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Loss of cytomegalovirus infectivity after treatment with castanospermine or related plant alkaloids correlates with aberrant glycoprotein synthesis

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Summary

Many plants contain polyhydroxyalkaloids which are potent inhibitors of glucosidases, enzymes involved in oligosaccharide trimming. These are important in determining the final configuration of specific glycoproteins. Human cytomegalovirus (CMV) encodes a number of glycoproteins, some of which ultimately reside in the outer envelope of the mature virion and are important for virus infectivity. Treatment with three polyhydroxyalkaloids, castanospermine (CAST), deoxynojirimycin (DNJ) and 2R,5R-dihydroxymethyl-3R,4R-dihydroxypyrrolidine (DMDP) blocked the growth of infectious virus, as determined by yield reduction and plaque reduction assays. However, in the presence of CAST, CMV infected cells continued to shed virions into the extracellular medium, as determined by electron microscopy. Envelope glycoproteins of virions produced after treatment with CAST (2.5 mM) were immunoprecipitated with a monoclonal antibody (F5) specific for the gcl family of glycoproteins. Analysis by PAGE-SDS showed an absence of gcl complex 2 (gp52 disulphide-linked to gp130) with a proportional increase in gcl complex 1 (gp52 disulphide-linked to gp95). The results indicated that gp130 alone, or linked to gp52, was important for CMV infectivity. As well as being potential targets for antiviral agents against CMV, inhibitors of glycoprotein trimming reactions may define components of the virion surface important for infectivity.

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**Introduction**

A number of viral glycoproteins with a range from 52 to 250 kDa are synthesized in cytomegalovirus (CMV) infected cells and are major components of the outer envelope (Stinski, 1976, 1977; Farrar and Oram, 1984). The envelope glycoproteins of CMV are considered to be important in virus infectivity and are known to induce the production of antibodies that neutralise the virus in vitro (Pereira et al., 1982; Britt, 1984; Rasmussen et al., 1985). Distinct families of glycoprotein complexes expressed on the virion envelope have been separated by ion-exchange chromatography (Kari et al., 1986) and recently designated gcI, gcII and gcIII with gcI comprising a major component (Gretch et al., 1988a). Under reducing conditions, SDS-PAGE analysis showed the gcI family consisted of glycoproteins of 50--55, 93--95 and 130 kDa, respectively (Kari et al., 1986; Farrar and Greenaway, 1986; Gretch et al., 1988a,b). In one of these studies (Farrar and Greenaway, 1986), immunoprecipitation of purified CMV envelope glycoproteins using a monoclonal antibody with specificity for a 52 kDa glycoprotein (Law et al., 1985) revealed three disulphide cross-bridged complexes of 150 kDa (52 kDa + 95 kDa), 180 kDa (52 kDa + 130 kDa) and 250 kDa, a possible multimeric form.

The final structure of oligosaccharides that constitute the N-linked glycan attached to CMV-encoded glycoproteins, will be dependent on a series of enzyme reactions which involve the removal or addition of sugar residues (see Fig. 1). Modification of the pre-formed N-linked glycan consisting of N-acetylglucosamine 2 mannose 7–9 glucose 3 initially involves the removal of the terminal glucose residues by processing alpha-glucosidase activities before further modification through a series of hydrolytic and transferase reactions (Furhmann et al., 1985) to high mannose and complex type oligosaccharides (Fig. 1). Certain polyhydroxyalkaloids extracted from plants are inhibitors of the glucosidase reactions (see Fig. 1) involved in the initial trimming of sugar residues (Saunier et al., 1982; Elbein et al., 1984; Fellows, 1986). They are castanospermine (CAST), deoxynojirimycin (DNJ) and 2R,5R-dihydroxymethyl-3R,4R-dihydroxypyrrolidine (DMDP) derivatives of octahydroxyindolizine, piperidine or pyrrolidine, respectively (Fellows, 1986). All these compounds have been found to be non-toxic to cells in culture at concentrations that effectively reduce glycoprotein processing (Fuhrmann et al., 1985; Tyms et al., 1987).

CMV is an important opportunistic pathogen in allograft recipients (Meyers et al., 1982; Betts, 1983), in AIDS patients (Tyms et al., 1988) and poses a major threat to the newborn after congenital infection (Stagno et al., 1975). In the present study we have investigated the inhibitory effects of polyhydroxyalkaloids on CMV growth in cell culture and shown that CAST, DNJ and DMDP inhibited the production of infectious virus. Using the monoclonal antibody F5, which is specific for gcI
Fig. 1. Sites of inhibition of polyhydroxyalkaloids (CAST, DNJ and DMDP) in the proposed sequence for the processing of peptide bound N-linked oligosaccharide chains: ●, N-acetylglucosamine; ○, mannose; ▲, glucose; □, galactose; ■, sialic acid; △, fucose. Reactions are catalysed by 1, α-glucosidase I; 2, α-glucosidase II; 3, α-mannosidase I; 4, N-acetylglycosaminyl transferase I; 5, α-mannosidase II; 6, N-acetylglycosaminyl transferase II; 7, fucosyl transferase; 8, galactosyl transferase; 9, sialyl transferase.

complexes (Law et al., 1985; Farrar and Greenaway, 1986), evidence is provided that inhibition of oligosaccharide trimming glucosidases results in the production of aberrant glycoproteins which correlates with a loss of virus infectivity. Such inhibitors may be important in the development of new antiviral strategies against CMV.

Materials and Methods

Cells and viruses

Human embryo fibroblast (HEF) cells were grown in Eagle’s minimal essential medium supplemented with 5% foetal calf serum (FCS), 9 mM sodium bicarbonate and 7 mM HEPES buffer. In maintenance medium (MM) the supplements were adjusted to 2% FCS, 13 mM sodium bicarbonate and 14 mM HEPES. Three different strains of CMV were used, a prototype strain AD169 and two clinical isolates: strain CMV Ir received from Dr. J. Booth, St. Georges Hospital Medical School and CMV 149, an isolate from an AIDS patient at St. Mary’s Hospital. Virus stocks were prepared and stored as described previously (Tyms and Williamson, 1980).
Inhibitors of glycoprotein trimming enzymes

CAST, DNJ, DMDP and also the alkaloid fagomine (FAG) were isolated from plants (Fellows, 1986). Fagomine is an alkaloid related structurally to DNJ. It is known to be an inhibitor of mammalian digestive isomaltase (Schofield et al., 1986) but its action on glycoprotein trimming enzymes has not been investigated. The structures of the compounds are shown in Fig. 2. The compounds are all soluble in water and appeared to be stable under culture conditions. Due to the restricted supply of compounds, the MM was only changed daily when the supernatant fluid was being harvested for detecting levels of infectious virus and pelleting of virions.

Plaque reduction assays

For plaque reduction assays, 24-well culture trays were seeded with HEF cells. Confluent cell monolayers were infected with virus to give 100 plaque-forming units (PFU) per well. After a 1 h adsorption period the inocula were replaced by 1.5 ml of MM incorporating 1% low gelling temperature agarose, supplemented with various concentrations of test compound. The cultures were incubated at 37°C, fixed with 5% formalin in phosphate buffered saline (PBS) after 6–8 days of infection and the cell monolayer stained with 0.3% methylene blue on removal of the overlay.

The mean plaque number was calculated from triplicate counts and plotted as percentage untreated control against the log_{10} drug concentration. The 50% effective dose (ED_{50}) was determined for each curve after linear regression analysis.

Titration of extracellular virus

The supernatant fluid harvested from infected cell cultures on successive days was clarified by centrifugation (3000 × g for 5 min) and the virus titrated on freshly seeded monolayers using an agarose overlay as described above.
Ultrastructural studies

Cells from representative cultures (about $1.5 \times 10^6$ cells) were removed by treatment with 0.01% pronase in PBS, washed in PBS and centrifuged. The cell pellet fixed for 2 h in 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3. Samples were washed for a minimum of 12 h in fresh cacodylate buffer before fixing for 2 h in 1% osmium tetroxide. After washing in 50% alcohol cell pellets were encased in agar. These blocks were dehydrated through a graded series of alcohols and embedded in Araldite using propylene oxide as the transitional fluid. Ultrathin sections were stained in alcoholic uranyl acetate and Reynolds lead citrate prior to examination in an Hitachi HU12A electron microscope.

Extraction of virion envelope and immunoprecipitation of glycoprotein complexes

The supernatant fluid from infected cell cultures, either untreated or treated with the glucosidase inhibitors, was clarified as described above and centrifuged for 1 h at 20000 rpm to pellet the virions. Pellets were resuspended in PBS and either used immediately or stored in liquid nitrogen. Resuspended virus was $^{125}$I-labelled by the method of Hunter and Greenwood (1962). Unincorporated isotope was removed by centrifuging and washing with PBS. Virion envelope components were extracted and immunoprecipitated as described previously (Farrar and Greenaway, 1986). Briefly, pelleted virus was treated with buffered 1% Triton X-100 solution for 20 min on ice with ultrasonication. Nucleocapsids were centrifuged at 20000 rpm for 1 h at 0°C and solubilised envelope components immunocomplexed using the monoclonal antibody F5 (Law et al., 1985) and protein A-sepharose beads (Pharmacia).

Polyacrylamide gel electrophoresis (PAGE)

The glycoprotein complexes were released from the protein A-sepharose beads by incubation with a sampling buffer containing 2% SDS, 0.08 M Tris HCl, 10% glycerol and bromophenol blue, pH 6.8, for 5 min at 90°C. For reducing conditions 0.1 M dithiothreitol was added to the sampling buffer. The solubilised samples analysed by SDS-PAGE in 7.5% running gels were subsequently dried and exposed to X-ray film (Fuji) at −70°C. The autoradiographs were scanned on a densitometric scanner (Shimadzu) to describe the complexes and compute the area under the curve.

Results

Effect of CAST on the growth of HEF cells

The toxicity of CAST for HEF cells was investigated by assessing the effects of CAST on cell growth. HEF cells were seeded at low numbers ($10^4$ cells/16 mm well) in the presence of 5 or 15 mM CAST or were left untreated and cells counted after six days of growth. No significant differences were recorded in the number of viable cells in the presence or absence of CAST. Because of the scarcity of compounds similar experiments were not conducted with DNJ or DMDP but toxicity was not observed during the course of experiments.
Effect of CAST on virus production

The growth characteristics of human CMV in HEF cells, determined by levels of extracellular virus, is illustrated in Fig. 3a. By replacing the MM daily, levels of infectivity represent the rate of virus production over a 24 h period. This reflected the continuous shedding of virions into the extracellular medium and was considered to be a suitable model to determine the effects of treatment with the glucosidase inhibitors. HEF cells were infected with AD169 virus at a multiplicity of infection (MOI) of 5 PFU/cell and treated with 2.5 or 5.0 mM CAST or left untreated. Titres of extracellular virus were measured beginning five days after infection when virus levels in the untreated controls approached maximum. Treatment with either concentration of CAST reduced extracellular virus levels by more than 100-fold when measured over a four day period (Fig. 3b).

Extracellular virus stock generated in drug-free medium was adjusted to 2.5 mM CAST by addition of concentrated drug or left untreated and incubated overnight at 37°C. The level of virus infectivity subsequently determined showed similar levels in CAST-treated ($8.6 \times 10^5$ PFU/ml) when compared to the untreated controls ($5.8 \times 10^5$ PFU/ml). This was evidence that CAST was not viricidal and therefore...
this failed to account for the reduction in virus infectivity in the presence of the alkaloid.

**Comparison of three glucosidase inhibitors on the infectivity of different CMV strains**

The effects of treatment with CAST or the two related piperidines DNJ and FAG on the production of extracellular virus were compared using low passage clinical isolates in conjunction with AD169 virus (Fig. 4). HEF cells were infected with CMV 149 or CMR Ir (MOI 1 PFU/cell) or AD169 virus (MOI 5 PFU/cell) and treated with 2.5 or 1.25 mM CAST, 2.0 mM DNJ, 2.0 mM FAG or left untreated. Extracellular virus was monitored on at least two successive days as described above. With all three viruses, treatment with CAST or DNJ reduced infectivity titres between 50- to 500-fold whereas FAG, the 2-deoxy form of DNJ, produced a less than 10-fold reduction, when compared to untreated controls.

**Dose-response effects of glucosidase inhibitors**

The ability of CAST and DNJ to reduce CMV growth was confirmed by a plaque-
Fig. 5. Dose-response curves for CAST ●, DNJ ○ and DMDP ▲ with CMV strain AD169. The error bars show one standard deviation from the mean value of % control determined from a triplicate series of counts.

Fig. 6. The electron micrograph of HEF cells infected with AD169 virus and treated with 2.5 mM CAST illustrates the skein-like (SK) structure of a nuclear inclusion with associated nucleocapsids (NC) which can be seen budding through the nuclear membrane (X) into an involuted segment of cytoplasm (IC) acquiring an envelope to form mature virions (MV). The changes in fine structure of CMV infected HEF cells treated with CAST were indistinguishable from those in the untreated, infected control cells.
reduction assay. The pyrrolidine DMDP was also investigated for antiviral activity by this method (Fig. 5). AD169 virus was used to infect cell monolayers at low MOI (about 0.001 PFU/cell) as described in Materials and Methods. Monolayers were overlayed with medium containing varying concentrations of CAST, DNJ or DMDP (0.06–2.0 mM). ED\textsubscript{50} values determined from dose response curves were similar for CAST (0.8 mM) and DNJ (1.0 mM) and both were lower than that recorded for DMDP (1.8 mM).

Electron microscopic studies

Cytochemical analysis of CMV-infected cells treated with 2.5 mM CAST had previously revealed cytomegalic cells with large intranuclear inclusions characteristic of late stages of virus growth. Fine structural analysis, by ultrathin sectioning and transmission electron microscopy (EM), of CAST-treated (2.5 mM) cells infected with AD169 virus (MOI 5 PFU/cell) confirmed the advanced state of the infection (see Mobberley et al., 1987) in the presence of the inhibitor. In both treated and untreated infected cells, skein-like inclusions with numerous nucleocapsids were observed in the nucleus. The acquisition of the viral envelope was observed at the nuclear membrane and mature virions were found in the cytoplasm particularly in the Golgi region. The presence of mature virions in infected cells treated with CAST is illustrated in Fig. 6.

Virions were pelleted (see Materials and Methods) from the supernatant fluid recovered from both CAST-treated and untreated cultures and examined by transmission EM after negative staining with phosphotungstic acid. No gross differences were observed in the number of enveloped virions present in either preparation even though the levels of infectivity were reduced by greater than 100-fold (results not shown). This lowered infectivity to particle ratio was a clear indication that the synthesis of aberrant virions occurred in CAST treated cells with reduced efficiency for infection.

Analysis of envelope glycoproteins of virions produced in the presence of CAST

In order to test for differences in the composition or structure of glycoproteins expressed by CMV in the presence of CAST, investigations were made on gC1 glycoprotein complexes found in the virus envelope and recognised by monoclonal antibody F5. This antibody is specific for a 52 kDa glycoprotein which is a common component of three glycoprotein complexes as described in the introduction. Confluent HEF cell monolayers were infected with AD169 virus (MOI 5 PFU/cell) and treated with 2.5 mM CAST or left untreated. After five days incubation the supernatant fluid was collected and replaced with fresh MM with or without CAST. Virions from three successive harvests were pooled and pelleted by ultracentrifugation. Whole virions were subjected to iodination and the envelope components solubilised, immunoprecipitated with monoclonal F5 and analysed by SDS-PAGE under non-reducing conditions. The autoradiographic images for glycoprotein complex 1 (150 kDa), complex 2 (180 kDa) and complex 3 (250 kDa) were identified in precipitates from virion envelopes produced in drug-free conditions. Virions formed in the presence of CAST were characterised by the absence of com-
Fig. 7. Extracellular virus shed from HEF cells infected with AD169 virus treated with 2.5 mM CAST or left untreated was collected on three successive days. Respective virus suspensions were pooled, virions pelleted and the envelope glycoproteins iodinated. After immune precipitation, radiolabelled glycoproteins were separated by SDS-PAGE in non-reducing conditions. Glycoprotein complex 1 (150 kDa), complex 2 (180 kDa) and complex 3 (250 kDa), described by Farrar and Greenaway (1986), were identified by autoradiography and sizes determined by molecular weight standards. The relative amount of each complex was measured using a Shimadzu scanning densitometer.

Complex 2 (Fig. 7). The densitometric scans shown in this figure enabled a comparison of the relative amounts of each complex associated with envelopes from treated and untreated virions. This is documented in Table 1 along with the composition of each glycoprotein complex. It was concluded that complex 2 was replaced in
TABLE 1
CMV Envelope glycoprotein complexes: composition after treatment with CAST

| Complex | kDa | Components | % Area under curve |
|---------|-----|------------|-------------------|
|         |     | Virus control | CAST |
| 1       | 150 | gp95 + gp52 | 45 | 75 |
| 2       | 180 | gp130 + gp52 | 25 | - |
| 3       | 250 | dimer of complex 1 | 12 | 14 |

* Farrar and Greenaway (1986).

CAST-treated virions by additional amounts of complex 1. It is deduced from these data that in the presence of drug the 130 kDa glycoprotein was not formed and this has been confirmed by SDS-PAGE analysis of glycoprotein precipitates under reducing conditions (results not shown). The absence of this envelope component or its complexed form correlated with the marked reduction in virus infectivity (Table 2) associated with infected cultures used in Fig. 7. This also illustrates the reversible nature of the CAST-induced changes by the raised levels of virus infectivity once the drug was removed.

Discussion

Inhibition of glycosylation per se has been considered for some years as a viable strategy for antiviral chemotherapy. Using tunicamycin, D-glucosamine, B-hydroxynorvaline or 2-deoxy-D-glucose, in vitro activity has been shown against influenza virus, Semliki forest virus (Kilbourne, 1959; Schwarz and Klenk, 1974; Schwarz et al., 1976), herpes simplex virus (Courtney et al., 1973) and most recently HIV (Blough et al., 1986). All these compounds affect the synthesis of the glycan precursor. Clinical efficacy was indicated when the 2-deoxy-D-glucose was used topically on women with recurrent genital herpes infection (Blough and Giuntol, 1979) but this is unlikely to form the basis of a systemic therapy.

Glucosidase activities in the endoplasmic reticulum are involved in the early

TABLE 2
Infectivity of virions used for glycoprotein analysis and demonstration of the reversible nature of CAST inhibition

| Culture conditions | Days after infection |
|--------------------|---------------------|
|                    | 5  | 6  | 12 |
| Drug-free          | 2.1 × 10^6 (100%)   | 6.9 × 10^7 (100%) | 1.48 × 10^7 (100%) |
| CAST (2.5 mM)*     | 7.6 × 10^7 (0.06%)  | 9.0 × 10^6 (0.13%) | 1.00 × 10^7 (100%) |

Numbers denote extracellular infectivity (PFU/ml) recovered from 5 × 10^6 cells infected with AD169 virus at 5 PFU/cell.

* Cell monolayers were washed and CAST-free added on day 7.
stages of glycoprotein processing but their major influence on the rest of the pathway in general allows for exploitation as selective antiviral targets. This could be possible by quantitative differences in glycosylation between host and virus encoded glycoproteins, by the nature and complexity of oligosaccharides expressed on the virion surface or by effects on post-translational events. The three glucosidase inhibitors CAST, DNJ and DMDP, shown to have activity against CMV in this study, along with a related compound methyl-DNJ, are known to inhibit the replication of mouse hepatitis virus (Repp et al., 1985), Sindbis virus (McDowell et al., 1987), murine (Sunkara et al., 1987) and human (Gruters et al., 1987; Tyms et al., 1987; Walker et al., 1987) retroviruses. In the case of influenza virus, treatment of infected cells with inhibitors of glucosidase activity induced structural alteration of the major surface glycoprotein (haemagglutinin) consistent with the retention of glucose residues. However, the presence of high mannose oligosaccharides on the haemagglutinin had little or no effect on virion assembly or on subsequent infectivity (Burke et al., 1984; Pan et al., 1983). This suggested that, in this case, the sugar composition was of little importance for interaction with the host-cell receptor. In the present study, treatment with CAST did not affect the shedding of CMV but the ability of cells to make infectious virus was significantly reduced and this is likely to be so for the other alkaloids except FAG which showed little effect. The observations with CAST were accounted for by a
modification of the glycoproteins expressed on the virion surface and, in particular, appeared to be related to the loss of one glycoprotein complex from the virion envelope.

Efficient transport of a virion glycoprotein precursor to the surface of cells infected with mouse hepatitis virus was dependent on processing glucosidase activity and inhibition by CAST or methyl-DNJ resulted in a drop in virus yield (Repp et al., 1985). Likewise, correct processing of oligosaccharides on a glycosylated polypeptide of murine leukaemia virus (MuLV) to the more complex type was essential for transport to the plasma membrane before cleavage into virion envelope glycoproteins (Pinter et al., 1984). As expected, CAST or DNJ had potent effect on the replication of MuLV (Sunkara et al., 1987). Removal of glucose residues was equally critical for proteolytic cleavage of Sindbis glycoprotein precursor P3 for efficient virus production but modification of high mannose oligosaccharides was also important (McDowell et al., 1987). This was of interest because most of the other viruses cited above, and HIV (Gruners et al., 1987), were resistant to mannose processing inhibitors such as deoxymannojirimycin or swainsonine. With CMV, the loss of gp130 on virions synthesized in the presence of CAST also indicated inefficient transport or cleavage of a precursor glycoprotein. In the studies by Farrar and Greenaway (1986) a component of the gcI family of glycoproteins present on the virion surface, gp130, was complexed with gp52 (complex 2), but the present study has shown that this complex was completely replaced by gp52 disulphide-linked with gp95 (complex 1) in the absence of gp130 (see Table 1).

In previous studies, a monoclonal antibody (41C2) was shown to immunoprecipitate all three components of gcI present on the virus (Kari et al., 1986) along with a 138 kDa cell-associated glycoprotein (Gretch et al., 1988b). This precursor molecule was found as a 158 kDa glycoprotein in CAST-treated cells which appeared to be due to the retention of glucose residues on the oligosaccharide side-chains. However, in the presence of the glucosidase inhibitor, a gp55 was identified in abundance with indications that components in the range of 92–130 kDa were also present. Inhibition of transfer of glycoproteins from the cis-face to the trans-face of the Golgi apparatus by treatment with the ionophore monensin completely blocked further post-translational modification of the 158kDa and 138 kDa precursors (Gretch et al., 1988b).

The exact relationship between the different components of gcI has not been fully defined. V8 protease mapping demonstrated close similarities between gp93 and gp130 but although their relationship to gp55 was uncertain, a common precursor for all gcI components seems likely (Gretch et al., 1988b). One explanation of the results of the present study, consistent with the work of Gretch and his colleagues (1988b), is presented in Fig. 8. This scheme proposes alternative pathways for the fate of the 158 kDa precursor which is influenced by the retention of the terminal glucose residues on the oligosaccharide side-chains. In the presence of CAST, neither the high mannose intermediate 138 kDa (Gretch et al., 1988) nor its proposed mature form, gp130 (Fig. 7) was generated, hence the inability to form complex 2. In contrast, gp55 and gp93 (complex 1) were present even after treatment with CAST (Fig. 7), and appear to be generated from the 158 kDa precursor identified
by Gretchen et al. (1988b). This would mean that the oligosaccharides on gp55 and gp93 were retained in a high mannose form. This may have contributed to the reduced infectivity levels, although complex oligosaccharides may not be a requirement for the infective process as demonstrated for influenza virus (Burke et al., 1984; Pan et al., 1983).

Even though the nature of gp130 remains to be fully characterised, the association of infectivity with the presence of a CMV surface component is of major interest. The study has not considered the effect of glucosidase inhibitors on the components of gcI and gcII (Gretch et al., 1988a). This needs to be established as all three families appear to provide epitopes important for virus neutralisation (Britt, 1984; Rasmussen et al., 1984; Rasmussen et al., 1985; Kari et al., 1986). The observations in the present study could have important implications in the search for therapeutics for CMV. Although not yet fully determined, glucosidase inhibitors were well tolerated in animals (Saul et al., 1985) and man (Schnack et al., 1986; Joubert et al., 1986) and are of interest as potential antiviral agents. Also, the identification and characterisation of specific glycoproteins or their complexes which are associated with infectivity could provide the basis for a subunit vaccine.

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