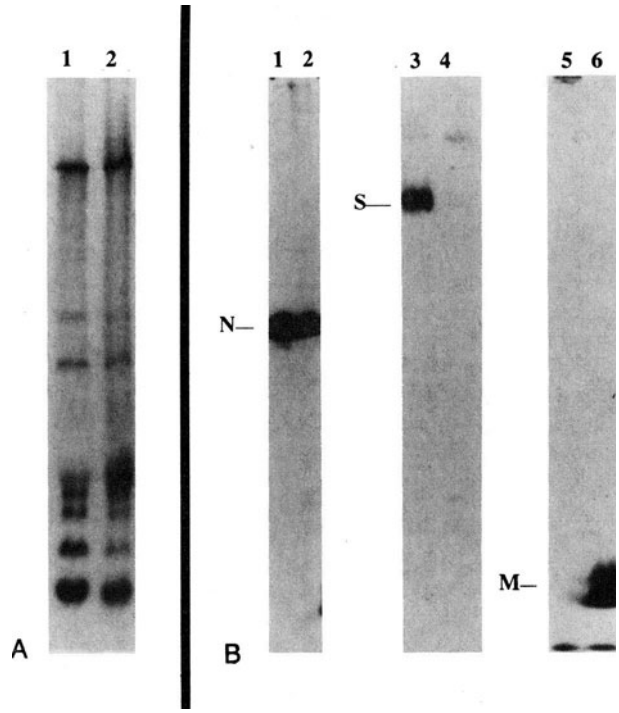

Figure 3. The effect of amantadine on MHV-specific RNA and protein synthesis. Panel A. Cultures were infected with MHV at a MOI=3 and incubated in the presence (lane 1) or absence (lane 2) of amantadine, labeled with ^{32}P-orthophosphate, and the RNA extracted and electrophoresed as described in Materials and Methods. Panel B. MHV-infected cultures were incubated in the presence (lanes 2,4,5) or absence (lanes 1,3,6) of 500 μM amantadine and labeled from 7-8 hours post infection with 100 μCi/ml ^{35}S- methionine. Cytoplasmic extracts were prepared and immunoprecipitated with antibodies to N (lanes 1and 2), S (lanes 3 and 4), and M (lanes 5 and 6) and resolved by SDS-PAGE..

cells were morphologically intact, and incorporation of ^{35}S-methionine into TCA-precipitable material was 75% of that in control cells when amantadine was added at time 0. The less potent inhibition of VSV replication by amantadine is consistent with published data (3).

This block in infectivity was also reflected in a decrease in the production of viral particles, as determined by banding in potassium tartrate gradients of ^{3}H-uridine labeled virus (not shown). However the inhibition in virus particle formation was considerably less than the inhibition in infectivity, suggesting that the particles produced in the presence of amantadine were less infectious.

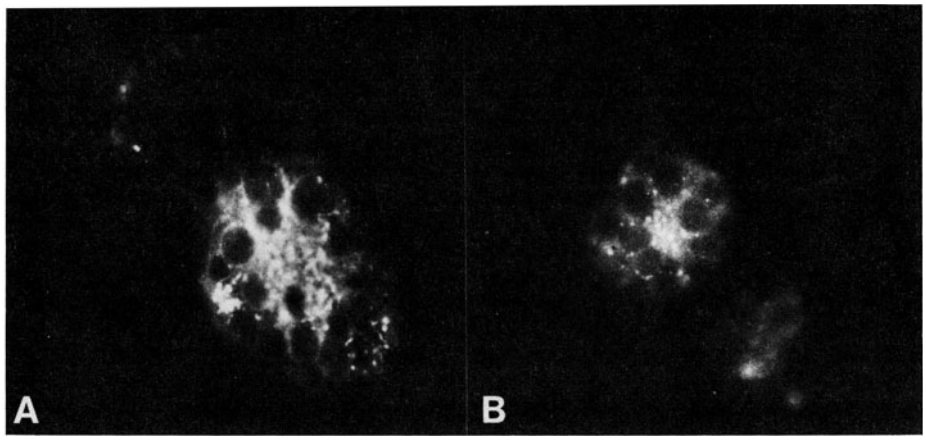

Figure 4. Immunofluorescent staining of amantadine treated cells. Cells were infected with MHV in the presence (Panel A) or absence (Panel B) of 500 μM amantadine, fixed at 7 hours post infection and stained with the anti-M monoclonal, J2.7.

Determination of the stage of replication effected by amantadine.

The above experiments suggested that amantadine exerted its effect on MHV replication at late times post infection. To further examine this, cells were infected with MHV-A59 and either treated with 500 μM amantadine from the time of infection or incubated in the absence of the drug. At seven hours post infection the cultures were labeled with ^{32}P-orthophosphate in the presence of actinomycin D. RNA was extracted and analyzed by gel electrophoresis. As shown in Figure 3A, there was no qualitative effect of amantadine on the species of MHV-specific RNAs synthesized, although the amount of virus-specific RNA was slightly decreased. Inhibition of MHV-specific RNA synthesis by amantadine was approximately fifty percent, as determined by actinomycin D resistant ^{3}H-uridine incorporation into TCA precipitatable material.

Since this degree of inhibition of MHV-specific RNA synthesis could not account for the observed effects on viral yield we then investigated the effect of the drug on virus-specific protein synthesis. Cells were infected with MHV-A59 and incubated for 7 hours in the presence or absence of 500 μM amantadine, labeled with ^{35}S-methionine for 60 minutes, and cytoplasmic extracts prepared. These extracts were analyzed by immunoprecipitation using antibodies to N, S, and M protein. As shown in Figure 3, Panel B, the accumulation of immunoprecipitable M and S glycoproteins was greatly diminished by amantadine. Pulse-chase experiments (data not shown) demonstrated that this decrease in MHV-specific glycoprotein accumulation was due to decreased synthesis, rather than enhanced turnover. In contrast to the large decrease in MHV glycoprotein synthesis in the presence of the amantadine, the amount of immunoprecipitatble nucleocapsid protein which accumulated was only moderately decreased (about 50%) by amantadine. The effect of amantadine on the accumulation of the M glycoprotein (Figure 4) and S protein (not shown) was confirmed by immunofluorescence microscopy.

DISCUSSION

We have demonstrated that amantadine inhibits MHV replication. The doses of amantadine required to exert this effect are one to two orders of magnitude higher than those required to inhibit influenza A replication. However, these doses approximate those reported to inhibit SFV and VSV by interfering with uncoating. For these viruses amantadine's mode of action appears to be due its accumulation in endosomes with a resultant increase in the pH of this cellular compartment. This increased pH interferes with a change in conformation of the VSV and SFV spike proteins, thereby interfering with the viruses' ability to escape the endosomes into the cytosol (2,3). Unlike the case for VSV or SFV, amantadine does not inhibit MHV uncoating since virus-specific RNA synthesis is not significantly inhibited by the drug.

The precise mechanism by which the accumulation of MHV-specific proteins, particularly glycoproteins, is inhibited by amantadine is unknown. Pulse-chase experiments have demonstrated that this represents a real decrease in synthesis of these proteins, not enhanced degradation in the presence of the drug. This decreased synthesis of the MHV glycoproteins is entirely out of proportion to the relatively minor, approximately 25%, inhibition of general protein synthesis which we observed with 500 μM amantadine. It cannot be accounted for by the 50% decrease in MHV-specific mRNA synthesis which we observed in the presence of the drug. It is possible that it is due to a decreased stability of membrane bound polysomes.

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