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Inhibition by Monensin of Human Cytomegalovirus DNA Replication

By

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With 6 Figures

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Summary

Monensin, at concentrations which depended on the multiplicity of infection, was found to prevent DNA replication of human cytomegalovirus (HCMV) as well as production of viral progeny in human foreskin fibroblasts. The drug did not affect DNA replication of herpes simplex virus. Inhibition of consecutive HCMV DNA synthesis was also observed following delayed addition of the drug within 12–24 hours postinfection, but was fully reversible upon its removal. Viral replication proceeded, however, without impairment in cultures treated with monensin prior to infection. Induction of viral DNA polymerase activity was not impeded by the inhibitor. Analysis of protein- and glycoprotein synthesis revealed that monensin interfered with the production of a number of HCMV-specific polypeptides. Furthermore, evidence was obtained that the drug may hinder intracellular transport of a 135 kd glycopolypeptide.

Introduction

The ionophore monensin, an antibiotic from streptomyces cinnamonensis, has found wide application recently for the study of the biosynthesis of viral glycoproteins (13, 14, 21, 38). Although there is evidence for its multiple sites of action (33) this compound apparently does not interfere, like e.g. tunicamycin, with the primary steps of glycosylation but with glycoprotein transport within the golgi apparatus and thus with the later stages of their processing (36). For enveloped viruses this mode of action implicates a significant reduction of release of progeny virus from

infected cells (14, 24). In addition, assembly may be affected of those viruses which bud from the outer plasma membran (12, 15). With regard to the herpesviruses which receive their envelope at the nuclear and inner cytoplasmic membranes (11) most authors agree that monensin mainly affects viral egress whereas viral morphogenesis is hardly impaired (13, 14). Furthermore, the expression in the presence of monensin of herpes simplex virus-induced surface membrane antigens as targets for immunolysis is either reduced or abolished (22, 42) while it appears to be unimpaired in bovine herpes virus-infected cells (38).

As compared with other members of the human herpesvirus group human cytomegalovirus (HCMV) presents several characteristic properties, e.g. pronounced species specificity, a very long replication cycle (4, 5), and a particular dependence on host cell functions (7, 20). Yet another property which distinguishes the HCMV host cell system is the sensitivity of virus-induced DNA replication to inhibitors of glycosylation, such as 2-deoxy-D-glucose and tunicamycin (28, 39) which are ineffective, on the other hand, on DNA replication in herpes simplex virus (HSV)-infected cells (28). Evidence was forwarded in this context for the HCMV system for an "early" virus-induced chromatin-associated glycoprotein as the target for the inhibitors (39).

In this investigation these initial observations are verified and extended by the use of monensin which was found also to prevent HCMV DNA replication. Furthermore, our experiments suggest that the drug may affect intracellular transport of a virus-induced glycoprotein.

Materials and Methods

Cells and Viruses

Human foreskin fibroblasts (HFF; generously donated by Dr. B. Fleckenstein, Erlangen, Federal Republic of Germany) were used between the 16th and 25th passage for all experiments as well as for virus propagation (28). The monolayers were cultivated in plastic flasks of various sizes (Nunc, Wiesbaden, Federal Republic of Germany; 25, 75, and 175 cm^2 corresponding to 1.5×10^6, 5×10^6 and 1.2×10^7 cells) in Eagle's minimum essential medium with Earle's salt solution (MEM, Gibco, Eggenstein, Federal Republic of Germany) supplemented with 200 units penicillin plus 50 µg gentamycin/ml and 10 per cent fetal calf serum (FCS). All experiments were performed with cultures partially arrested by serum deprivation (0.2 per cent FCS for 72 hours), beginning on average 3 days after the cultures reached confluency (3, 31). The Towne strain of HCMV (5) was propagated on confluent HFF at a multiplicity of infection (MOI) of 0.1 using 2 per cent FCS. The KOS strain of herpes simplex virus type 1 (HSV-1; 26) was propagated in the same cells in serum-free medium. Virus stocks of HCMV and HSV-1 were prepared as described previously (4, 26). For purification HCMV was collected from the cell-free culture medium and subjected to centrifugation on gradients of 20—70 per cent sucrose (6).

Experimental infections of HFF were performed with stock virus at the desired MOI as indicated in Results. Under the conditions used about 70—95 and 50 per cent of the HCMV- or HSV-infected cells, respectively, contained viral antigen (28). Virus titers were

determined by the endpoint dilution method combined with indirect immunofluorescence for viral antigen (4, 31).

Isotopic Labelling Procedures

For pulse labelling of DNA, ^{3}H-thymidine (sp.act. 21 Ci/mmol) was used at 5 μCi/ml culture medium. Protein and glycoprotein labelling was performed with ^{35}S-methionine (sp.act. 1120 Ci/mmol) and ^{3}H-mannose (sp.act. 54 Ci/mmol) or ^{3}H-glucoseamine (sp.act. 36 Ci/mmol), using 10 μCi and 5 μCi/ml of culture medium which was either depleted of methionine or, in the case of tritiated sugars, in which glucose was replaced by sodium pyruvate (18, 34). Labelling of glycoproteins of purified HCMV *in vitro* with ^{3}H-borohydride (sp.act. 17 Ci/mmol) was carried out as described by LUUOKONEN *et al.* (19). All radiochemicals were purchased from Amersham Buchler (Frankfurt, Federal Republic of Germany).

Analysis of DNA

DNA was extracted from labelled cells following lysis with 1 per cent sodium dodecyl sulphate (SDS) by phenol/chloroform/isoamyl alcohol treatment (10, 31). The DNA content of the samples was estimated by the method of GILES and MYERS (9). Separation of viral and host cell DNA was achieved by isopycnic centrifugation in neutral CsCl (mean density 1.71 g/ml) where HFF DNA bands at a density of 1.699 g/ml, HCMV DNA at 1.717 g/ml and HSV-1 DNA at 1.725 g/ml (28).

Determination of DNA Polymerase Activity

To determine DNA polymerase activity HFF (5×10^6 cells) were subjected to the desired treatment, washed twice with cold phosphate buffered saline (PBS) and harvested in PBS by scraping at 48 hours p.i. prior to solubilization in 0.5 ml TNT buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl and 0.5 per cent Triton X-100) and sonication at maximum setting for 2×10 seconds with a Branson sonifier. Following sedimentation of the insoluble material at $2000 \times g$ the supernatant cellular extract was examined for DNA polymerase activity. The basic assay contained in 200 μl (10, 27): 100 μg of bovine serum albumin (BSA), 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), 100 μg of activated calf thymus DNA (41), 0.1 mM of dATP, dCTP, dGTP, 10 μM ^{3}H-TTP as the labelled substrate (sp.act. 1000 ct/min/pmol), and 20 μl of cellular extract corresponding to about 30 μg of protein. KCl and/or inhibitors were supplemented at the concentrations indicated in Results. Incubations were performed in duplicate assays for 30 minutes at 37° C prior to determination of acid insoluble radioactivity (see below).

Polyacrylamide Electrophoresis in the Presence of Sodium Dodecyl Sulphate (SDS-PAGE) and Fluorography

SDS-PAGE for separation of polypeptides was performed according to LÄMMLI (16) as described by GALLWITZ *et al.* (8) using either total cellular or cytoplasmic and nuclear extracts. For preparation of extracts HFF (1.2×10^7 cells) were washed twice with cold PBS, harvested by scraping in PBS and solubilized in 1 ml TNT supplemented with 0.5 per cent sodium deoxycholate (DOC). Total extracts were obtained by subsequent sonication and sedimentation of the insoluble material (see above). To prepare subcellular extracts sedimentation of nuclei followed directly after treatment with TNT-DOC buffer prior to an additional wash in 1 ml of the same buffer. The combined supernatants were pooled representing the cytoplasmic extract. The nuclear pellet was resuspended in 1 ml TNT-DOC buffer and subjected to sonification followed by sedimentation of the insoluble material. The supernatant was used as the nuclear extract. After electrophoresis the slab gels were subjected to fixation, staining (39) and fluorography according to the procedure of

BONNER and LASKEY (1) using Rotifluoreszint D (Roth, Karlsruhe, Federal Republic of Germany) for impregnation. The latter steps were omitted when further analysis included immunoblotting.

Immunoblotting

For immunoblotting (29, 37), electrotransfer of polypeptides from slab gels after SDS-PAGE to nitrocellulose sheets (BA 85, Schleicher & Schüll, Dassel, Federal Republic of Germany) was performed at 35 V and 250 mA in a chamber constructed in our laboratory (30). The transfer buffer consisted of 20 mM Tris-HCl, pH 8.3, 150 mM glycine and 20 per cent methanol. Indirect immunostaining was carried out at dilutions between 1 : 20 to 1 : 50 of anti-HCMV hyperimmune serum (Biotest Pharma, Dreieich, Federal Republic of Germany) or human reconvalescent sera, tested for the presence of HCMV-specific IgG by standard enzyme-linked immunosorbent assays (ELISA), as the first antibody, and horseradish peroxidase-conjugated rabbit antihuman IgG (Dakopatts, Hamburg, Federal Republic of Germany) as the second antibody at a dilution of 1 : 500. 3,3'-Diaminobenzidine was used as the substrate.

Determination of Acid-precipitable Radioactivity

Trichloroacetic acid (TCA) precipitable radioactivity of labelled macromolecules was determined after transfer of aliquots to glass fiber filters (GF/C, Schleicher & Schüll, Dassel, Federal Republic of Germany) which were successively washed with 10, 5 per cent TCA, ethanol and ether (30) followed by counting in a toluene-based scintillation cocktail (Quickszint, Roth, Karlsruhe, Federal Republic of Germany).

Determination of Protein Content. Chemicals

Protein content in cellular extracts was estimated by the method of LOWRY *et al.* (17). Phosphonoacetic acid (PAA) and monensin were purchased from Sigma, Deisenhofen, Federal Republic of Germany, tunicamycin from Calbiochem, La Jolla, U.S.A.

Results

Inhibition by Monensin of Infected Cell DNA Synthesis

In a typical experiment for the determination of infected cell DNA synthesis in the presence of monensin, parallel cultures of HFF (5×10^6 cells) were infected by HCMV (MOI of 1) and pulse labelled with ^{3}H-thymidine (5 μCi/ml) during the late phase of the consecutive infectious cycle (28). Prior to infection HFF were subjected to serum starvation (3, 31) to avoid cellular stimulation in addition to that by HCMV. Under these conditions concentrations above 1.5 μM effectively abolished virus-mediated induction of precursor incorporation (Table 1, Exp. 1). When a higher MOI was used, the drug concentration had to be raised (Table 1, Exps. 2 and 3) to obtain a comparable suppression of ^{3}H-thymidine uptake. For all subsequent experiments an MOI of 1 was chosen.

In contrast to this, herpes simplex virus (HSV)-induced DNA synthesis proved to be resistant to monensin even at relatively high concentrations (Table 1, Exp. 4). This differential effect of monensin on the two herpes virus-host systems compares well with that of tunicamycin (Table 1, Exps. 1 and 4)

Table 1. *Effect of monensin on induction of DNA synthesis by HCMV, HSV or serum*

Induction by	MOI	Drug treatment	Precursor incorporation (cpm/µg DNA in % of control[b])
Exp. 1[a]			
0.2 % FCS	–	–	23
HCMV	1	–	100[b]
HCMV	1	1.5 µM monensin	16
HCMV	1	6 µM tunicamycin	13
Exp. 2[a]			
HCMV	3	–	100[b]
HCMV	3	1.5 µM monensin	91
HCMV	3	4.5 µM monensin	39
HCMV	3	15 µM monensin	9.7
Exp. 3[a]			
HCMV	10	–	100[b]
HCMV	10	1.5 µM monensin	112
HCMV	10	4.5 µM monensin	41
HCMV	10	15 µM monensin	8.3
Exp. 4[a]			
HSV		–	100[b]
HSV	0.5	7.5 µM monensin	111
HSV	0.5	6 µM tunicamycin	117
Exp. 5[a]			
0.2 % FCS	–	–	39
10 % FCS	–	–	100[b]
10 % FCS	–	7.5 µM monensin	12

[a] Pulse labelling with ^{3}H-thymidine (5 µCi/ml) was from 48–60 hours p.i. in *Exp. 1* and *5*, from 60–72 hours p.i. in *Exp. 2* and *3*, and from 12–24 hours p.i. in *Exp. 4*. Culture medium p.i. contained 0.2 per cent FCS

[b] 100 per cent corresponded to 20,200, 19,800, 12,700, 24,500, and 18,800 ct/min/ µg of DNA in *Exp. 1–5*, respectively

which also prevents HCMV-mediated induction of ^{3}H-thymidine incorporation but fails to inhibit that triggered by HSV (28).

Serum-mediated induction of precursor uptake, on the other hand, was again sensitive to the action of monensin (Table 1, Exp. 5), whereas arrested HFF were not inhibited (not shown). Subsequent analysis of infected cell DNA by isopycnic centrifugation in neutral CsCl revealed that inhibition of the HCMV-host cell system in the presence of monensin was indeed due to prevention of thymidine incorporation into viral DNA (Fig. 1 A and B). Labelling of HSV DNA, on the other hand, was not affected by the drug (Fig. 1 C and D).

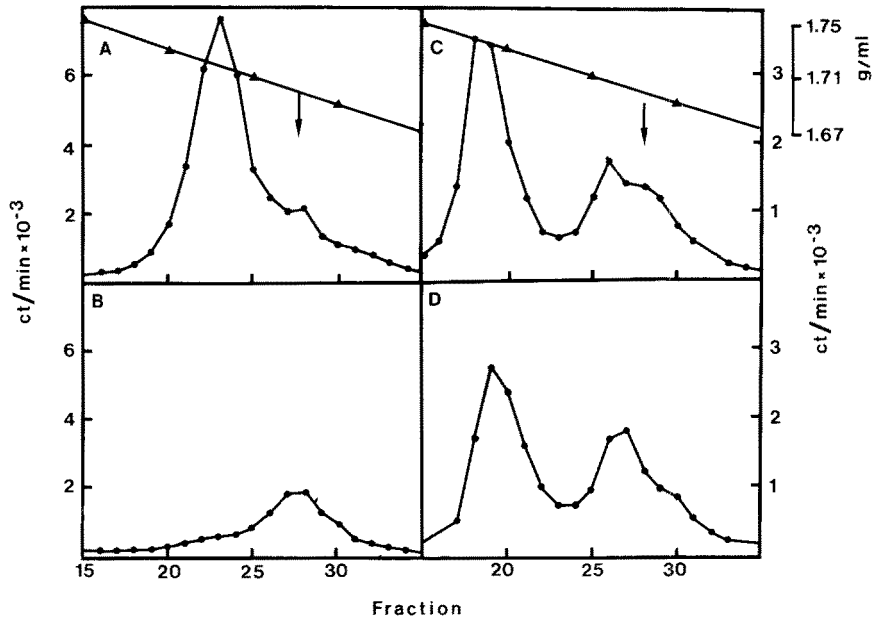

Fig. 1. Isopycnic centrifugation in neutral CsCl (mean density 1.71 g/ml) of DNA extracted from untreated (*A* and *C*) and monensin-treated (*B* and *D*) HCMV- (*A* and *B*) or HSV-infected (*C* and *D*) HFF pulse labelled with ^{3}H-thymidine (5 μCi/ml) from 60–72 or 12–24 hours p.i., respectively. The monensin treatment (4.5 μM for HCMV-infected and 7.5 μM for HSV-infected cultures) was initiated 1 hour p.i. and extended until harvest of the cells. 7 and 4 μg DNA from HCMV- and HSV-infected cells, respectively, were analyzed per gradient. The arrows indicate the position of ^{14}C-labelled marker DNA from HFF which was included in each gradient

As expected from these observations, the drug also prevented the production of intra- as well as extracellular HCMV progeny (not shown).

Viral DNA Synthesis in Pretreated HFF and Reversibility of the Drug Effect

To examine whether pretreated cells support viral replication, confluent serum-starved HFF were exposed to various concentrations of monensin prior to virus infection and subsequently pulse labelled with ^{3}H-thymidine during the interval of expected viral DNA synthesis (28; Fig. 2 A). Pretreatment with drug concentrations up to 7.5 μM had no inhibitory effect on consecutive precursor uptake under our conditions. Likewise, analysis of the DNA showed that synthesis of viral DNA was essentially unimpaired, as compared with the control (Fig. 2 A, w/o and monpre). Pretreatment with higher drug concentrations (e.g. 15 μM) which induced signs of beginning cytotoxicity, led to a reduced ^{3}H-thymidine incorporation into viral DNA (not shown).

In a further experimental setup cultures were infected and subsequently kept in the presence of monensin (4.5 μM) for 48 hours. At this time the drug was removed the cultures refed with fresh medium without inhibitor and subjected to three sequential pulses with tritiated thymidine for 24 hour intervals each, to monitor DNA synthesis (Fig. 2 B). This protocol again did not prevent subsequent recovery of viral DNA replication within 48–72 hours after removal, an observation which was based on the comparative analysis by isopycnic centrifugation in CsCl of DNA samples from untreated and drug-treated infected cultures labelled during corresponding pulse intervals (Fig. 2 B, w/o and monint).

Effect of Monensin During the Infectious Cycle

The following experiments served to determine differences in sensitivity of HCMV-induced DNA synthesis to the drug during the infectious cycle. For this purpose serum-starved HFF were infected as described above, drug addition (4.5 μM), however, followed only delayed at 12, 24, 36, 48 and 60 hours p.i. prior to pulse labelling from 60–72 hours p.i. (Fig. 3).

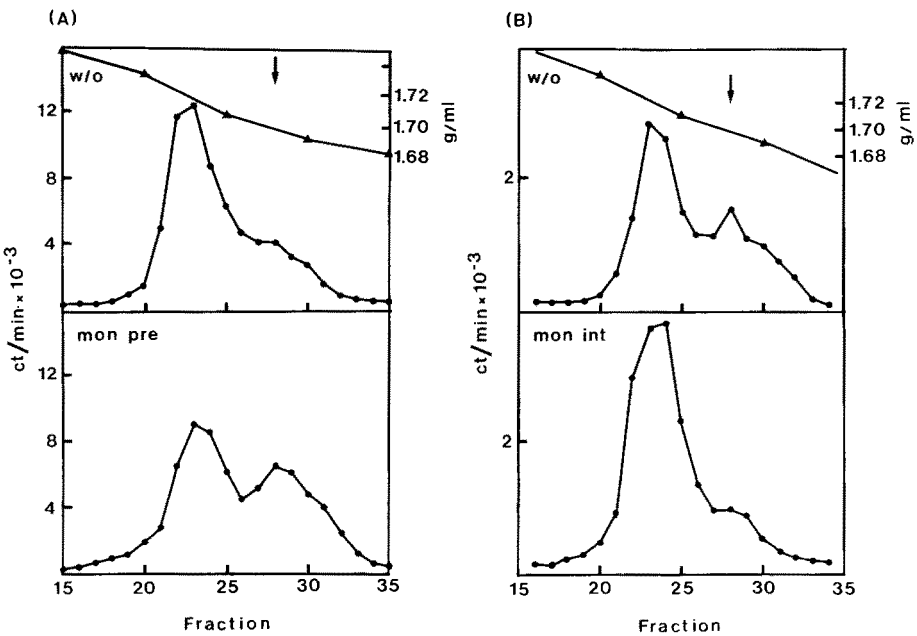

Fig. 2. Isopycnic centrifugation in neutral CsCl (mean density 1.71 g/ml) of pulse labelled DNA extracted from *A* HCMV-infected HFF which were left untreated *(w/o)* or exposed to monensin (*monpre;* 7.5 μM) prior to infection and pulse labelled (5 μCi ^{3}H-thymidine/ml) from 60–72 hours p.i.; and from *B* HCMV-infected HFF which were left untreated *(w/o)* or exposed to monensin (*monint;* 4.5 μM) from 1–48 hours p.i. Pulse labelling with tritiated thymidine (5 μCi/ml) was from 48–72 hours *(w/o)* and 96–120 hours p.i. *(monint)*. 8 and 3 μg of DNA were analyzed per gradient in *A* and *B*, respectively. The arrows indicate the position of ^{14}C-labelled marker DNA from HFF which was included in each gradient

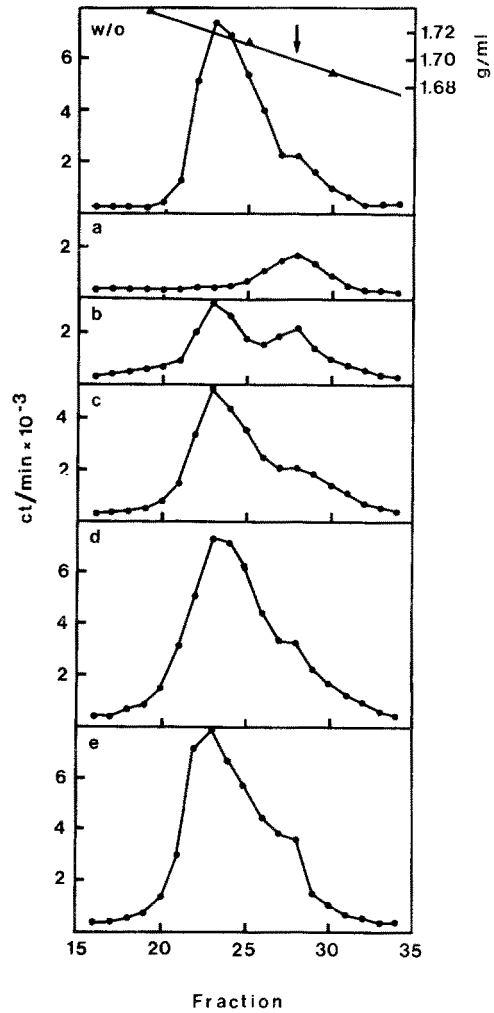

Fig. 3. Isopycnic centrifugation in neutral CsCl (mean density 1.71 g/ml) of DNA extracted from pulse labelled (5 μCi ^{3}H-thymidine/ml from 60–72 hours p.i.) HCMV-infected HFF which were left untreated *(w/o)* or exposed to 4.5 μM of monensin starting at 12 hours *(a)*, 24 hours *(b)*, 36 hours *(c)*, 48 hours *(d)* and 60 hours *(e)* p.i., respectively, until harvest of the cultures at the end of the pulse interval. 6 μg of DNA were analyzed per gradient. The arrow indicates the position of ^{14}C-labelled marker DNA from HFF which was included in each gradient

Untreated infected parallel cultures were labelled during the same interval as a control (Fig. 3 w/o). This experimental approach showed that drug addition until 12 hours p.i. was effective in complete abolishment of consecutive viral DNA synthesis (Fig. 3 a). When added before 36 hours p.i. monensin reduced (Fig. 3 b and c), drug addition at later times did not impair precursor incorporation into viral DNA (Fig. 3 d and e).

Induction of Viral DNA Polymerase Activity in the Presence of Monensin

To exclude the obvious possibility of suppression by monensin of induction of the viral DNA polymerase, cell extracts were prepared from drug-treated infected HFF at 48 hours p.i. and their activities compared with those of appropriate control extracts (Table 2). Under the conditions used monensin did not impede induction of enzyme activity showing the characteristic properties with regard to *in vitro* PAA-sensitivity and salt stimulation. In addition, the drug did not inhibit DNA polymerase activity in the *in vitro* assay (Table 2).

Table 2. *DNA polymerase activity in extracts from monensin-treated HCMV-infected HFF*

Induction by	Inhibitor treatment of cell cultures[a]	Units[b] of DNA polymerase activity / 100 µg protein of cell extract			
		w/o KCl	+ PAA[c]	+ KCl[c]	KCl + monensin[c]
0.2 % FCS	–	0.42	0.52	0.32	–
HCMV + 0.2 % FCS	–	42.9	13.2	72.6	79.2
HCMV + 0.2 % FCS	0.7 mM PAA	25.7	8.06	44.5	–
HCMV + 0.2 % FCS	4.5 µM monensin	39.9	13.5	64.9	63.8
HCMV + 0.2 % FCS	6 µM tunicamycin	42.5	23.4	117.7	–

[a] Infected cultures were treated with the inhibitors from 1–48 hours p.i.

[b] One unit of DNA polymerase is defined as the activity that catalyzes the incorporation of 1 pmol of ^{3}H-TTP into an acid-insoluble form in 30 minutes

[c] The concentration used in the DNA polymerase assay were 70 µM PAA, 100 mM KCl and 1.5 µM monensin, respectively

Viral Protein and Glycoprotein Synthesis in Drug-treated HFF

From the observed suppression of HCMV DNA synthesis one should also expect an impairment in the presence of monensin of expression of (late) viral polypeptides. To prove this conjecture parallel cultures (5×10^{6} cells) of infected HFF were subjected to drug treatment and pulse labelled with ^{35}S-methionine (Fig. 4 A) or with tritiated sugars (Fig. 5) from 60–72 hours p.i. At the concentrations used monensin prevented the virus-mediated increase in precursor uptake, i.e. the specific radioactivities of the protein samples from drug-treated infected cells equaled those from uninfected cells in the case of ^{35}S-methionine-labelled extracts (approximately 1500 cpm ^{35}S/µg protein), and were decreased to about 50 and 20 per cent, respectively, of those of the control cells following labelling with tritiated sugars (390 and 830 cpm ^{3}H/µg protein for ^{3}H-mannose- and ^{3}H-glucosamine-labelled extracts, respectively). Analysis by SDS-PAGE and fluorography revealed for the methionine-labelled extracts that monensin inhibited synthesis of the most prominent polypeptides (Fig. 4 A, lanes b and e) of 135 and 67 kilo-

daltons (kd) which are assumed to represent known major viral proteins (23, 35, 40). As compared to the cultures exposed to PAA (Fig. 4 A, lane c; 30) monensin treatment also prevented labelling of polypeptides of about 100 and 50 kd (Fig. 4 A, lanes c and e). These observations were essentially

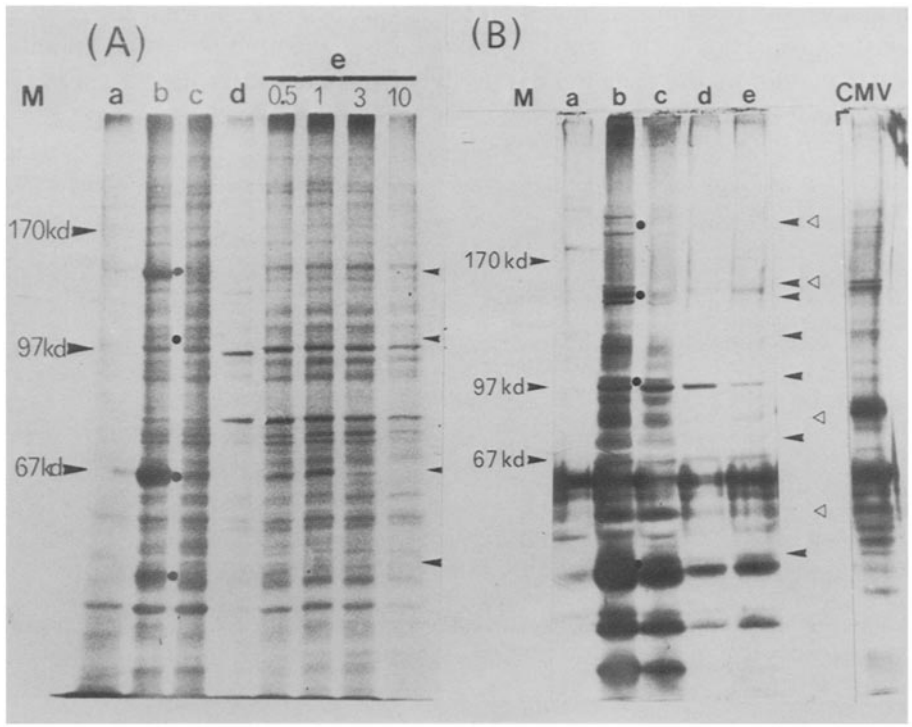

Fig. 4. *A* Fluorogram of SDS-PAGE of extracts from pulse labelled (10 μCi ^{35}S-methionine/ml from 60–72 hours p.i.) uninfected *(a)* and HCMV-infected *(b–e)* HFF which were left untreated *(a, b)* or exposed to 0.7 mM PAA *(c)*, 6 μM tunicamycin *(d)* and various concentrations of monensin (*e;* 0.75, 1.5, 4.5 or 15 μM), respectively. Drug treatment was from 1 hour p.i. until harvest of the cells at the end of the pulse interval. Care was taken to analyze comparable amounts of protein (50 μg) in each track. The circles indicate the positions of virus-induced polypeptides, the arrows on the right-hand side the positions of several suppressed by monensin. The molecular weights *(M)* on the left-hand side indicate electrophoretic mobilities of reduced alpha-2-macroglobulin (170 kd), phosphorylase b (97 kd) and bovine serum albumin (67 kd). *B* Immunoblot with HCMV-specific antiserum of SDS-PAGE of extracts from uninfected *(a)*, HCMV-infected untreated *(b)*, PAA-treated *(c)*, tunicamycin- *(d)*, or monensin-treated (*e;* 4.5 μM) infected HFF and of a partially purified HCMV preparation *(CMV)*. Drug treatment was from 1 hour p.i. until harvest of the cells at 72 hours p.i. 50 μg of protein were analyzed in each track, except in track *(CMV)* where 10 μg were loaded. The circles indicate the positions of major virus-induced polypeptides also recognized during biosynthetic labelling in *A*, the arrows on the right-hand side several suppressed by monensin, the open triangles label the positions of assumed late polypeptides affected by the drug. The molecular weights *(M)* indicate the positions of the blotted marker proteins used in *A*

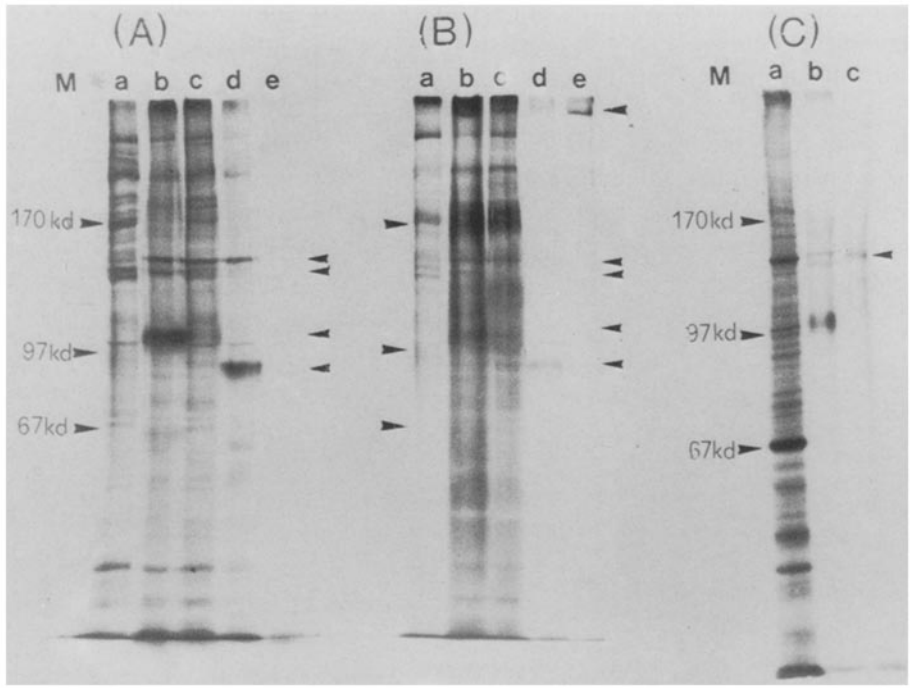

Fig. 5. *A* and *B* Fluorograms of parallel SDS-PAGE of extracts from pulse labelled (5 μCi ^{3}H-mannose/ml *(A)* or 5 μCi ^{3}H-glucosamine/ml *(B)* from 60–72 hours p.i.) uninfected *(a)* and HCMV-infected *(b–e)* HFF which were left untreated *(a, b)* or exposed to 0.7 mM PAA *(c)*, 4.5 μM monensin *(d)* or 6 μM tunicamycin *(e)*. Drug treatment was from 1 hour p.i. until harvest of the cells at the end of the pulse interval. Approximately 50 μg of protein was loaded in each track. The arrows on the right-hand side indicate the positions of polypeptides that were affected by the presence of monensin or tunicamycin. The molecular weight markers *(M)* indicated on the left-hand side correspond to those listed in the legend of Fig. 4 A. *C* Fluorogram of parallel SDS-PAGE of extracts from untreated HCMV-infected ^{35}S-methionine-labelled *(a)* or ^{3}H-mannose-labelled HFF (*b*; see Figs. 4 A and 5 A) and of a partially purified HCMV preparation labelled *in vitro* with ^{3}H-borohydride *(c)*. 50 μg of protein were loaded in *a* and *b* each, 10 μg in *c*. The arrow on the right-hand side indicates the position of a virus-induced 140 kd polypeptide. The molecular weights *(M)* on the left-hand side indicate the positions of the marker proteins used in *A* and *B*

verified and extended by immunoblotting of the samples with human HCMV- specific reconvalescent sera (Fig. 4 B). The latter technique showed in addition suppression by the drug of two polypeptides in the range of 200 kd and a further two of about 115 and 85 kd, respectively (Fig. 4 B, lane e). Of the three viral polypeptides recognized in the molecular weight range of approximately 130/140 kd (130, 135 and 140 kd; Fig. 4 B, lane b), the amounts of the 130 and 140 kd products appeared to be particularly reduced in the presence of monensin (Fig. 4 B, lane e). Comparison with the polypeptides stained in a parallel blot of a preparation of partially purified

virus suggested that the drug apparently lowered synthesis of several viral structural proteins (Fig. 4 B, lanes e, open triangles and CMV), e.g. those of 200, 140, 80 and about 60 kd.

Analysis of the extracts after labelling with tritiated sugars (Fig. 5) showed that monensin interfered with glucosamine- but not mannose incorporation into a 140 kd product (Fig. 5 A and B, lanes b–d). Uptake was largely reduced, on the other hand, with both precursors into a 130 kd glycopolypeptide (Fig. 5 A and B, lanes b and d). Interestingly, the main glycoprotein of the ^{3}H-borohydride labelled purified virus preparation migrated

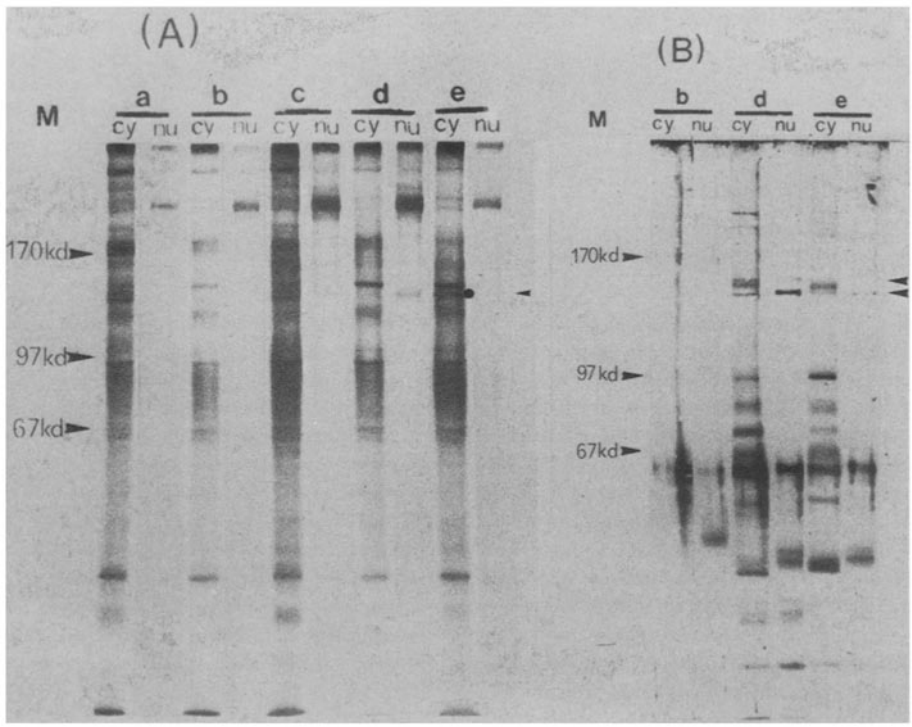

Fig. 6. *A* Fluorogram of SDS-PAGE of cytoplasmic *(cy)* and nuclear *(nu)* extracts from uninfected *(a, b)* and HCMV-infected *(c–e)* HFF pulse labelled with ^{3}H-mannose plus ^{3}H-glucosamine (2.5 μCi/ml of each precursor) from 2–24 hours p.i. *(a–e)* and subsequently chased without (*b* and *d*) or in the presence of monensin (*e*; 4.5 μM) until 48 hours p.i. 50 μg of protein of cytoplasmic extracts and 30 μg of nuclear extracts were analyzed in the respective tracks. Circle and arrow in *e* indicate the position of a virus-induced 135 kd polypeptide. The molecular weight markers *(M)* used were those listed in Fig. 4 A. *B* Immunoblot with HCMV-specific antiserum of SDS-PAGE of the identical cytoplasmic *(cy)* and nuclear *(nu)* extracts (*b, d,* and *e*) as in *A* from chased cells. The amounts of protein loaded in *b, d* and *e* corresponded to those given in *A*. The arrows in *e* indicate the positions of polypeptides which were affected in the presence of monensin. The molecular weight indications *(M)* correspond to the positions of the blotted marker proteins

slightly faster than 140 kd (Fig. 5 C, lane c). Furthermore, drug treatment abolished precursor uptake into the 100 kd glycoprotein but resulted in induction of a glycosylated polypeptide of about 85/90 kd (Fig. 5 A and B, lane d). Tunicamycin-resistant incorporation was obvious only in the very high molecular weight range after labelling with ^{3}H-glucosamine (Fig. 5 B, lane e).

Effect of Monensin on Intracellular Distribution of a Virus-induced Polypeptide

The observation that sensitivity of HCMV DNA synthesis to delayed addition of monensin is restricted to the initial phase of the infectious cycle supports the speculation that intracellular transport of a glycopolypeptide may be involved. It was thus attempted to prove this assumption by the following experimental approach: HCMV-infected HFF were labelled with tritiated sugars for 24 hours p.i. (Fig. 6 A, lane c) and subsequently subjected to a chase in the presence of monensin until 48 hours p.i. prior to cell fractionation (Fig. 6, lane d). Uninfected as well as infected cultures chased without the inhibitor were included as controls (Fig. 6 A, lanes b and d). Analysis of the cytoplasmic and nuclear extracts by SDS-PAGE and fluorography (Fig. 6 A) showed that cytoplasmic extracts from HCMV-infected cells chased in the presence of the drug retained a glycopolypeptide in the range of 135 kd (Fig. 6 A, lane e, cy) which was apparently chased into the nuclear fraction in the absence of the inhibitor (Fig. 6 A, lane d, nu). Immunoblotting (Fig. 6 B) with HCMV-specific antisera of the extracts from chased cultures again revealed decreased amounts in the presence of monensin of viral polypeptides of 130 and 140 kd (see above), but did not support the assumption from the parallel fluorogram (Fig. 6 A) that monensin affected the intracellular transport of a 135 kd virus-specific product.

Discussion

The use of inhibitors for the analysis of complex biological systems is often hampered by adverse side effects. This possibility has to be considered allthemore when results are obtained which do not feature an immediate consequence of the known mechanism of the inhibitor action. In the case of the drug used here several observations argue against its toxicity at the concentrations used, e.g. by irreversible damage of cellular polypeptide synthesis: i) Neither treatment of cultures prior to infection, intermediary exposure of infected cells nor addition of monensin during the infectious cycle prevented resumption or progress of consecutive HCMV DNA synthesis. In addition, monensin showed no inhibitory effect on viral DNA synthesis in HSV-infected HFF. ii) Analysis of the influence of monensin on viral polypeptide synthesis as determined by precursor incorporation and

immunblotting favors a relatively selective effect which is particularly obvious when a protocol of delayed drug addition was used (e.g. Fig. 6 B). Furthermore, monensin effected specific and consistent changes of glycoprotein synthesis in infected cells which indeed reflected its action on posttranslational protein modification, e.g. relative resistance of ^{3}H-mannose incorporation as compared to that of ^{3}H-glucosamine.

Of the main virus-induced glycoproteins (gp) labelled by ^{3}H-mannose, i.e. gp 140, 130 and 100, monensin allowed incorporation only into gp 140 whereas labelling of gp 130 and 100 is abolished, and a new strongly labelled polypeptide of about 85/90 kd is induced. (Minor bands of lower molecular weight appearing after sugar labelling are not considered here). By ^{3}H-glucosamine incorporation additional gp were revealed in the range of 150–160 kd. Monensin prevented appearance of radioactive bands at the high molecular weight position (150–160 kd) as well as in the 130 and 100 kd position, but again allowed some uptake into a glycopolypeptide of 85/90 kd. In view of Pereira's elaborate analysis of the polymorphism of HCMV-specific glycoproteins (25) which was performed also using reducing conditions for electrophoretic analysis, gp 140 and 85/90 whose synthesis appears unimpaired in the presence of monensin, may represent partially processed immature forms of gpA (A_2, A_3) and gpB (B_2), respectively. The recent reports of RASMUSSEN *et al.* (32) and BRITT and AUGER (2) support the view that maturation of the gpA complex involves cleavage of complexed precursors. Monensin may be an experimental aid to define the influence of posttranslational protein modification on final gpA processing.

Our observations (Figs. 4 and 5) suggest that a major gp of 140 kd is made "early" after infection in the absence of viral DNA synthesis. Its relationship to the main viral envelope gp which migrates slightly faster under our conditions remains to be determined.

Taken together, the interference by monensin with HCMV-induced glycopolypeptide—as well as polypeptide synthesis (Fig. 4) appears to be much more pronounced than those described for HSV-infected cultures (13). As for the particular inhibitory effect of monensin on viral DNA synthesis which distinguishes HCMV from HSV, the underlying mechanism is difficult to assess. Previous observations on prevention of HCMV DNA synthesis by glycosylation inhibitors (28, 39), as well as the findings described here suggest that glycosylated products participate in the control of viral DNA synthesis. Our observation that monensin impedes synthesis of several virus-specific polypeptides including structural proteins may thus reflect a secondary effect of the drug. It appears pertinent in this context to define the specificity (host- or virus-specific) of the 135 kd gp whose intracellular distribution is affected by monensin. Analysis of glycoprotein processing in serum-stimulated HFF whose DNA synthesis is equally sensitive to the action of monensin is hoped to shed light on this question.

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