Antiviral Research 14 (1990) 75–86
Elsevier

AVR 00432

Tromantadine inhibits a late step in herpes simplex virus type 1 replication and syncytium formation

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(Received 6 February 1990; accepted 10 April 1990)

Summary

Addition of tromantadine after virus penetration inhibited HSV-1 induced syncytium formation and virus production in HEp-2 and VERO cells and acted additively with neutralizing antibody in blocking virus spread and cytopathology. Inhibition of syncytium formation in VERO cells infected with 0.01 pfu/cell of HSV-1 GC+ was observed at a concentration >25 μg/ml. The extent of inhibition was dependent upon the multiplicity of infection and cell type. Tromantadine inhibited a late event in HSV-1 replication which appeared to be sensitive to cycloheximide. Reversal of the inhibitory effect of tromantadine on syncytium formation required new protein synthesis. HSV-1 gB, gC, and gD were synthesized in the presence of tromantadine and could be detected on the cell surface by immunofluorescence. Tromantadine most likely inhibits a cellular process that is required for syncytium formation, such as glycoprotein processing, which occurs after the synthesis of the fusion protein but before its expression on the cell surface.

Tromantadine; HSV-1; Syncytium formation; Fusion

Introduction

Tromantadine (*N*-1-adamantyl-*N*-[2-(dimethylamine) ethoxy]acetamide hydrochloride, (Merz & Co., Frankfurt, F.R.G.) is a topical antiherpetic drug that is commercially available in some European countries, Asia, and South America (Salva, 1986;

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Hellgren and Hermann, 1983; Ostheimer et al., 1989). Tromantadine has two independent actions on herpes simplex virus type 1 (HSV-1) replication: early, during virus binding or penetration (Rosenthal et al., 1988); and late, after the initiation of DNA synthesis (Rosenthal et al., 1982).

HSV can spread extracellularly, through intracellular bridges and by syncytium formation and, as a result, escape antibody control (Lodmell et al., 1973). As a result, antiviral drugs are a useful adjunct to immune control of HSV infections.

Herpes simplex virus type 1 produces syncytia by fusion from within (FFWI) (Gallaher et al., 1980). Induction of FFWI by HSV-1 requires replication of the virus and viral glycoprotein synthesis (Manservigi et al., 1977; Keller, 1976). HSV-1 glycoproteins gB and gD are important for induction of syncytia (Cai et al., 1988; Marsden, 1987; Spear, 1985) and their synthesis and glycan processing are initiated 6–9 h post infection (p.i.) (Campadelli-Fiume et al., 1988; Kousoulas et al., 1978; Bzik et al., 1982). HSV-1 induced cell-cell fusion can be blocked by inhibiting glycoprotein synthesis or processing (Spear, 1985; Campadelli-Fiume and Serafini-Cessi, 1985) or by altering the fusion properties of the viral and cellular proteins and membranes involved in the process.

In this report we show that addition of tromantadine at 1 h post infection, after the initial tromantadine sensitive-event in the HSV replicative cycle has taken place, reduces both the number and size of HSV-1 induced syncytia. Tromantadine does not block the synthesis or cell surface expression of the viral glycoproteins but new protein synthesis is required to reverse its inhibitory effect on syncytium formation. Tromantadine also acts additively with neutralizing antibody in blocking virus spread.

Materials and Methods

Cells and virus

VERO and HEp-2 cells were grown in Eagle's minimum essential medium (Auto-Pow) supplemented with 7.5% fetal calf serum, 5% horse serum, 1% non-essential amino acids (GIBCO), 0.03% glutamine and 10 U penicillin/10 μg streptomycin/0.48 mg gentamycin/ml. Maintenance medium was prepared as above, except that it contained 2% newborn calf serum.

HSV-1 strains GC+ Miyama (GC+) and D129 were originally obtained from Richard Dix, Miami. Stocks of these viruses were grown in VERO cells at 34°C and harvested at 24–48 h post infection (p.i.). HSV-1 strains D129 and GC+ form syncytia. Virus titers were determined by plaque assay in VERO cells using maintenance medium containing 0.5% methylcellulose as an overlay and stained with 0.5% crystal violet in 70% ethanol.

Antisera

Neutralizing anti-HSV antiserum, prepared by immunizing New Zealand white

rabbits with Triton X-100 extracts of HSV-1 KOS infected cell membranes, had a titer sufficient to neutralize >100 pfu at a 1/200 dilution. Monoclonal antibody directed to gB was a gift from N. Balachandran, Philadelphia. Monospecific rabbit antibodies to gC and gD were gifts from G. Cohen, Kansas City. FITC-goat anti-mouse antibody was from HyClone and FITC-goat anti-rabbit antibody was from Sigma.

Determination of the size of syncytia

VERO and HEp-2 cells grown on 12 mm diameter coverslips were infected at an MOI of 0.01 with HSV-1 GC+ for 1 h at 37°C. The virus inoculum was replaced with maintenance medium containing varying concentrations of tromantadine and incubated at 34°C for 23 h. The conditions of infection yielded 100–300 syncytial foci per coverslip. The cells were fixed and stained with crystal violet, mounted with glycerol and viewed. The outlines of the syncytia were manually traced and their areas determined by computer assisted analysis using the BIOQUANT™ (R & M Biometrics, Nashville, TN) program. The cell equivalent area was determined by dividing the average area of ten syncytia by the average area of ten uninfected single cells of the same monolayer to give an estimate of the average number of cells involved in each syncytium.

Time course of tromantadine and cycloheximide action on HSV-1 induction of syncytia

The protocol for this study was adapted from Kousoulas et al. (1978, 1982). HEp-2 cells grown in 24-well plates were incubated with 500 plaque forming units of HSV-1 GC+ for 1 h at 37°C for the plaque reduction type assay. The inoculum from each cell monolayer was replaced with 1 ml maintenance medium and untreated or treated with either tromantadine (final concentration 200 μg/ml) or cycloheximide (20 μg/ml final concentration) at various times p.i. The concentration of cycloheximide was empirically determined to allow inhibition and reversibility of the inhibition of syncytium formation. The cells were stained with crystal violet at 24 h p.i. and the number of syncytia with >6 nuclei per cell were counted.

Alternatively, VERO cells infected at an MOI of 0.04 of HSV-1 strain GC+, were untreated or treated initially with tromantadine (200 μg/ml) at 1 h p. i. and the treatment was continued or the medium was replaced by new medium [with or without cycloheximide (25 μg/ml)] at 12 h p.i. The monolayers were stained at 48 h p.i. and the number and size of syncytia were evaluated.

Immunofluorescence

HEp-2 cells grown on coverslips were infected at a MOI of 0.01 of HSV-1 GC+ and treated at 1 h p.i. with 300 μg of tromantadine in 1 ml medium. At 24 h p.i., the unfixed cells were washed and then incubated with antibody specific for gB, gC or gD at 37°C for 1 h. Viable cells were stained to limit access of the antibody to the cell surface. Similar results were obtained upon longer incubation periods at 4°C.

Antibody binding to the cells was visualized with FITC-goat anti-immunoglobulin antibody. Cells were washed and then fixed with acetone prior to viewing.

Results

Tromantadine inhibits virus production and reduces the number and size of syncytia

Tromantadine treatment reduced the number and size of syncytia induced by HSV-1 GC+ or D129 in either HEp-2 or VERO cells when added at 1 h after infection. The drug treatment also reduced plaque and virus production. All tromantadine treatments in this study were initiated 1 h after infection to separate the drug effects on syncytia from those on the early tromantadine-sensitive steps in virus replication. Rounding or loss of cells from the monolayer indicative of toxicity was not observed at the treatment levels of tromantadine.

The efficacy of the drug on syncytium formation was dependent on MOI. Complete inhibition of syncytium formation could be observed upon tromantadine treatment (200 μg/ml) of VERO or HEp-2 cells infected at an MOI of 0.01 of HSV-1 GC+. At an MOI of 0.1 or 1, syncytium formation was reduced from +++++ (100% cellular involvement in syncytia) to + (<25%) following treatment with tromantadine at 400 μg/ml, but only to ++ (25%) and +++ (50%), respectively, with tromantadine at 200 μg/ml.

Addition of tromantadine 1 h p.i. also inhibited virus production, as determined by plaque assay of the extracellular medium, but to a lesser extent than syncytium formation. Virus was assayed at 12 h p.i. to determine tromantadine action on a single cycle of virus replication. A 10–20% reduction was observed upon treatment with tromantadine at 50–200 μg/ml, a 40% reduction at 300 μg/ml and 55% reduction at 400 μg/ml. In addition, reductions in plaque number were noticed in several of the experiments described in this report.

Computer assisted image analysis was used to compare the effects of tromantadine on syncytial size (Fig. 1). Reduction of syncytial size in infected HEp-2 cells occurred at a concentration greater than 50 μg/ml and complete inhibition of syncytium formation was observed at 200 μg/ml. Syncytial size was represented as the average area of 10 representative syncytia divided by the average area of 10 uninfected cells of the same monolayer. Maximal inhibition of syncytium formation in infected VERO cells required a tromantadine concentration of 300 μg/ml. Tromantadine treatment reduced the size of syncytia to five cell equivalents for HEp-2 cells and to ten cell equivalents for VERO cells. Monolayers of uninfected, treated or untreated cells, normally contain a small number of cells with three to six nuclei.

Time course of tromantadine inhibition of syncytium formation

The time when tromantadine initiates inhibition of syncytium formation was determined by adding a fusion-inhibitory concentration of tromantadine (200 μg/ml) at different times p.i. and then evaluating the number of syncytia at 24 h p.i., the earliest time syncytia were discernable (Fig. 2). Addition of the drug for up to

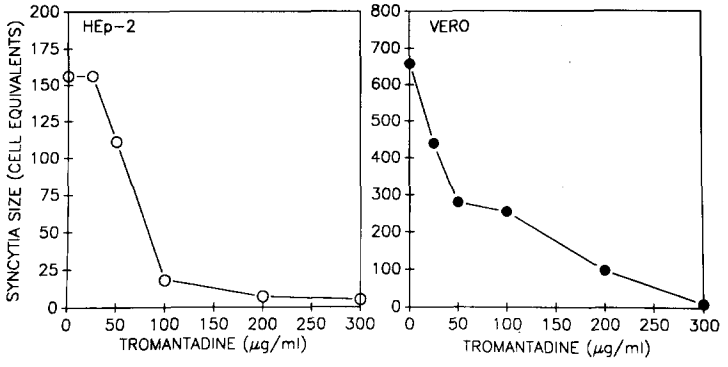

Fig. 1. Tromantadine treatment reduces the size of syncytia. VERO or HEp-2 cells grown on 12-mm coverslips were infected at 0.01 MOI with HSV-1 GC+ and treated with various concentrations of tromantadine 1 h p.i. The monolayers were fixed and stained with crystal violet 24 h later. Individual syncytia were traced and the areas calculated by BIOQUANT™ (R & M Biometrics, Nashville, TN) computer assisted image analysis. The size of the syncytia is represented by the average number of cell equivalents per syncytium. Cell equivalents were defined as the ratio of the average areas of ten syncytia divided by the average area of ten individual cells from the same monolayer.

9 h p.i. completely inhibited syncytium formation in GC+ infected HEp-2 cells. Small syncytia (>6 nuclei) were observed and distinguishable in cells treated with tromantadine at 10 h p.i. or later. Inhibition of protein synthesis with cycloheximide (20 μg/ml) blocked syncytium formation when added up to 7 h p.i. The number of syncytia observed in the cell monolayers treated with tromantadine after 9 h p.i. or treated with cycloheximide after 7 h p.i. were similar to those for untreated,

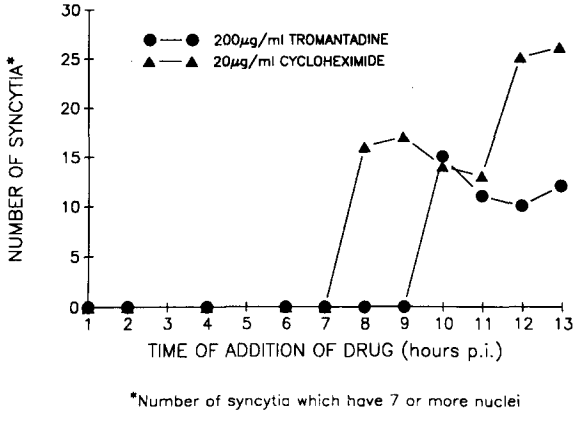

Fig. 2. Time course of tromantadine and cycloheximide inhibition of HSV-1 syncytium formation. HEp-2 cells grown in 24 well tissue culture plates were infected with 500 plaque forming units of HSV-1 GC+. At varying times p.i., the medium was replaced with new medium containing either 200 μg/ml tromantadine or 20 μg/ml cycloheximide. The cells were fixed and stained with 0.5% crystal violet (in 70% ethanol) at 24 h p.i. The syncytia containing more than six nuclei were counted.

infected monolayers. Initial production of gB occurs at approximately 7 h p.i. (Spear, 1985). The shift in time-dependent sensitivity between the cycloheximide and tromantadine actions suggests that tromantadine inhibits a step subsequent to the synthesis of the fusion protein(s).

Requirement for new protein synthesis to reverse tromantadine inhibition of syncytium formation

VERO cells infected with HSV-1 GC+ were untreated or initially treated with 200 μg/ml tromantadine for 12 h (at 1 h p.i.), the drug was removed, subsequent protein synthesis was inhibited with 25 μg/ml cycloheximide or not, and the cells were evaluated at 48 h p.i. Extensive syncytium formation occurred in cell monolayers which had received no tromantadine or from which tromantadine had been removed after the initial 12 h treatment. The latter result indicates that tromantadine inhibition is spontaneously reversed with time. However, no syncytia and a 200-fold plaque reduction were observed in cell monolayers treated with cycloheximide after the initial 12 h tromantadine treatment period. No syncytia and a 2000-fold plaque reduction were observed in cells treated with tromantadine for 47-h p.i. Thus, cycloheximide treatment prevented the spontaneous recovery from the tromantadine treatment to yield results similar to those obtained if tromantadine had been present during the entire period. Similar experiments showed that ammonium chloride inhibition of syncytium formation was reversible in the presence of cycloheximide indicating that in this case new protein synthesis was not required to reverse the inhibition of syncytium formation (Kousoulas et al., 1978, 1983b).

Expression of HSV-1 glycoproteins on the surface of tromantadine treated cells

Cell surface expression of gC, gB and gD on untreated and tromantadine-treated HSV-1 (GC+)-infected cells was examined by indirect immunofluorescence using monoclonal antisera directed to gB (gift from N. Balachandran) and monospecific rabbit antibody to gC and to gD (gifts from G. Cohen). No immunofluorescence was obtained in uninfected cells or with normal mouse or rabbit serum controls (data not shown).

Syncytia were apparent in infected, untreated HEp-2 cells and could be identified as large cells with >100 nuclei (Fig. 3A–F). A fluorescent outline of each syncytium was readily observable following immunofluorescent staining with each of the monospecific antisera directed against gB, gC and gD (Fig. 3A–C). In contrast, only small foci of infection with 5 to 15 cells, but not syncytia, were present in cells treated with 200 μg/ml of tromantadine (Fig. 3G–L). These cells still express the gC, gB, and gD glycoproteins on their cell surface as indicated by immunofluorescence (Fig. 3G–I). This shows that tromantadine inhibited syncytia formation and limited the spread of the virus but did not prevent viral glycoprotein synthesis or their transport to the cell surface.

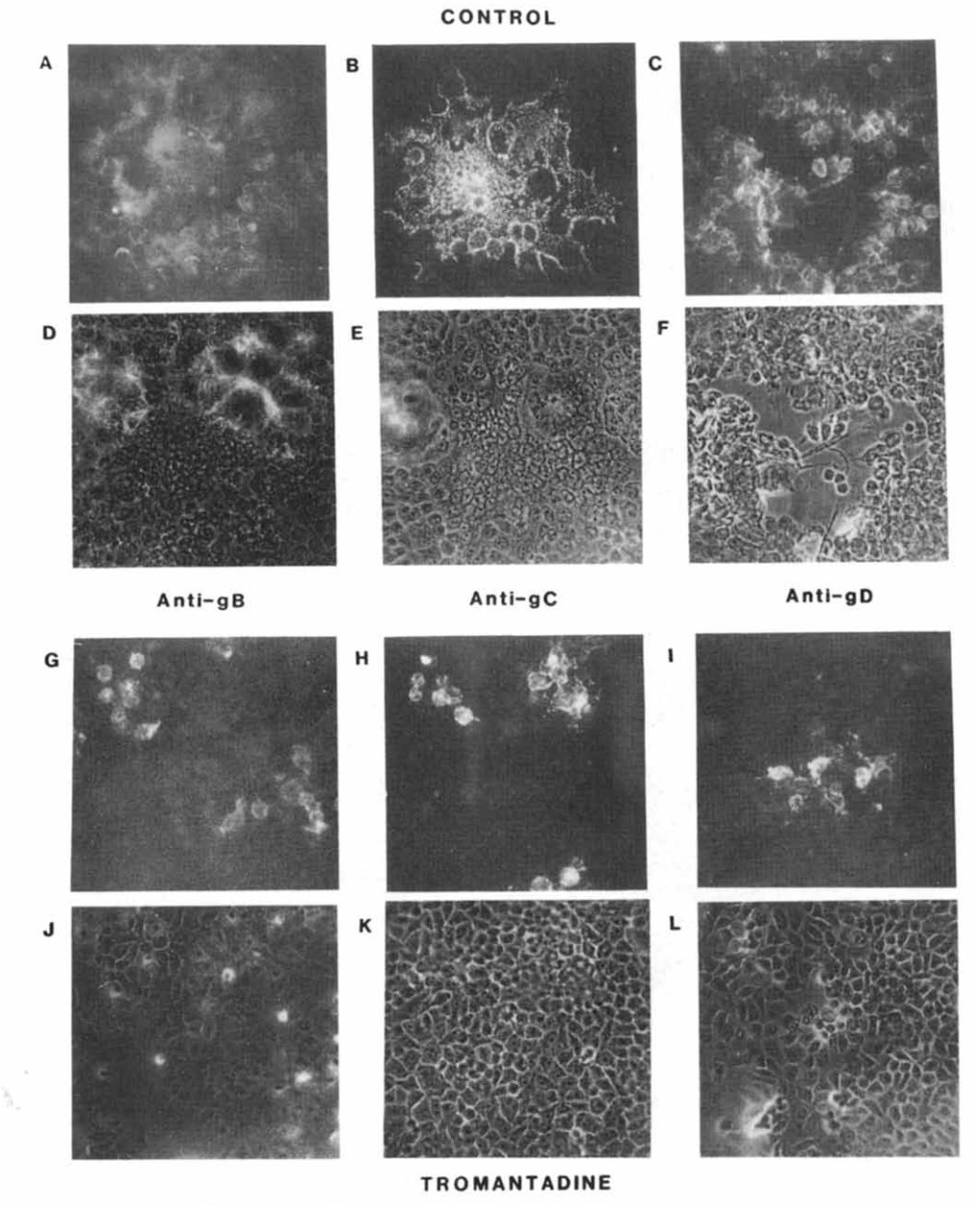

Fig. 3. Immunofluorescence of HSV-1 glycoproteins on the cell surface. HEp-2 cells were grown on coverslips and infected with HSV-1 GC+ at 0.01 MOI. The indicated monolayers were treated with 200 μg/ml tromantadine. At 24 h p.i. the monolayers were washed, incubated with primary anti-glycoprotein antibody and then incubated with FITC-labeled second antibody. The antibodies were added to unfixed cells to limit labeling to the cell surface. The monolayers were fixed with acetone and then viewed in bright field (D–F, J–L) or by fluorescence (A–C, G–I). Fields representative of the plaque morphology

Additive effect of tromantadine and neutralizing antibody

The effect of tromantadine in combination with neutralizing antibody on HSV-1 GC+ infection of VERO cells was tested (Fig. 4). Antibody added 1 h p.i., limited the number but did not prevent syncytia formation. Addition of 300 μg/ml tromantadine at 1 h p.i. inhibited syncytia formation and significantly reduced the number of virus plaques in the monolayer but some were observable as small non-syncytial plaques. No cytopathological effect was observed in the monolayers which received both tromantadine and antibody. Similar results were obtained upon treatment of cell monolayers infected with HSV-1 GC+ or MP clone 2 (data not shown). The combination of antibody and tromantadine appeared to block both the extracellular and intracellular spread of virus to protect the monolayer.

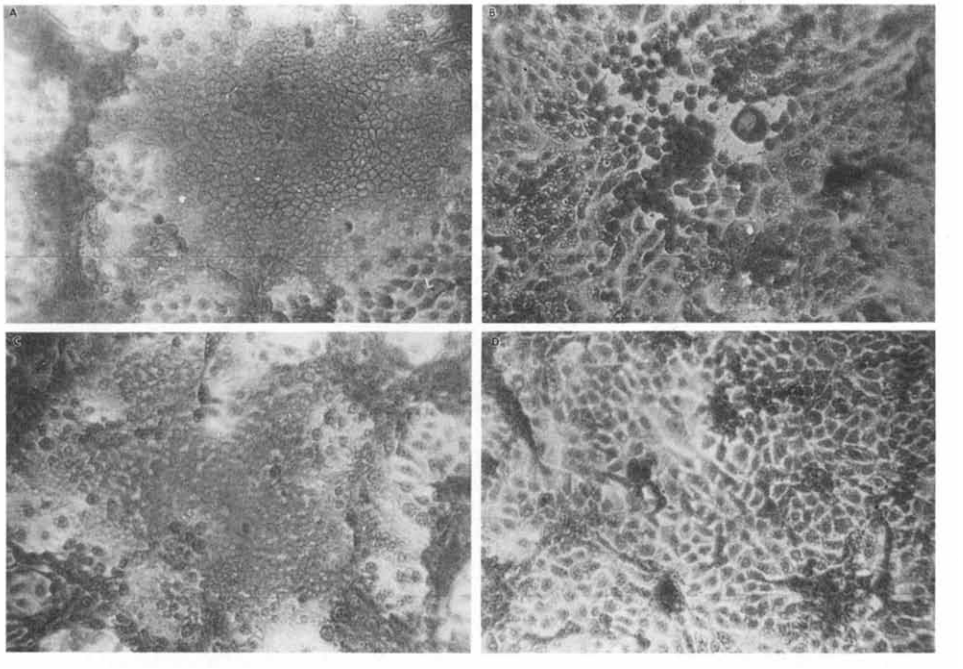

Fig. 4. Additive effect of tromantadine and neutralizing antibody. VERO cells were infected with 100 pfu of HSV-1 D129 and (A) untreated or treated at 1 h p.i. with (B) 300 μg/ml tromantadine, (C) neutralizing rabbit anti-HSV sera or (D) both tromantadine and anti-HSV. The cells were fixed and stained with crystal violet 48 h after infection and fields representative of the plaque morphology present in the monolayer were photographed.

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present in the monolayer were photographed. A–F: untreated, infected cells; G–L: tromantadine treated, infected cells. A, D, G, J: reacted with monoclonal anti-gB; B, E, H, K: reacted with rabbit monospecific anti-gC; C, F, I, L: reacted with rabbit monospecific anti-gD.

Discussion

Tromantadine inhibits HSV-1 GC+ induction of syncytium formation and virus production, when added after the initial tromantadine-sensitive step in virus replication. The inhibition was manifest in more than one cell type and has also been observed for other HSV-1 strains, varicella zoster and pseudorabies virus (data not shown).

Tromantadine inhibition of cell-cell fusion is observed in the presence of virus replication. Syncytium formation is inhibited at drug concentrations which also inhibit virus production, but syncytium formation is more sensitive to tromantadine treatment. The concentration of tromantadine required to inhibit syncytium formation is larger than that required for some agents, such as monensin (Kousoulas et al., 1983a) and cyclosporine A (McKenzie et al., 1987) but smaller than for 2-deoxy-D-glucose (Knowles and Person, 1976), ammonium chloride or adamantanone (Kousoulas et al., 1978; 1983b). However, comparisons between different studies even with the same drug (Hu and Hsiung, 1989) are difficult, because of the influence of the virus strain, cell type and multiplicity of infection on drug efficacy. Different HSV-1 strains also have different propensities towards syncytium formation which also differ from cell type to another (Keller et al., 1970).

HSV-1 induced fusion from within requires viral glycoprotein synthesis and glycan processing (Marsden, 1987; Spear, 1985; Datema, 1987). The tromantadine-sensitive step for inhibition of syncytium formation occurs late in the replication cycle, approximately 2 h after the synthesis of the protein(s) required for cell-cell fusion. Glycoproteins gB, gC and gD are still formed and expressed on the cell surface in the presence of tromantadine, despite the inhibition of syncytium formation. These glycoproteins are involved in syncytium formation and virus binding, and are also expressed at different times during the replication cycle (Marsden, 1987).

The characteristics of the tromantadine inhibition of syncytium formation are different from those of other inhibitors of HSV-induced fusion. Unlike monensin, which inhibits syncytium formation by blocking the processing and translocation of the fusion glycoproteins to the cell surface (Johnson and Spear 1982; Kousoulas et al., 1983a), tromantadine inhibits syncytium formation but not the synthesis or cell surface expression of HSV-1 glycoproteins. Reversal of tromantadine inhibition of syncytium formation requires new protein synthesis which suggests that tromantadine affects the synthesis or structure of a protein required for fusion. This is unlike ammonium chloride (Kousoulas et al., 1978; 1982) and cyclosporin A (McKenzie et al., 1987), even though both cyclosporin A (McKenzie et al., 1987) and tromantadine (Cheetham and Epand, 1987) interact with and disrupt membrane structure.

Other studies in our lab (Ickes, 1989; submitted for publication) indicate that tromantadine blocks the processing of the carbohydrate portion of the viral glycoproteins. HSV-induced fusion and virus production can be inhibited by inhibitors of glycoprotein synthesis (Kumarasamy and Blough, 1984; Bzik et al., 1982; Ludwig et al., 1974; Datema et al., 1987). Glycoprotein processing also occurs within the 2-h period which follows synthesis of the HSV fusion protein and the tromantadine

sensitive step in syncytium formation.

The effect of tromantadine on syncytium formation must be achieved by a different mechanism than for HSV attachment and penetration, since the latter processes do not require synthetic events.

Tromantadine inhibition of syncytium formation demonstrates an alternative approach to controlling HSV infections. Syncytium formation and direct cell-cell spread are important means by which HSV and other viruses escape antibody detection and elimination (Lodmell et al., 1973). In cell culture, the activities of tromantadine and neutralizing antibody appear to add up to prevent virus spread and cytopathology. Thus, the combination of an antiviral agent such as tromantadine with neutralizing antibody may help in controlling the HSV infection.

Acknowledgements

This work was supported by a grant from Merz and Co., Frankfurt, F.R.G. and the Ohio Board of Regents Research Challenge Program 1987–1989 funds. We thank Karen Janiga, Jeanette Killius and Darlene Walro for their assistance.

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