Inhibition of Dengue Virus Replication by Amantadine Hydrochloride

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The effect of amantadine hydrochloride (1-adamantanamine hydrochloride) on dengue virus replication was examined in vitro. Amantadine decreased the titers of all four types of dengue viruses grown in LLC-MK2 cells by greater than 90% at concentrations of 50 μg/ml. There was no evidence for any cytopathic effect of the drug at concentrations less than 100 μg/ml. Studies of the time of addition showed that the antiviral effect was maximal when drug was added to virus cultures immediately after the viral adsorption period. In addition, amantadine caused a marked reduction in the growth of dengue virus type 2 in both human and rhesus peripheral blood leukocytes without affecting cell viability. These findings demonstrate that amantadine significantly inhibits the replication of dengue viruses in vitro and indicate a need to determine the efficacy of this drug against dengue virus infections in vivo.

Dengue viruses are a major cause of morbidity and mortality among children in tropical Asia (5). The dengue viruses constitute a subgroup of group B arboviruses (flaviviruses) and occur as four distinct antigenic types (14). Humans infected with any of these serotypes exhibit symptoms ranging from relatively mild dengue fever to the more severe dengue hemorrhagic fever and life-threatening dengue shock syndrome (10). The severe aspects of dengue virus disease have been associated with secondary heterologous dengue virus infections, suggesting that immunopathological processes play a significant role in both dengue hemorrhagic fever and dengue shock syndrome (6, 9).

At the present time, an effective vaccine against dengue hemorrhagic fever is not available. Vaccine development in the dengue system is faced with theoretical difficulties, since a vaccine virus that induces only homotypic protection could itself sensitize and predispose an individual toward dengue hemorrhagic fever and dengue shock syndrome (23). As a result, the need for effective control measures for dengue infections has stimulated a search for chemotherapeutic agents against this disease.

Amantadine hydrochloride (1-adamantanamine hydrochloride) has been reported to possess antiviral activity against a wide variety of viruses (15). This present study examines the efficacy of amantadine against dengue virus infections and demonstrates that the drug significantly inhibits the replication of dengue viruses in vitro.

MATERIALS AND METHODS

Virus and cell cultures. The following strains of dengue viruses recovered in BS-C-1 cells (African green monkey kidney cells) or in suckling mice from acute-phase sera from patients with dengue hemorrhagic fever in Thailand or the Philippines were chosen for study: dengue virus type 1 (strain 16007), type 2 (strain 16681), type 3 (strain 16562), and type 4 (strain H-241) (13, 14). Viruses were propagated in rhesus monkey kidney cells (LLC-MK2) maintained with Eagle basal medium with Earles salts and 10% calf serum as previously described (11). Briefly, 4 to 6 days postinfection, cells were lysed by freeze-thawing, and the suspension was clarified by centrifugation at 2,000 × *g* for 15 min. Virus was concentrated by ultracentrifugation at 100,000 × *g* for 4 h in a 10 by 10 angle head rotor in a Spinco L-65 ultracentrifuge (Beckman Instruments, Inc., Spinco Division, Fullerton, Calif.). After resuspension of the pellet by sonication, agamma heat-inactivated calf serum was added to a final concentration of 25%, and virus suspensions were stored at −70°C. Virus was assayed by a modification of a plaque method in LLC-MK2 cells (12). Three-day-old LLC-MK2 cell monolayers grown in 1-ounce (ca. 30-ml) prescription bottles (Brockway Glass Co. Inc., Brockway, Pa.) were rinsed with Hanks balanced salt solution (Microbiological Associates, Bethesda, Md.) and inoculated with 0.2 ml of virus sample for 90 min at 37°C on a rocker platform (Bellco Glass, Inc., Vineland, N.J.). The inoculum was then poured off, and cells were overlayed with 1% purified agar (BBL Microbiology Systems, Cockeysville, Md.) containing Eagle basal medium and final concentrations of 10% calf serum, 2 mM L-glutamine, 200 U of penicillin per ml, 200 μg of streptomycin per ml, 0.1% sodium bicarbonate, and neutral red diluted to 1: 24,000. The bottles were incubated in the dark at 37°C for 7 days, and the plaques were counted. Each sample was assayed in triplicate.

Leukocyte cultures. Heparinized whole blood was collected by venipuncture from rhesus monkeys (*Macaca mulatta*) and normal adult human volunteers. Mononuclear leukocytes were isolated on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden; sodium diatrizoate, Winthrop, New York, N.Y.) density gradients (3), washed 3× in phosphate-buffered saline (pH 7.4),

1× in Hanks balanced salt solution and suspended in RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) containing 20 mM HEPES (N-2-hydrox-yethylpiperazine-N'-2-ethanesulfonic acid; Calbi-ochem, San Diego, Calif.), 0.2% sodium bicarbonate, 2 mM L-glutamine, 200 U of penicillin per ml, and 200 μg of streptomycin per ml immediately before infection with dengue virus.

Infection of leukocytes. A 0.6-ml amount of the leukocyte suspension (10^7 cells per ml) was incubated with dengue virus type 2 (multiplicity of infection = 0.1 to 1.0) and anti-dengue type 4 serum (1:200 final dilution) in a total volume of 1 ml for 90 min at 37°C. The inoculum was then washed off, and cells were resuspended at 2×10^6/ml in RPMI 1640 supplemented with 2% fetal calf serum. Cultures (1 ml) were incubated at 37°C in a 5% CO_2-in-air atmosphere for 3 to 5 days, then harvested and kept at −70°C for further use. Virus growth was assayed in LLC-MK2 cells as described above. Amantadine hydrochloride (lot no. 5042-175, E.I du Pont de Nemours, Newark, Del.) was added at the times indicated in figure legends.

Infection of LLC-MK2 cells. Cells were washed 1× with Hanks balanced salt solution and inoculated with dengue virus types 1 to 4 (multiplicity of infection = 0.01 to 0.1) for 90 min at 37°C. The inoculum was poured off, and 5 ml of virus maintenance medium (Eagle basal medium supplemented with 2% calf serum) was added to each 1-ounce bottle. Aliquots of culture medium were removed daily and frozen at −70°C for future viral growth analysis. For drug studies, amantadine was dissolved in sterile distilled water and added at times indicated in the figure legends.

Viability studies. Viability of leukocytes was determined by trypan blue dye exclusion as previously described (18). Briefly, viability was determined by adding 50 μl of 0.1% trypan blue to 0.2-ml cultures, staining for 30 s, and terminating by the addition of 0.1 ml of 4% acetic acid. Two hundred cells from each culture were counted twice, and replicate cultures of each experimental variable were analyzed to obtain accurate percentages (±3%) of the viable cells in all cultures.

Statistical analysis. Student's t test was used to evaluate the significance of observed differences between experimental and control groups.

RESULTS

Dose-response studies. Figure 1 shows the effect of increasing concentrations of amantadine on the growth of dengue virus type 2 in LLC-MK2 cells. At a concentration of 50 μg/ml, dengue virus titers were suppressed by more than 99% ($P < 0.05$). Amantadine did not cause any observable cytotoxic effects at this concentration. However, generalized rounding and destruction of the cell sheet in both uninfected and dengue-infected LLC-MK2 monolayers were seen with amantadine concentrations equal to or greater than 100 μg/ml.

Inhibition of dengue virus synthesis. Table 1 indicates the susceptibility of the four
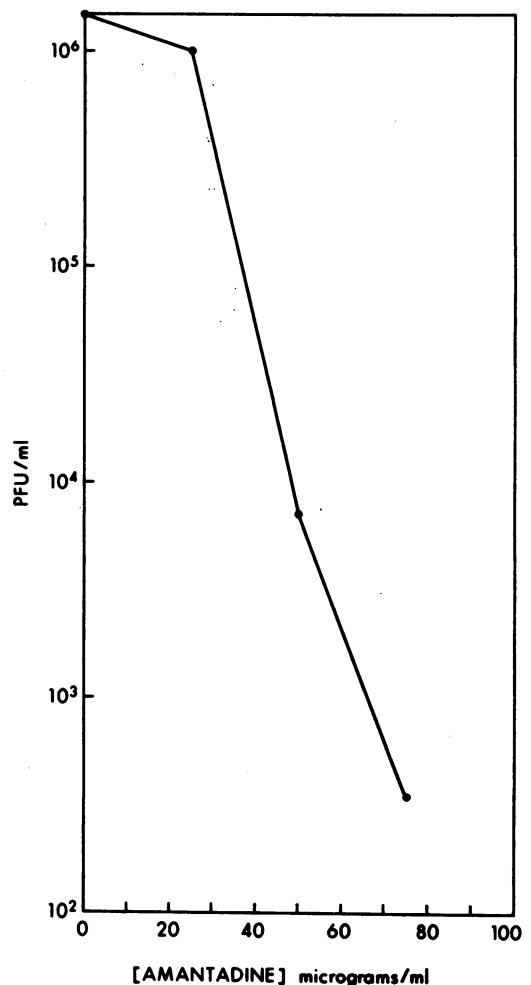

FIG. 1. Dose-response curve of amantadine on the infection of LLC-MK2 cells with dengue virus type 2. Drug was added immediately after the viral adsorption period. At 5 days postinfection, aliquots of culture fluids were harvested and assayed in LLC-MK2 cells for determination of virus titer.

types of dengue viruses to amantadine. At a concentration of 50 μg/ml, amantadine inhibited the replication of dengue virus types 1 to 4 by at least 90%. Whereas the differences in virus titers between untreated and drug-treated samples of each virus were all statistically significant ($P < 0.05$), there was no statistical difference in the amount of inhibition by amantadine observed when comparing virus types. Thus, amantadine effectively inhibits the replication of all four dengue virus types in vitro.

Time of addition studies. Table 2 describes the effect of amantadine on the replication of dengue virus type 2 in LLC-MK2 cells when the drug was added at various times after the 90-

TABLE 1. *Inhibition of dengue virus replication by amantadine[a]*

Dengue virus type	Drug[b]	PFU/ml[c]	% of untreated
1	−	6.42×10^6	100
1	+	1.83×10^4	0.29
2	−	1.02×10^6	100
2	+	9.46×10^3	0.93
3	−	1.25×10^5	100
3	+	5.0×10^3	4.00
4	−	2.62×10^5	100
4	+	4.41×10^3	1.68

[a] Dengue viruses were infected into LLC-MK2 cells, and culture fluids were harvested 5 days postinfection and assayed for released virus in an LLC-MK2 plaque assay as described in the text.

[b] Amantadine was added immediately after the viral adsorption period at a concentration of 50 µg/ml.

[c] Values indicate the mean plaque forming units (PFU) from three replicate experiments.

TABLE 2. *Effect of time of addition of amantadine on dengue virus type 2 replication in vitro*

Time of addition[a]	PFU/ml[b]	% of untreated
Control (no drug)	1.24×10^7	100
0	8.25×10^3	0.07
1	4.73×10^4	0.38
4	1.04×10^6	8.39
8	2.11×10^6	17.02
24	1.33×10^7	107

[a] Dengue virus type 2 was infected into LLC-MK2 cells as described in the text. Amantadine (50 µg/ml) was added at the indicated hours post-infection and remained on the cells until culture fluids were harvested for analysis of virus growth on day 5.

[b] Values indicate the mean plaque-forming units (PFU) per milliliter from three replicate experiments.

min viral adsorption period. Although amantadine reduced virus titers when added 4 to 8 h post-infection, there was a markedly diminished effect of the drug when addition was later than 1 h postinfection. When treatment was delayed for 24 h, amantadine did not effect the replication of dengue virus type 2 in LLC-MK2 cells.

Inhibition of dengue virus replication in PBL. The effect of amantadine on dengue virus replication in peripheral blood leukocytes (PBL) was examined, since a wide array of data point towards the leukocyte as a probable site of dengue replication in vivo (2, 7, 8, 19, 20, 21). Table 3 describes the effect of amantadine on the replication of dengue virus type 2 in both rhesus and human PBL. At a concentration of 50 µg/ml, amantadine significantly ($P < 0.05$) reduced the growth of dengue virus in both PBL systems. Dose-response studies on the effect of amantadine on dengue virus growth in both PBL sys-

TABLE 3. *Effect of amantadine on the replication of dengue virus type 2 in human and rhesus PBL[a]*

PBL	Drug[b]	PFU/ml[c]	% of untreated	Mean % viable[d]
Human	−	8.17×10^3	100	93
Human	+	3.10×10^2	3.79	90
Rhesus	−	1.92×10^3	100	92
Rhesus	+	1.22×10^2	6.35	88

[a] PBL were infected with dengue virus type 2 as described in the text. Amantadine was added immediately after the viral adsorption period. Cultures were harvested after incubation at 37°C in a 5% CO_2-in-air atmosphere for 4 days.

[b] Concentration of amantadine = 50 µg/ml.

[c] Values indicate the mean plaque-forming units (PFU) per milliliter of four replicate experiments.

[d] Viability was determined by trypan blue dye exclusion.

tems indicated that the drug significantly reduced virus titers at concentrations of 25 µg/ml (data not shown). Viability studies, by trypan blue dye exclusion, demonstrated that amantadine did not decrease the viability of cultured PBL from either rhesus or human at concentrations less than 100 µg/ml.

DISCUSSION

The present studies demonstrate that amantadine hydrochloride significantly inhibits the replication of dengue viruses in vitro. With an LLC-MK2 tissue culture system, the growth of all four dengue virus types was markedly reduced at drug concentrations well below cytotoxic levels. Addition of the drug immediately after the 90-min viral adsorption period produced maximal inhibition of virus replication. Finally, in cultures of PBL from either rhesus or human donors, amantadine effectively reduced the growth of dengue virus type 2 without affecting cell viability.

Several lines of evidence support the hypothesis that mononuclear leukocytes play an integral role in the viral immunopathogenesis of dengue infections. Histopathological examination of patients dying of dengue hemorrhagic fever revealed marked depletion of lymphocytes in the thymic dependent areas of lymph nodes and spleen (1, 2), and dengue viral antigens have been found on the surfaces of their mononuclear leukocytes (2). Virus recovery studies on dengue-infected rhesus monkeys and human beings showed that dengue virus was associated in tissues rich in leukocytes (20, 21). In addition, peripheral blood leukocytes from dengue-immune rhesus monkeys and humans supported dengue virus replication in vitro, whereas PBL cultures from nonimmune donors were not permissive (7, 8, 19).

Our findings demonstrated that amantadine significantly reduced the growth of dengue viruses in both rhesus and human PBL. Viability studies by trypan blue dye exclusion revealed no significant differences between drug-treated and untreated controls at concentrations of drug which inhibited virus replication. These viability data compared favorably with other previous reports examining the toxicity levels of amantadine and its analogs on PBL in vitro (18, 22). Thus, amantadine is effective in limiting the growth of dengue viruses in the cells which appear to play a prominent role in the pathogenesis of dengue virus infections.

Although amantadine inhibits the replication of a wide variety of viruses in vitro, the exact mode of action of the drug has yet to be determined (15). Most studies have concluded that the drug is not virucidal at concentrations active in tissue culture (16) and acts early in the virus replicative cycle either by blocking virus penetration into the host cell or suppressing the uncoating of the virus particle (4, 17). Dose response studies have shown that amantadine causes gross cytopathic effects on a wide variety of cells at concentrations greater than 100 μg/ml (15). Similarly, in the dengue virus-LLC-MK2 system, 50 μg of amantadine per ml was well tolerated by the cells, and cytopathic effects were first observed at concentrations of 100 μg/ml.

Time of addition studies have indicated that the effectiveness of amantadine is dependent on the specific virus-cell system used. With an influenza-chicken enbryo fibroblast system, amantadine did not reduce the growth of virus when added later than 30 min after the viral adsorption period (16). However, studies with lymphocyte choriomeningitis virus in L-929 cells demonstrated that amantadine reduced virus titers when added as late as 20 h postinfection (24). For dengue virus replication in vitro, it is now apparent that although the drug reduces virus replication when added 4 to 8 h postinfection, maximal inhibition occurs when the drug is present immediately after the viral adsorption period. Furthermore, unlike the influenza system where amantadine inhibits the replication of type A viruses but not type B viruses (15), the growth of all 4 types of dengue viruses is reduced when cultured in the presence of amantadine.

Since severe dengue virus disease has been associated with secondary heterologous dengue virus infections (6, 9), the development of an effective vaccine against dengue hemorrhagic fever has been hampered by the possibility of sensitizing individuals towards more severe dengue virus disease by injection with a homotypic virus vaccine (23). As a result, other methods for controlling dengue virus infections are now being more closely examined. We are currently studying the efficacy of amantadine and its analogs against dengue virus infections in animal models.

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