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Antiviral Research 47 (2000) 29–40

Antiviral Research

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Retinoic acid reduces the yield of herpes simplex virus in Vero cells and alters the *N*-glycosylation of viral envelope proteins

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Received 13 December 1999; accepted 20 March 2000

Abstract

Treatment of Vero cells with all-*trans*-retinoic acid (RA) decreased the production of infectious herpes simplex virus (HSV) by 1000–10 000-fold when compared with control cultures. Levels of total HSV envelope glycoproteins gB, gC and gD produced following RA treatment, were comparable with those found in control cultures. Following 24 h of RA treatment, lower molecular weight variants of gB, gC and gD were produced in addition to the typical molecular mass of each protein found in control samples. Between 24 and 48 h of RA treatment, the proportion of the lower molecular mass variants increased. When control and RA treated samples were incubated with peptide *N*-glycosidase F (PNGase F), which removes *N*-glycosylated sugars, the molecular weights of the respective gB, gC and gD proteins produced were comparable in both the groups, indicating that RA did not alter the primary sequence of viral proteins during protein synthesis or increase viral protein proteolysis. RA treatment increased [^{3}H]mannose incorporation into glycoproteins in HSV infected cells but did not change [^{3}H]glucosamine incorporation. We conclude that RA treatment does not reduce the synthesis of three major viral envelope glycoproteins but alters their *N*-glycosylation and postulate that the inhibitory effect of RA is related to its action on *N*-glycosylation.

Keywords: Retinoic acid; Herpes simplex virus; Glycosylation

1. Introduction

The incidence of genital herpes simplex virus (HSV) infections, due to both HSV-1 and HSV-2,

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0166-3542/00/$ - see front matter
PII: S0166-3542(00)00090-5

has been steadily increasing and consequently so has the prevalence of neonatal herpes with its potentially severe complications leading to neurodevelopmental disability or even death (Brown et al., 1997). Transmission of HSV-1 and HSV-2 to newborns most commonly occurs during birth when HSV present in the cervical or vaginal secretions come into contact with the neonate (Whitley, 1994; Brown et al., 1997). The longer the duration and the greater the quantity of viral shedding, at the time of birth, the greater the risk of vertical (mother to infant) transmission. Primary HSV infection near the time of labor carries a ten-fold greater risk of neonatal infection than recurrent infections and has been associated with the shedding of 10^6–10^8 plaque forming units of virus (Whitley, 1994) in comparison with recurrent infections in which 10^2 plaque forming units are shed (Whitley, 1994).

Vitamin A and related compounds (retinoids) have been found to stimulate immune response and promote epithelial barrier integrity. In addition to their role in cell growth, differentiation and interaction, Vitamin A deficiency results in an impaired response to infection (Ross, 1992; Zhao and Ross, 1995; Ross and Stephensen, 1996). HSV ocular pathology in Vitamin A deficient rats is more severe (Nauss et al., 1985) and when rabbits with normal levels of Vitamin A are given parenteral supplements, the severity of HSV keratitis is reduced (Starr et al., 1981). Previous work from our laboratory has shown that retinoic acid (RA) inhibits HSV-1 replication in Vero cells (Isaacs et al., 1997).

Dietary retinoids are converted to the active all-*trans*-RA and 9-*cis*-RA forms which have been shown to alter the replication of a number of viruses including HIV (Poli et al., 1992; Turpin et al., 1992; Yamaguchi et al., 1994; Maciaszek et al., 1998), HSV (Isaacs et al., 1997), cytomegalovirus (CMV) (Angulo et al., 1995, 1998), Epstein–Barr virus (Deshmane et al., 1993; Sista et al., 1995) and hepatitis B virus (HBv) (Huan and Siddiqui, 1992; Hsu et al., 1993). The primary mechanism of action of RA and 9-*cis* RA is mediated by binding to nuclear RA receptors (RARα, β, γ) and retinoid X receptors (RXRα, β, γ) which are transcription factors activated by ligand binding (Kastner et al., 1995; Chambon, 1996). In addition to attaching to DNA binding sites, RA receptor complexes in some instances interact directly with viral transcription factors (Sista et al., 1995). Other effects of RA are nongenomic including retinoylation, which is the covalent binding of the retinoyl moiety to protein (Takahashi and Breitman, 1992; Myhre et al., 1996).

The previously reported decrease in HSV infectivity following exposure to RA could, therefore, be the consequence of a number of different mechanisms resulting in the production of fewer virus particles or the production of noninfectious particles. Both of these possibilities could result from the interaction of RA nuclear receptors with the cellular or HSV genomes or transcription factors or direct interaction with HSV proteins not involved in replication. There is abundant evidence that preventing *N*-glycosylation of HSV-1 enveloped glycoproteins using tunicamycin leads to the production of noninfectious virus particles (Katz et al., 1980; Kousoulas et al., 1983). The overall integrity and number of virus particles is maintained but infectivity is reduced by a factor of 10^3 (Katz et al., 1980).The present studies were undertaken to determine if RA altered the *N*-glycosylation of the HSV-1 envelope glycoproteins. The viral proteins gB and gD are essential for HSV adsorption to and penetration of cells (Manservigi et al., 1977; Johnson and Ligas, 1988; Kuhn et al., 1990; Herold et al., 1991). Since earlier studies have shown that preventing the *N*-glycosylation of gB leads to its intracellular accumulation and degradation (Norrild and Pedersen, 1982; Glorioso et al., 1983; Chatterjie et al., 1990) and that mutants lacking gB are not infectious (Manservigi et al., 1977) it was hypothesized that if RA altered the glycosylation of viral proteins in Vero cells then this could account for the reduced HSV-1 infectivity. The HSV-1 glycoproteins produced in the presence of RA showed altered glycosylation patterns and surprisingly increased protein incorporation of mannose, but not glucosamine, which may indicate an inhibition of glycoprotein processing.

2. Methods

2.1. Cells and virus

Vero cells (African green monkey kidney cell line) were purchased from the American Type Culture Collection, Rockville, MD and were grown as monolayer cultures in RPMI 1640 medium containing glutamine (BioWhittaker, Walkersville, MD), 0.075% $NaHCO_3$, 0.2% gentamicin and 10% inactivated fetal bovine serum (Atlanta Biologicals, Inc., Norcross, GA) (Isaacs et al., 1997). The maintenance medium for Vero cells was as described above but with 1% fetal bovine serum. HSV-1 (strain F1) was obtained from Dr Rubenstein (New York State Institute for Basic Research) and grown in Vero cells. Vero cells were used to determine HSV-1 infectivity by measuring the cytopathic effect of the virus as previously described (Isaacs et al., 1997).

2.2. Infection of cells and preparation of cell lysates

Vero cell monolayers were grown in 6 cm plastic dishes (Falcon, Oxnard, CA) and infected with HSV-1 at a multiplicity of infection (MOI) of ten tissue culture infective doses ($TCID_{50}$) per cell. Virus was adsorbed for 1 h at 37°C in RPMI 1640 medium containing 1% fetal bovine serum. The virus was then removed and RPMI 1640 with 1% fetal bovine serum was added to the monolayers. Cells were then treated with either all-*trans*-RA (7.5 μg/ml) (Sigma, St Louis, MO), tunicamycin (10 μg/ml) (Calbiochem, San Diego, CA) or monensin (1 μM) (Sigma) for 24 or 48 h after infection. Cells were washed with ice-cold phosphate buffered saline (PBS: 10 mM, pH 7.4, Sigma) and lysed with 1X sample buffer (62.5 mM Tris pH 6.8, 2% sodium dodecyl sulfate (SDS), 5% β-mercaptoethanol, 10% glycerol, pH 6.8). Samples were utilized for western blot analysis.

2.3. Labeling of glycoproteins

Vero cells infected with HSV-1 were treated with RA for 4 h and then labeled with either 10 μCi/ml of D-[1,6-^{3}H]glucosamine hydrochloride or 20 μCi/ml of D-[2,6-^{3}H]mannose (American Radiolabeled Chemicals, St Louis, MO) for 19 h. Cells were extracted with 1X sample buffer and proteins were precipitated with trichloroacetic acid onto glass fiber filters. The precipitated proteins were then counted in a scintillation counter.

2.4. Immunoprecipitation and electrophoresis of viral proteins

Cell extracts were precipitated with a polyclonal antibody made in rabbit against HSV-1 (Accurate Chemical, Westbury, NY Cat # AXL237). Cell extract supernatants (500 μl) were mixed with 3 μl of polyclonal antibody and allowed to react overnight at 4°C. The immuneprecipitates were collected with protein A-sepharose beads (Sigma) washed with IP buffer C (0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1% NP40, 0.15 M NaCl, 1 mM EDTA, 0.1% gelatin, 0.1% sodum azide, 1X protease inhibitors (Kascsak et al., 1987), 0.05 M Tris, pH 8.0) and LiCl buffer (0.1 M Tris pH 8.4, 0.5 M LiCl, 0.1% βmercaptoethanol, pH 8.4) (Caughey and Raymond, 1991) and then released from the beads by boiling for 4 min in 1X sample buffer.

Samples of SDS-solubilized cell extracts or immune precipitates were loaded onto 7.5 or 12% polyacrylamide gels cross-linked with Bis (Laemmli, 1970) and electrophoresed at 100 V. Following electrophoresis the proteins were transferred to PVDF membranes (Bio–Rad) using a Mini Trans-Blot Cell (Bio–Rad) at 70 V for 3 h. Samples on PVDF membranes were incubated with monoclonal antibodies against gB, gC or gD in PBS containing 3% bovine serum albumin at 4° overnight followed by incubation with alkaline phosphatase conjugated goat anti-mouse immunoglobulin G and M (BioSource, Camarillo, CA) in 3% bovine serum albumin for an additional 2 h at 37°C. Western blots were developed by the addition of BCIP/NBT (Sigma) in alkaline phosphatase buffer (0.1 M Tris, 0.1 M NaCl, 5 mM MgCl2, pH 9.5).

2.5. Incubation of HSV proteins with glycosidase

Following immuneprecipitation samples were treated with peptide *N*-glycosidase F (PNGase F) (Calbiochem, San Diego, CA) according to the manufacturer's protocols. Samples were incubated for 3 h at 37°C in a total volume of 50 μl containing five units of PNGase F.

2.6. Viral protein synthesis in the presence of retinoic acid

Vero cell cultures in 25 cm^2 flasks were either untreated (control) or treated with RA at 12.5 μg/ml for 24 h prior to the addition of virus. Cultures were infected with HSV-1 at an MOI of 10 for 1 h in the presence or absence of RA. Cells were maintained in the presence or absence of RA for the 24-h incubation period in RPMI-1640 containing 1% fetal calf serum (FCS). At that time, cultures were extensively washed with PBS, cells removed using a rubber policeman and disrupted by probe sonication. Total protein concentration in the cell extracts was determined by a micro Bradford assay (Pierce, Rockford). Protein extracts were bound to ELISA plates (Nunc Star Well MaxiSorp) at 10 or 20 μg/ml in PBS at 4°C overnight. Plates were washed in PBS and unbound sites blocked using PBS containing 1% normal goat sera. Antibodies to HSV-1 were added to triplicate wells at two-fold serial dilutions starting at 1:1000. The following HSV-1 antisera were employed: Mabs 1103, 1104 and 1105 (Goodwin Institute) and human polyclonal antibody to HSV-1 (Flow Laboratories, Rockville, MD). Antibodies were bound at 37°C for 2 h. Plates were washed in PBST and goat anti-mouse or goat anti-human antibody conjugated with alkaline phosphatase (1:1000, BioSource, Camarillo, CA) added for 1 h at 37°C. Plates were washed, substrate (*N*-phenylphosphate) added and reactivity monitored using a Cambridge 7520 ELISA reader (Cambridge Technologies, Waterford, MA).

Table 1
Inhibition of HSV-1 replication[a]

Treatment	$TCID_{50}$ incubation time (h)	
	24	48
Control	$10^{6.70} \pm 0.37$	$10^{7.25}$
RA	$10^{2.70} \pm 0.37$	$10^{4.25}$
Tunicamycin	$< 10^{3.00} \pm$ [0b]	ND[c]
Monensin	$10^{5.75} \pm 0.31$	ND[c]

[a] Vero cells were infected with HSV-1 and incubated for 24 or 48 h. The numbers in the 24 h experiments are the mean ± S.D. of five separate experiments. Data for the 48 h experiment are representative of numerous replicates and are the infectivity data for the 48 h results in Fig. 2. RA, tunicamycin and monensin were used at concentrations of 12.5, 10 μg/ml and 1 μM, respectively.

[b] The cytopathic effect caused by active virus could not be distinguished from the changes in cell shape caused by tunicamycin at sample dilutions up to 1/100.

[c] Not done.

3. Results

3.1. Inhibition of HSV-1 replication by RA and glycosylation inhibitors

Earlier studies from our laboratory have shown that RA reduced the production of infectious HSV-1 particles in Vero cells (Isaacs et al., 1997). In the present study the ability of RA to inhibit HSV-1 replication was compared with that of tunicamycin and monensin which reduce *N*-glycosylation and 0-glycosylation, respectively (Table 1). After 24 h of RA treatment the viral titer was 3–4 $\log_{10}$ lower than in the untreated cultures. Tunicamycin treatment produced a decrease in viral infectivity comparable to that of RA. Monensin reduced viral infectivity by less than 1 $\log_{10}$. Examination of RA and tunicamycin treated cultures 24 h following viral infection showed that the appearance of the RA treated cells was similar to tunicamycin cultures, in that there was very little cytopathic effect (rounded cells), but untreated and monensin treated cultures had many more rounded cells (results not shown). These results show that RA reduces the production of HSV-1 infectivity in Vero cells as effectively as an *N*-glycosylation inhibitor. In the period between 24 and 48 h following HSV-1 infection viral production increased in both RA

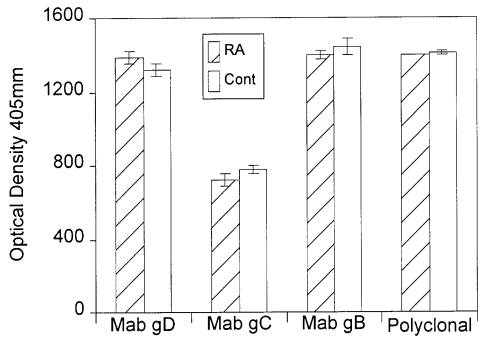

Fig. 1. RA does not reduce the synthesis of HSV-1 envelope glycoproteins. Vero cell cultures were pretreated with RA for 1 h prior to infection with HSV-1 (MOI 10). Cultures were maintained in the presence of RA for the entire 24-h incubation period. Control cultures (C) were processed as above but without RA. Washed cells were disrupted by sonication and added to ELISA plates at 10 μg/ml. The indicated antibodies were added at a 1:1000 dilution and the assay conducted as described in Section 2. Each sample represents the average (±S.E.) of three separate experiments. RA treatment decreased the $TCID_{50}$ by 3 $\log_{10}$.

treated and control cells but there were still 3 $\log_{10}$ fewer infectious virus particles in the RA treated culture. By 48 h after tunicamycin treatment of uninfected Vero cells, significant cytopathology was produced which was not seen in the RA treated cells (results not shown), indicating that while RA and tunicamycin both inhibit HSV-1 production, RA is far less cytotoxic than tunicamycin.

3.2. Effect of RA on HSV-1 envelope glycoprotein synthesis

To provide an insight into the mechanism of RA inhibition of HSV-1 infectivity, the production of envelope glycoproteins was measured by standard ELISA using monoclonal antibodies which react to primary amino acid protein sequences and whose immunoreactivity is not affected by protein glycosylation (Fig. 1). Production of the HSV-1 glycoproteins gB, gC and gD was not decreased following RA treatment for 24 h post infection. Similar results were obtained when a polyclonal antibody directed against total viral envelope protein was used. Again comparable levels of viral protein synthesis were observed in both RA treated and control cultures. To ensure that these measurements reflected sufficient solubilization of viral membrane glycoproteins capture ELISA assays were also done (results not shown) and following the capture of individual glycoproteins by the appropriate monoclonal antibody the results were identical to those found using the standard enzyme linked immunosorbent assay (ELISA). Thus RA treatment did not reduce envelope glycoprotein synthesis even though the same conditions reduced HSV-1 infectivity by 3–4 $\log_{10}$ (Table 1).

3.3. Effect of RA and glycosylation inhibitors on the HSV-1 envelope glycoproteins

Western blot analysis was performed at 24 and 48 h following viral infection to determine whether RA changed the molecular weight of the viral envelope glycoproteins gB, gC and gD (Fig. 2). After a 24 h incubation the gB protein showed one band with an apparent molecular weight of 99 kDa in the control cultures (Fig. 2A). Treatment with RA produced multiple bands one of which had the same apparent molecular weight (99 kDa) as the control but also numerous bands of lower molecular mass. The lowest band in the RA treated sample overlapped with the gB band produced following tunicamycin treatment with an apparent molecular mass of 93 kDa. Treatment with the 0-glycosylation inhibitor monensin did not alter the molecular weight of the gB protein. When RA was incubated with HSV-1 for 48 h and compared with a 48-h control, a marked difference in the gB banding patterns was seen. The 48-h control lane showed a major 99 kDa band at the same molecular mass as the 24-h control and an additional minor band at 98 kDa. The major band in the RA treated sample at 48 h was at 93 kDa and there was also a continuum of bands of increasing molecular weight up to one at 99 kDa which was the same molecular weight as the 48-h control. These results show that in the period from 24 to 48 h following viral infection RA caused an accumulation of a lower molecular weight gB as compared with the control. When the 24 and 48 h RA treated samples are compared it can be seen that there is an increasing shift in

gB molecular weight from that seen in controls that to seen in tunicamycin treated samples.

When the gC envelope glycoprotein was examined following a 24 h incubation different banding patterns in the presence and absence of RA were seen (Fig. 2B). The control sample had a major band with a molecular mass of 90 kDA and minor bands at 99 and 71 kDa. Following treatment with RA the 90 kDa band was considerably decreased and a continuum of bands with decreasing molecular mass was produced extending from 90 to 71 kDa. A molecular mass of 71 kDa is the major and highest molecular mass band produced after tunicamycin treatment. Following tunicamycin treatment two minor bands were also seen at 70 and at 51 kDa, the latter representing completely unglycosylated gC protein. Monensin treatment produced the same molecular mass bands as the control. After 48 h the banding of the control sample was unchanged. The RA treated sample continued to show a continuum of bands from 90 to 71 kDa, but the heaviest band was now at 71 kDa and not 90 kDa, as seen at 24 h.

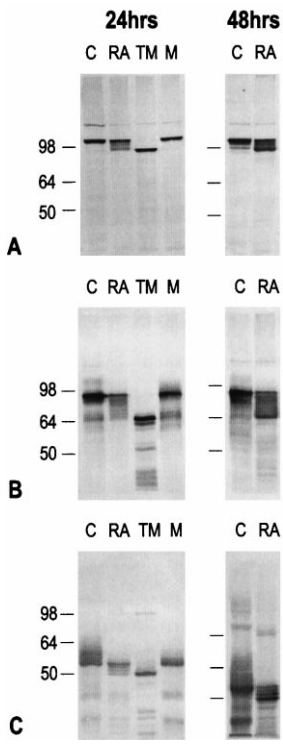

Fig. 2. RA treatment of Vero cells alters the molecular weight of HSV-1 envelope glycoproteins. Vero cell cultures were pretreated with RA (12.5 μg/ml), tunicamycin (10 μg/ml) (TM) or monensin (1 μM) (MN) for 30 min prior to the addition HSV-1 (MOI 10). Cultures were maintained in the presence of the appropriate compound for 24 or 48 h. Control cultures (C) were processed as above but lacked inhibitors. After a 24 or 48 h incubation period cell extracts were electrophoresed and transferred to PVDF membranes. Viral envelope proteins, (A) gB, (B) gC and (C) gD were detected using monoclonal antibodies to each protein (see Section 2).

The gD envelope glycoprotein showed changes in molecular weight consistent with those seen with gB and gC following RA treatment (Fig. 2C). After 24 h untreated HSV-1 infected cells had gD bands ranging in molecular weight from 53 to 60 kDa. The RA treated sample had a major band at 53 kDa as well as a series of lower molecular weight bands between 53 and 47 kDa. Tunicamycin treatment produced a band with a molecular weight of 48 kDa which was similar to the lowest molecular weight band following RA treatment. Monensin treatment produced a major band at 54 kDa but no lower molecular weight bands. When the virus was allowed to grow for 48 h the gD control was similar to the 24 h culture and most of the protein was at 53 kDa. The RA treated sample showed the same molecular weight spread as at 24 h but the major band was now at 47 kDa indicating an accumulation of much lower molecular weight protein.

The molecular shifts seen with gB, gC and gD following RA treatment could reflect an alteration in viral protein primary structure or a change in protein post translational maturation or modification. Further experiments were done to distinguish between these two possibilities.

3.4. Glycosidase treatment of HSV-1 envelope glycoproteins

The molecular weights of gB, gC and gD were examined in control and RA treated cultures following exposure to the glycosidase PNGase F which removes *N*-linked sugars (Fig. 3). Control and RA treated samples not incubated with PNGase showed similar banding patterns to the 24 h samples in Fig. 2 in that RA treated samples had

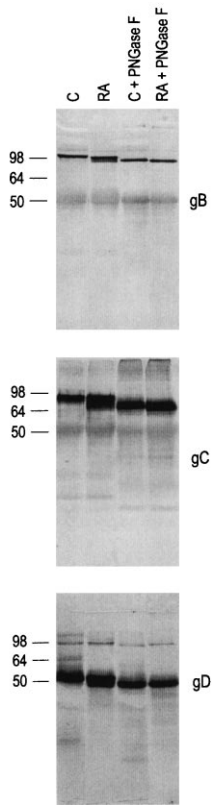

Fig. 3. Treatment of HSV-1 glycoproteins gB, gC and gD with PNGase F following exposure to RA. HSV-1 proteins from control (c) or RA treated cells were immunoprecipitated with a polyclonal antibody as described in the Section 2. Control and RA treated samples were both separated into two groups and half of each sample was treated with PNGase F. Following electrophoresis and western blotting, viral proteins were detected using monoclonal antibodies against gB (top panel), gC (middle panel) and gD (bottom panel).

an increased number of lower molecular weight bands when compared with the control. However, following incubation with PNGase F control and RA samples of gB, gC and gD showed single bands with the same molecular weights. This indicates that the altered molecular weights of HSV-1 glycoproteins produced in RA treated cells did not result from an alteration in primary sequence during synthesis nor from proteolysis but from altered *N*-glycosylation. Studies done in our laboratory with the glycosidase endoH, which removes high mannose and hybrid *N*-linked oligosaccharides but not complex *N*-linked sugars, gave the same result as the PNGase F (results not shown). This suggests that RA treatment prevents the conversion of *N*-linked sugars to the complex form. Since carbohydrates are added to the viral proteins using the cellular glycosylation machinery, the question remained as to whether the RA effect was a generalized phenomenon affecting all glycoproteins or whether the effect was directed specifically at viral proteins.

3.5. Glycosylation of the proteins in RA treated cells

In order to determine how the glycosylation of proteins might be altered in RA treated Vero cells, glycoproteins in HSV-1 infected cells were labeled with [^{3}H]glucosamine or [^{3}H]mannose (Fig. 4). The data show that increasing concentrations of RA are paralleled by increasing [^{3}H]mannose incorporation into protein and decreasing production of viral infectivity. At an RA concentration of 7.5 μg/ml, HSV-1 infectivity has

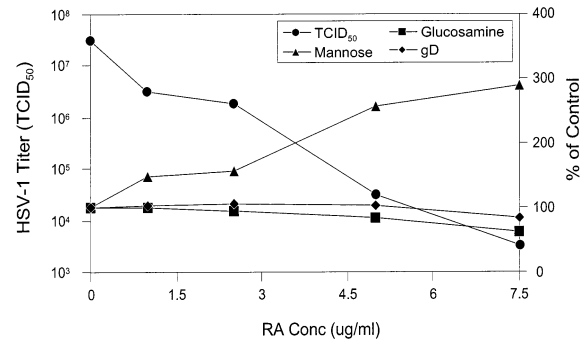

Fig. 4. Effect of varying concentrations of RA on [^{3}H]glucosamine and [^{3}H]mannose incorporation into glycoproteins in HSV-1 infected Vero cells. Vero cells were treated with the indicated concentration of RA and then 4 h later labeled with either [^{3}H]glucosamine or [^{3}H]mannose for 19 h. Proteins were extracted from parallel cultures of HSV-1 infected cultured Vero cells and counted to determine either [^{3}H]glucosamine or [^{3}H]mannose incorporation. The gD protein was measured by ELISA, as described in Fig. 1, and the results from the [^{3}H]glucosamine and [^{3}H]mannose cultures were averaged. $TCID_{50}$s were determined using the supernatants from the [^{3}H]glucosamine and [^{3}H]mannose labeled cultures and averaged. The control (100%) values for mannose, glucosamine and gD were 84 414 cpm, 326 261 cpm and 1264 absorbance units, respectively.

dropped by 4 $\log_{10}$ but the incorporation of mannose has increased by almost 300%. However, glucosamine incorporation is barely reduced at 5 μg/ml RA and drops by 40% at 7.5 μg/ml while the production of the viral gD protein is decreased by only 15% at a RA concentration of 7.5 μg/ml. The increased mannose incorporation coupled with no or a relatively small drop in glucosamine incorporation suggests that glycosylation processing is altered following the addition of *N*-linked oligosaccharides as a single unit (Kornfeld and Kornfeld, 1985).

4. Discussion

RA treatment of HSV-1 infected Vero cells reduces the production of infectious virus while producing gB, gC and gD envelope glycoproteins with lower molecular weights than in the control samples. Western blot analysis done 24 and 48 h post infection showed that RA caused an increasing accumulation of gB, gC and gD proteins with varying molecular weights suggesting that RA interfered with protein glycosylation. When gB, gC and gD molecules were enzymatically treated to remove *N*-linked sugars, the molecular weight patterns of control and RA treated samples were the same, again indicating that RA alters the *N*-glycosylation of three major HSV envelope proteins and that the multiple bands found for the gB, gC and gD viral glycoproteins were not the result of proteolysis.

HSV envelope glycoproteins are processed in a similar fashion to cellular glycoproteins with an early high mannose *N*-glycosylated form that is transported to the golgi where it is converted to the complex type and in some instances 0-linked glycosylation also occurs (Johnson and Spear, 1983; Roizman and Sears, 1993). The addition of normal high-mannose *N*-linked oligosaccharides is a minimal requirement for HSV infectivity (Campadelli-Fiume et al., 1982; Kousoulas et al., 1983; Roizman and Sears, 1993). Preventing *N*-glycosylation of HSV-1 envelope proteins reduces the production of infectious virus (Katz et al., 1980; Kousoulas et al., 1983). However, despite a drop of 2–3 $\log_{10}$ in infectious yield, the number of envelope HSV-1 particles is only decreased 30–40% (Katz et al., 1980). Nonglycosylated HSV-1 particles produced by tunicamycin treatment, adsorbed to permissive cells as well as infectious virus, however, nonglycosylated virus particles were less able to penetrate the plasma membrane and cause infection (Spivak et al., 1982; Svennerholm et al., 1982). Monensin, which inhibits the addition of O-linked oligosaccharides, and the processing of high-mannose *N*-linked oligosaccharides to the complex type, does not substantially reduce the specific infectivity of virus particles (Johnson and Spear, 1982). Conversion of high mannose glycans into complex-type glycans seems to be required for the release of HSV from infected cells but not for infectivity (Johnson and Spear, 1982; Serafini-Cessi et al., 1983).

HSV glycoproteins have been shown to play an important role in the viral replication cycle. Glycoproteins gB, gC and gD, which were examined in this study, have been implicated in viral attachment to receptors, whereas penetration across the plasma membrane involves gB and gD (Manservigi et al., 1977; Johnson and Ligas, 1988; Kuhn et al., 1990; Herold et al., 1991). A number of studies (Norrild and Pedersen, 1982; Glorioso et al., 1983; Chatterjie et al., 1990), suggest that improperly glycosylated gB is either degraded intracellularly or unable to integrate into the viral envelope leading to the production of noninfectious virions. Nonglycosylated gC protein conversely maintains its functional stability and is transported to the cell surface (Norrild and Pedersen, 1982; Glorioso et al., 1983; Chatterjie et al., 1990). Studies of unglycosylated gD have shown that whether or not gD is transported to the cell, the surface is cell type dependent (Norrild and Pedersen, 1982; Glorioso et al., 1983; Chatterjie et al., 1990). Mutant cells with mature gD protein but lacking gB protein are not infectious indicating that the presence of gD cannot promote cell penetration by itself (Manservigi et al., 1977). The present study shows that RA interferes with the *N*-glycosylation of gB and gD and it is known that these two herpes virus proteins are necessary for virus entry into cultured cells (Ligas and Johnson, 1988; Roop et al., 1993). HSV glycoproteins gH and gL, which were not exam-

ined in this study, are also essential for the HSV entry into cells and changes in their glycosylation patterns could also result in decreased viral infectivity (Roop et al., 1993). Therefore, changes in the glycosylation of gB, gD, gH or gL singly or in concert resulting from RA treatment may cause the reduction in infectious HSV production observed in this study.

It has been shown that interfering with the processing of glycoproteins, as suggested for RA, decreases the specific infectivity of many enveloped viruses. HIV virions produced from interferon (IFN)-treated cells are 100–1000-fold less infectious than an equal number of virions from control cells (Hansen et al., 1992). Both the IFN-treated and control cultures produced comparable numbers of virions, DNA, RNA and p24 antigen, but IFN treatment resulted in the decreased infectivity due to defective processing of the gp 160 glycoprotein (Hansen et al., 1992). When HIV infected cells are treated with an α-glucosidase inhibitor the processing of gp 120 *N*-linked oligosaccharides is impaired, leading to a decrease in virus infectivity but not virus output (Fischer et al., 1995, 1996). A similar situation is found with vesicular stomatitis virus (VSV) and murine leukemic viruses in which the IFN treatment interferes with the viral envelope glycoprotein processing and assembly leading to the production of noninfectious virions (Billiau et al., 1978; Singh et al., 1988). RA treatment of HSV infected cells is likely to result in a phenomenon analogous to IFN treatment of HIV, VSV or murine leukemic viruses and α-glucosidase treatment of HIV. A potential mechanism by which RA altered glycosylation could prevent the production of functional viral glycoproteins is by interfering with their folding. For example the interaction between gB and the molecular chaperone calnexin is dependent upon proper glycoprotein processing (Yamashita et al., 1996). Calnexin interacts with proteins following chain termination, during which time, proteins acquire disulfide bonds and assemble into oligomeric complexes (Bergeron et al., 1994). While the binding of high mannose gC and gD to calnexin is an early event and short lived, gB binding in contrast is much more prolonged and sensitive to disruption (Yamashita et al., 1996). If RA treatment interferes with an early stage of gB glycosylation and consequently the gB-calnexin association, it could lead to the production of noninfectious HSV particles. Although, many proteins interact with calnexin only a small group show folding and secretion defects when the interaction is inhibited (Elbein, 1991) indicating that many glycoproteins can use alternative pathways of folding and assembly in the endoplasmic reticulum. It is noteworthy that both the G protein of VSV and the influenza virus hemaglutinin (HA) use calnexin, but also that the former is strictly dependent on calnexin for proper folding while the latter can also fold independently (Elbein, 1991; Hammond and Helenius, 1994). It remains to be determined how the *N*-glycosylation of viral proteins, such as HSV-1 gB, is altered by RA. A recent study (Meissner et al., 1999) shows that the RA treatment leads to increased mannose incorporation and glycosylation of cellular proteins in a mouse teratocarcinoma cell line. However, the results presented in the present study show that the RA treatment leads to increased mannose incorporation into glycoproteins but has no affect on glucosamine incorporation. Since, initially mannose and glucosamine are added to proteins as part of a single unit (Kornfeld and Kornfeld, 1985), the current results could reflect a decreased removal of mannose during glycoprotein processing due to the inhibition of mannosidase activity in the endoplasmic reticulum and golgi or possibly decreased glucosidase activity in the endoplasmic reticulum. Interfering with sugar removal from viral proteins and, therefore, in some instances the subsequent addition of new sugars could account for the multiple molecular weight bands seen following the RA treatment. However, the present results do not rule out the possibility that some of the multiple gB, gC and gD glycoprotein bands seen at 24 and 48 h (Fig. 2) following the RA treatment result from an effect similar to TM, which prevents the en block glycosylation on some sites of viral envelope glycoproteins. Further studies are required to determine the structure of envelope glycoprotein carbohydrate chains produced in the RA treated cells.

There is a need for a topical agent that reduces viral shedding or leads to the production of non-infectious HSV particles during birth (Carmack and Prober, 1993; Forsgren and Malm, 1996). Although the risk from primary HSV infection is greater than that of recurrent infection, since there is greater viral shedding in primary infections, both instances contribute about 50% of perinatal infections. Most maternal primary and recurrent infections are asymptomatic and thus preventing neonatal exposure is difficult. If RA could be used in a topical microbicide prior to labor to reduce the shedding of infectious virions, vertical transmission of HSV could be reduced at minimal risk to either the mother or child. The viral inhibitory properties of RA on HSV and perhaps other enveloped viruses may be useful in a variety of antiviral applications. Towards this end, studies are currently ongoing, using human cell lines (SY5Y cells) and the preliminary data suggests a similar inhibitory effect on HSV replication.

Acknowledgements

This work was supported by a grant to CEI from the National Institutes of Health (AI 39061) and by the New York State Office of Mental Retardation and Developmental Disabilities. We are grateful to Anna Parese for her assistance in the preparation of the manuscript and to Maryellen Cafaro for graphics preparation.

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