Hantaan Virus Replication: Effects of Monensin, Tunicamycin and Endoglycosidases on the Structural Glycoproteins

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SUMMARY

The monovalent ionophore monensin, which interferes with cellular transport pathways, and the antibiotic tunicamycin, which prevents glycosylation of newly synthesized proteins, were used to examine Hantaan virus particle formation and polypeptide synthesis. Viral replication in the presence of either drug resulted in reduced antigen production as well as reduced yields of both intracellular and extracellular infectious virus. Analysis of viral polypeptides synthesized in the presence of the drugs suggested differential effects of monensin and tunicamycin on Hantaan virus. Although reduced levels of the three major structural proteins were detected with increasing concentrations of monensin, the electrophoretic migrations of the polypeptides synthesized were unaltered. In contrast, after tunicamycin treatment, G1 was no longer detectable and G2 displayed both a quantitative reduction and an apparent molecular weight reduction of approximately 3000. Both G1 and G2 were sensitive to endoglycosidases H and F with resultant electrophoretic mobility shifts corresponding to molecular weights of approximately 7000 for G1 and 3000 for G2. Oligosaccharides appeared to be mostly, but not entirely, of the high-mannose type.

INTRODUCTION

Hantavirus is the proposed name of a new and separate genus of the family Bunyaviridae comprising Hantaan and serologically related viruses (Schmaljohn et al., 1985). These biochemically and antigenically similar viruses are believed to be associated with a large group of human diseases collectively known as haemorrhagic fever with renal syndrome (HFRS). Viruses within this genus have three-segmented, single-stranded, negative-sense RNA genomes enclosed in ribonuclease-sensitive nucleocapsids (Schmaljohn et al., 1983; Schmaljohn & Dalrymple, 1983). Virion particles contain two envelope glycoproteins, designated G1 and G2, with apparent molecular weights of 68000 and 55000 respectively, a single nucleocapsid protein of approximately 50000 mol. wt. and a large polypeptide (200000 mol. wt.) which may represent the virion-associated polymerase (Schmaljohn et al., 1983; Schmaljohn & Dalrymple, 1984; Elliott et al., 1984). Examination of the kinetics of synthesis of Hantaan virus proteins within infected cells revealed that at a high multiplicity of infection, viral polypeptides could first be detected at 6 h post-infection, quantitatively increased up to 24 h, then remained constant up to 56 h (Schmaljohn & Dalrymple, 1984).

Little is known about the carbohydrate components of Hantaan virus envelope proteins, and even less about their relative importance for virus infectivity and maturation. The morphogenesis of most viruses in the Bunyaviridae involves budding into smooth vesicles at or near the Golgi complex (for review, see Bishop & Shope, 1979), but the maturation pathway of Hantaan virus has been difficult to define, mostly because of an inability to visualize viral morphogenesis by means of electron microscopy even when all cells can be demonstrated to possess viral antigen by fluorescent antibody staining (unpublished observations). While this frustrating paradox cannot be explained at present, it does not appear to be a general property of
all Hantaviruses, since recent studies employing thin-section and immuno-electron microscopy have indicated that various strains of Hantaviruses isolated in China mature in cisternae of the endoplasmic reticulum, rather than at the plasma membrane, in a manner similar to that described for other viruses in the Bunyaviridae family (Hung et al., 1983a, b, 1985). The morphogenesis of Hantaviruses may be somewhat different from typical Bunyaviridae, however, in that little or no association with the Golgi apparatus was observed, budding through cytoplasmic vesicles was only rarely observed, and large cytoplasmic viral inclusions were common (Hung et al., 1983b, 1985).

If the processing pathways of glycoproteins ultimately determine the site of viral budding, as suggested by Boulan & Pendergast (1980), then information concerning the assembly of viruses can also be obtained by examination of such pathways. For example, the glycoproteins of plasma membrane-maturing viruses, such as Semliki Forest virus and vesicular stomatitis virus, are rapidly processed from their site of synthesis in the rough endoplasmic reticulum, through the Golgi apparatus, and to the plasma membrane (for review, see Klenk & Rott, 1980). In contrast, the structural proteins of Uukuniemi virus, a member of the Bunyaviridae, have been shown to accumulate in the Golgi complex; this observation led to a proposal that deficient transport of glycoproteins to the plasma membrane may determine the site of virus maturation (Kuismanen, 1984).

Most information concerning the role of glycoprotein processing in virus assembly has come from studies employing glycosylation and transport inhibitors for disruption of various steps of glycosylation. Two inhibitors which have proved to be valuable tools for studying interrupted glycosylation pathways are the monovalent ionophore monensin and the antibiotic tunicamycin. Monensin has been reported to inhibit intracellular transport of membrane glycoproteins by blocking the release of secretory vesicles from Golgi membranes as well as to interfere with fusion of Golgi vesicles with the plasma membrane (Tartakoff & Vassalli, 1978). For viruses such as the Bunyaviridae, which appear to have an intimate association with the Golgi apparatus, such perturbation of its normal functions might be expected to alter the production and release of viral particles and perhaps result in the accumulation of virions or viral antigens within infected cells.

Unlike monensin, which only indirectly affects glycosylation, tunicamycin inhibits formation of \(N\)-acetylglucosamine–lipid intermediates, thus directly preventing asparagine-linked glycosylation of newly synthesized proteins (Tkacz & Lampen, 1975). Examination of virus yield and polypeptide synthesis in the presence of tunicamycin should therefore provide clues to the importance of \(N\)-linked oligosaccharide addition in viral replication.

To gain a better understanding of the role of the envelope glycoproteins in Hantaan virus morphogenesis, we have examined the effects of monensin and tunicamycin on virus antigen production, particle formation, infectivity and polypeptide synthesis. We have also performed a preliminary study of the type and amount of oligosaccharide residues attached to Hantaan virus envelope proteins.

**METHODS**

**Cells, viruses, medium and sera.** Hantaan virus strain 76-118 was propagated and assayed in Vero E6 cells (American Type Culture Collection C1008) as previously described (Schmaljohn et al., 1983; Schmaljohn & Dalrymple, 1983). Cell cultures were maintained in Eagle's MEM (Earle's salts) (EMEM) supplemented with 10% foetal bovine serum and penicillin and streptomycin at concentrations of 100 units and 100 \(\mu\)g per ml respectively. Hyperimmune mouse ascitic fluid (HMAF) was prepared by the method of Brandt et al. (1967) using infected suckling mouse brain suspensions to immunize outbred ICR mice.

**Monensin and tunicamycin treatments.** Monensin (Sigma) was dissolved in 95% ethanol to yield a concentration of 10 \(\mu\)M, and tunicamycin (Boehringer Mannheim) was dissolved in dimethyl sulphoxide (Sigma) to yield a concentration of 10 mg/ml. The drugs were subsequently diluted to experimental concentrations in EMEM. To assess the yield of infectious virus, 12-well tissue culture plates (22 mm diam.) were infected at a multiplicity of 10 p.f.u./cell with Hantaan virus and incubated at 37 °C for 4 h. The inocula were removed and cell monolayers were washed three times with physiological saline prior to addition of 1.0 ml EMEM containing 1% dialysed foetal bovine serum and monensin at concentrations of 0, 10\(^{-8}\) M, 10\(^{-6}\) M or 10\(^{-7}\) M or tunicamycin at a concentration of 2
Infectivity assays were performed at 24 h intervals on cell culture supernatants as previously described (Schmaljohn et al., 1983). Cell-associated infectivity was measured by scraping cells into physiological saline, freezing at −70 °C, thawing at 37 °C, mixing vigorously, pelleting cell debris at 13,000 g and assaying the resultant cell lysates. Intracellular antigen was examined by indirect immunofluorescence of cell monolayers grown on 16 mm diam. glass cover slips, utilizing dilutions of HMAF and fluorescein-conjugated goat anti-mouse antibody. Cover slips were mounted in phosphate-buffered glycerol and examined with a Zeiss epifluorescence microscope.

Radiolabelling of viral proteins. Four h prior to assay, intracellular viral proteins were radiolabelled with 100 µCi/ml [3H]leucine (Amersham) or 100 µCi/ml [35S]methionine in EMEM without leucine or methionine (Gibco) and containing the indicated amounts of monensin or tunicamycin. Cells were solubilized in 0.5 ml NET buffer (10 mM-Tris-HCl pH 7.4, 1% Triton X-100, 250 mM-NaCl, 1 mM-EDTA) containing 20 µg/ml aprotinin and 100 µg/ml α-2-macroglobulin (Sigma), nuclei were removed by centrifugation at 12,000 g, and viral proteins were immune-precipitated by incubation with 5 µl HMAF at 4 °C for 12 h followed by addition of Protein A-Sepharose (Sigma) in NET buffer. After a further incubation of 30 min, pellets were collected by centrifugation for 2 min at 12,000 g, washed four times in NET buffer, and once in 10 mM-Tris–HCl pH 7.4. Virion proteins were radiolabelled for 24 h prior to harvest, with 100 µCi/ml [3H]leucine in the presence of monensin and supernatants were directly immune-precipitated as above. Samples were boiled for 2 min in protein sample buffer (2.5% SDS, 5% 2-mercaptoethanol, 10% glycerol, 62.5 mM-Tris-HCl pH 6.7, 0.25% bromophenol blue) and analysed by electrophoresis in 12.5% acrylamide gels crosslinked with N,N‘-diallyltartardiamide (Bio-Rad) using conditions previously described (Schmaljohn et al., 1983).

Sucrose gradient sedimentation and radioimmune assay. Hantaan virus-infected cells were treated with 10⁻⁶ M monensin or 2 µg/ml tunicamycin for 12 h prior to harvest. Released viral particles were assayed by layering cell supernatants directly onto 8 ml 10 to 70% (w/v) sucrose gradients prepared in buffer (0.01 M-Tris-HCl pH 7.4, 0.1 M-NaCl, 0.001 M-EDTA) and centrifuging at 40,000 r.p.m. in a Beckman SW41 rotor for 90 min. Gradients were fractionated and examined for viral antigen by solid-phase radioimmunoassay as previously described (Schmaljohn et al., 1983).

Endoglycosidase H (Endo H) and F (Endo F) treatments. Intracellular viral proteins were radiolabelled with 200 µCi/ml [35S]methionine (Amersham) or 400 µCi/ml [2-3H]mannose (Amersham) from 24 to 28 h post-infection. Cell monolayers were solubilized in NET buffer and viral proteins immune-precipitated as above. The final wash samples were suspended into 50 µl Endo H buffer (0.15 M-Tris–HCl pH 8.8, 1% SDS, 1% 2-mercaptoethanol) or 50 µl Endo F buffer (75 mM-Na₂HPO₄/NaH₂PO₄ pH 6.1, 38 mM-EDTA, 0.75% NP40, 0.1% SDS, 1% 2-mercaptoethanol), boiled for 3 min, and cooled to room temperature. One-hundred µl buffer containing 300 mM-disodium citrate pH 5.5 and 50 mM-EDTA was added to samples to be treated with Endo H. The indicated concentrations of Endo H or F (New England Nuclear) were then added and samples were incubated at 37 °C overnight. Prior to electrophoresis, proteins were concentrated by precipitation with an equal volume of 20% TCA, incubated on ice for 10 min, recovered by centrifugation at 12,000 g and the pellets washed twice in cold acetone.

**RESULTS**

**Infectious virus and viral antigen production in the presence of monensin**

The effect of monensin on both released and intracellular Hantaan virus infectivity was examined by propagating virus in the presence of 0, 10⁻⁵, 10⁻⁶ or 10⁻⁷ M-monensin. Virus infectivity was quantified by plaque assay. As displayed in Fig. 1(a), a monensin concentration of 10⁻⁵ M completely inhibited infectious virus release, 10⁻⁶ M reduced infectivity, and 10⁻⁷ M slowed the production of infectious particles and slightly reduced virus yield. Similar results were observed within infected cells, except that 10⁻⁷ M-monensin-treated cultures achieved infectivity titres equivalent to those of untreated cultures with no delay (Fig. 1b). Both infected and uninfected cells treated with 10⁻⁵ or 10⁻⁶ M-monensin developed cytopathic effects by 3 and 4 days after treatment, respectively; thus, values beyond this are not shown. No toxicity appeared to be associated with 10⁻⁷ M-monensin throughout the experiment.

The accumulation of antigen within infected cells during monensin treatment was monitored by indirect fluorescent antibody staining (Fig. 1c). Monensin concentrations of 10⁻⁵ and 10⁻⁶ M drastically reduced the amount of viral antigen present in infected cells, whereas viral antigen in 10⁻⁷ M-monensin-treated cells was quantitatively indistinguishable from that observed in untreated cells. Since accumulation of intracellular Hantaan virus antigen closely paralleled infectious virus production, the data suggest that monensin inhibited viral protein production, resulting in reduced extracellular virus yields.
Fig. 1. Infectious virus yield in the presence of monensin. The effects of monensin on infectious Hantaan virus released from infected cells (a) or produced within infected cells (b) were assayed by plaque formation on Vero E6 cell monolayers. Accumulation of viral antigen within cells (c) was monitored by indirect fluorescent antibody (FA) staining. An arbitrary scale of 1 +, 2 +, 3 + or 4 + indicates approximately 0 to 10%, 10 to 50%, 50 to 75% and 75 to 100% fluorescent antibody-positive cells, respectively. Concentrations of monensin of 0 (○), 10⁻⁵ (■), 10⁻⁶ (▲) and 10⁻⁷ (○) M were examined.

Fig. 2. Comparison of the effects of monensin and tunicamycin on virus production. Hantaan virus-infected Vero E6 cells were treated with 2 µg/ml tunicamycin or 10⁻⁶ M-monensin for 12 h prior to assay at days 1 to 6 after infection. Infectious virus in the cell supernatant (a) or within infected cells (b) was assayed by plaque formation. Release of non-infectious particles was examined by sedimentation of cell supernatants through linear sucrose gradients. The results displayed in (c) represent supernatant at 3 days post-infection. Gradient fractions were assayed for the presence of viral antigen by solid-phase radioimmunoassay. ●, No treatment; ○, tunicamycin; ▲, monensin.
Hantaan virus glycoproteins

Comparison of the effects of tunicamycin and monensin on the production of virus particles

Infectious virus yields from cell cultures treated for 12 h with monensin (10^{-6} M) or tunicamycin (2 μg/ml) were compared at various times after infection. Such short-term treatment of cells with the inhibitors allowed examination of infectivity yields without detectable drug-induced cytopathic effects. Even with these relatively brief treatment conditions, both inhibitors significantly reduced the amount of infectious virus released from and contained within infected cells (Fig. 2a, b).

To examine the possibility that only infectivity was inhibited and non-infectious particles were still released, cell supernatants were sedimented through sucrose gradients and fractions examined for the presence of virus antigen by radioimmunounassay. Although sedimentable viral antigen was recovered from the supernatants of untreated cells, little antigen could be detected in supernatants of cells treated with either monensin or tunicamycin (Fig. 2c). These results were confirmed by recovery of an identical peak of radiolabelled, sedimentable particles from infected cell cultures pretreated with [5,6-3H]uridine in the absence, but not in the presence, of monensin or tunicamycin (data not shown). These data demonstrate that not only was the release of infectious particles inhibited, but also that extrusion of viral particles in general was blocked.

Comparison of polypeptides synthesized in the presence of tunicamycin or monensin

To determine whether the monensin- and tunicamycin-induced inhibition of infectious virus was accompanied by processing defects which might be reflected in electrophoretic mobility shifts of viral polypeptides, radiolabelled polypeptides synthesized in cells treated with the inhibitors were examined. The effects of tunicamycin were assessed by pulse-labelling viral polypeptides in the presence of increasing concentrations of the drug. As the concentration of tunicamycin increased, a noticeable decrease in the amount of viral glycoproteins was observed. Although G1 could no longer be observed at concentrations of tunicamycin in excess of 1 μg/ml, a limited amount of G2 with a decrease in mol. wt. of about 3000 was still apparent (Fig. 3). The diffuse nature of untreated G1 and G2 may indicate the utilization of more than one glycosylation site on each polypeptide and consequently the simultaneous presence of proteins in more than one glycosylation state. While we have no direct evidence for this, the gradual sharpening of the G2 band (Fig. 3) to a single species at the increased tunicamycin concentrations, may be consistent with the stripping of sugars at more and less accessible sites.

A non-viral polypeptide with a mol. wt. of approximately 80000 was also precipitated from both infected and uninfected Vero E6 cells treated with tunicamycin and was not pursued further (Fig. 3, arrow).

Recently, it was found that tunicamycin is not a single compound but instead is composed of several homologues, some of which inhibit only glycosylation, while others inhibit protein synthesis as well (Mahoney & Duksin, 1979). Although decreased protein synthesis may be responsible in part for the observed absence of G1 and reduction in the amount of G2, it is not likely to be the only factor, since the amount of unglycosylated nucleocapsid protein decreased only slightly even at the highest concentration of tunicamycin tested. These data indicate either that non-glycosylated Hantaan virus envelope proteins are not synthesized in the presence of tunicamycin, or are not stable without attached oligosaccharides. Both possibilities will be discussed.

The effects of monensin on viral polypeptide production were examined by immune precipitation of intracellular and extracellular proteins synthesized in the presence of three concentrations of monensin at various times after infection. In the absence of monensin, virus-specific polypeptides could be detected within infected cells 1 day post-infection and in cell supernatants by 2 days (not shown). In the presence of monensin, viral polypeptides were first detected intracellularly by 2 days and extracellularly by 3 days (Fig. 4). Cell-associated polypeptides synthesized in the absence of monensin or in the presence of 10^{-7} M-monensin appeared quantitatively similar, with a slight increase in polypeptides detected in treated cultures at days 4 and 5. Monensin concentrations of 10^{-6} and 10^{-5} M, however, appeared to inhibit drastically or completely the synthesis of viral proteins. As in the infectivity studies
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Fig. 3. Hantaan virus polypeptides synthesized in the presence of increasing concentrations of tunicamycin. Infected Vero E6 cells were pretreated for 5 h with 0 to 10 μg/ml of tunicamycin as indicated, prior to radiolabelling for 4 h with [35S]methionine in the presence of tunicamycin. Viral polypeptides were immune-precipitated and analysed by PAGE. The arrow indicates a non-viral protein (see text). C, uninfected control; NC, nucleocapsid protein.

detailed above, neither infected nor uninfected cells treated with 10⁻⁵ or 10⁻⁶ M-monensin could be examined at later times due to the cytotoxic effects of monensin. In contrast to the apparent increase in the amount of intracellular virus-specific polypeptides detected following treatment with 10⁻⁷ M-monensin, extracellular viral polypeptides were greatly reduced by all concentrations of monensin tested. Highly radiolabelled, extracellular material which was non-specifically precipitated from supernatants of both infected and uninfected cells at the higher monensin concentrations and with increasing periods of treatment obscured detection of viral polypeptides in some supernatant samples and was believed to be cell debris associated with the disruptive effects of monensin on normal cell functions. Nevertheless, it was apparent from these data that electrophoretic migration of Hantaan virus polypeptides synthesized in monensin-treated or untreated cells were indistinguishable, and suggested that all conditions allowing viral polypeptide synthesis resulted in normal Hantaan virus proteins. While both the tunicamycin- and the monensin-induced reduction in viral particle formation appeared to be at the level of synthesis of polypeptides, only tunicamycin resulted in altered electrophoretic migration of one of the glycoproteins, indicative of inhibition of oligosaccharide addition.

Deglycosylation by Endo H and Endo F treatment

The characteristics of the sugar residues attached to the G1 and G2 proteins of Hantaan virus were examined by enzymic cleavage. Several naturally occurring endoglycosidases have been described with specificities for N-linked oligosaccharides. Endo H (endo-β-N-acetylglucosaminidase H) is specific for neutral oligosaccharides, particularly of the high-mannose type, while endoglycosidase F cleaves both high-mannose and complex sugars from asparagine-linked glycoproteins (Tarentino et al., 1974). Following immune precipitation, Hantaan virus proteins from infected cell lysates were treated with increasing concentrations of Endo H or Endo F and examined by polyacrylamide gel electrophoresis. Both enzymes produced a relative mol. wt. decrease in G1 of approximately 7000 and in G2 of approximately 3000, while the molecular weight of the nucleocapsid protein appeared unchanged (Fig. 5a, b).

To determine whether all sugar moieties on Hantaan virus proteins could be removed with Endo H and consequently were entirely of the high-mannose type, viral proteins were radiolabelled with [3H]mannose and digested with the two endoglycosidases (Fig. 5c). Most, but
Fig. 4. Influence of monensin on viral polypeptide synthesis. Hantaan virus-infected Vero E6 cells were treated with monensin from 4 h to 5 days after infection. Intracellular viral polypeptides were radiolabelled with [3H]leucine for 4 h and virion polypeptides for 24 h prior to harvest and were immune-precipitated and analysed by PAGE at days 1 to 5. Lanes 1 and 2, cell controls with 0 or 10^{-5} M-monensin. Lanes 3, 4, 5, 6, infected cells with 0, 10^{-7}, 10^{-6} or 10^{-5} M-monensin. Lane 7, uninfected cell supernatant with no monensin. Lanes 8, 9, 10, infected cell supernatant with 0, 10^{-7} or 10^{-6} M-monensin.
Fig. 5. Endoglycosidase treatment of Hantaan virus proteins. (a, b) Intracellular polypeptides were immune-precipitated from Hantaan virus-infected Vero E6 cell cultures radiolabelled with [35S]methionine, treated with (a) Endo H (0 to 0.3 μg/sample) or (b) Endo F (0 to 1.0 units/sample), and examined by PAGE. Spurious polypeptide bands were occasionally observed between G1 and G2 and beneath the nucleocapsid band and were believed to be due to non-specific protease degradation which occurred during the 20 h incubation of these samples at 37 °C. (c) Hantaan virus polypeptides were radiolabelled with [2-3H]mannose prior to treatment with Endo H (0.2 μg) or Endo F (0.5 unit). Lanes 1 and 3, 14C-methylated protein markers (Amersham); mol. wt. × 10^-3. Lane 2, purified Hantaan virion proteins radiolabelled with [35S]methionine. Lane 4, Hantaan virion proteins radiolabelled with [3H]mannose. Lane 5, Endo H digestion of [3H]mannose-labelled polypeptides. Lane 6, Endo F digestion of [3H]mannose-labelled polypeptides.

not all, of the sugar could be removed with Endo H, as indicated by the diminution of radiolabel as well as the shift in apparent mol. wt. following enzyme digestion (lane 5). In contrast, almost no sugar label remained following Endo F treatment (lane 6). A small amount of 3H label was apparently metabolized into amino acids, as indicated by labelling of the nucleocapsid protein (which remained constant in both enzyme-treated samples). This consequently served as a convenient marker for quantification. A scanning densitometer tracing of the autoradiograph of a non-digested sample (Fig. 5c, lane 4) showed that the nucleocapsid band accounted for 7.5% of the total detectable radiolabel as calculated by integration of the three major peaks. Using this value as a constant for scanning densitometric tracings of lanes 5 and 6, Endo H digestion was calculated to remove 87% of total label from G2 and 58% from G1, while Endo F digestion removed 96% of G2 and 97% of G1. These data collectively suggest that the sugar residues on Hantaan virus envelope glycoproteins are predominantly, but not entirely, of the high-mannose type.

DISCUSSION

The glycoproteins of viruses have been shown to play major roles in virus assembly, transport, infectivity and antigenicity. The precise role of the sugar residues in these processes is still unclear, but the requirement for correct glycosylation appears to vary for different viruses (for review, see Klenk & Rott, 1980). It is clear, however, that the primary and secondary structure of the polypeptide dictates whether and what type of glycosylation is necessary. Experimentally, it is possible to trace the pathway of a newly synthesized protein through the intracellular compartments by examining its glycosylation pattern. For example, it is known that
oligosaccharides containing glucosamine and mannose are transferred \textit{en bloc} from a polyisoprenol derivative to the polypeptide in the endoplasmic reticulum. Glucosyl and mannose residues are subsequently trimmed and terminal sugar residues are added in the smooth endoplasmic reticulum and Golgi apparatus. Removal of mannose residues determines whether a high-mannose or complex type sugar structure will be present on the viral glycoprotein (Klenk & Rott, 1980). Because the Bunyaviridae have been shown to mature in the endoplasmic reticulum at or near the Golgi complex, rather than at the cell surface as is the case for most enveloped viruses (Bishop & Shope, 1979), disruption of glycosylation processes involved in transfer of the core sugars and transport through the Golgi apparatus can provide clues to the process of morphogenesis by allowing examination of specific maturation events. Two drugs useful for such studies are the antibiotic tunicamycin and the ionophore monensin.

We have examined the effects of tunicamycin and monensin on (i) the yield of released and intracellular virus, (ii) the accumulation of viral antigen within cells and (iii) the polypeptides synthesized within cells and released into cell supernatants. Not only was the production of infectious virus particles inhibited by both drugs, but also the release of assembled particles in general appeared to be blocked. Examination of viral antigen and polypeptides produced in the presence of the drugs indicated that the reduced yield of viral particles was directly correlated with a reduction in the amount of viral polypeptides rather than to any obvious glycoprotein processing defect. Although increasing concentrations of monensin and treatment times did not result in the accumulation of intracellular viral antigens detectable by fluorescent antibody staining, low concentrations of monensin ($10^{-7} \text{M}$) did appear to produce slightly increased levels of intracellular Hantaan virus polypeptides. Concomitantly decreased levels of extracellular polypeptides may indicate a block in virus assembly. Despite the reduced quantities of Hantaan virus polypeptides observed following monensin treatment, all viral polypeptides which were synthesized were indistinguishable from those produced in untreated cells. These results differ from those reported for another bunyavirus, La Crosse virus, for which the monensin-induced reduction of infectious virus was accompanied by a defect in G1 protein processing. This defect resulted in accumulation of a polypeptide with an altered glycosylation pattern that appeared to be an intermediate in the normal glycosylation pathways (Madoff & Lenard, 1982).

In contrast to the uniform reduction in quantities of Hantaan virus polypeptides observed with monensin treatment, a selective reduction in envelope glycoproteins could be detected in infected cells after tunicamycin treatment. This effect was particularly pronounced with the G1 protein which appeared to be more highly glycosylated. Three explanations for this observation might be (i) polypeptides were not synthesized, (ii) unglycosylated proteins exhibited decreased solubility or sustained conformational changes which made them unrecognizable to antibodies used for immune precipitation or (iii) the unglycosylated protein may be preferentially degraded by endogenous cellular proteases. Although some of the components of tunicamycin are known to inhibit protein synthesis in general, the continued synthesis of Hantaan virus nucleocapsid protein argues against this explanation. Furthermore, similar experiments performed using the A1, B2, C2 and D2 homologues of tunicamycin (Boehringer Mannheim) which have been shown not to inhibit protein synthesis (Mahoney & Duksin, 1979), produced results identical to those shown in Fig. 3. Altered solubility in non-ionic detergents such as Triton X-100 has been shown to occur with unglycosylated proteins of vesicular stomatitis and Sindbis viruses (Leavitt \textit{et al.}, 1977; Gibson \textit{et al.}, 1979). While decreased solubility may have occurred after tunicamycin treatment, it did not prevent polyclonal or monoclonal antibodies from recognizing viral proteins in fluorescent antibody or radioimmunoassays (data not shown), and so presumably would not have affected immune precipitation. A precedent for the third possibility is the demonstration that certain unglycosylated forms of viral glycoproteins can have different stabilities within the same host cell due to endogenous cellular proteases (Schwarz \textit{et al.}, 1976). Degradation of the unglycosylated haemagglutinin protein of fowl plague virus was reported to occur in infected chick cells and could be partially counteracted by treatment of infected cells with the protease inhibitor TLCK which allowed accumulation of the carbohydrate-free precursor of the haemagglutinin protein (Schwarz \textit{et al.}, 1976). Preliminary results from our laboratory have indicated that pretreatment of infected cells with the general protease inhibitor
α-2-macroglobulin (10 mg/ml) and the serine protease inhibitor aprotinin (1 mg/ml) does allow increased and reproducible detection of G1 and G2 (although not the levels detectable in cultures not treated with tunicamycin), suggesting that host or virus-specified protease degradation may be a factor in the reduced levels of Hantaan virus glycoproteins observable following tunicamycin treatment (data not shown).

The type and amount of sugar residues present on viral glycoproteins can also help to identify maturation events. Two broad classes of asparagine-linked oligosaccharides (complex or high-mannose) are found on mature glycoproteins. Often both types are attached to the same polypeptide chain. The nature and extent of glycosylation of bunyavirus proteins may reflect their unique site and mode of morphogenesis in association with the Golgi apparatus. This was evidenced by novel oligosaccharides which appeared to be intermediate between the high-mannose and complex structures found in association with the glycoproteins of Uukuniemi, Inkoo and La Crosse viruses (Kuismanen et al., 1982; Madoff & Lenard, 1982; Pesonen et al., 1982; Kuismanen, 1984). Enzymes such as Endo H, which cleaves only high-mannose residues, and Endo F, which cleaves both high-mannose and complex residues, have proved useful for determining the basic structure of oligosaccharides. Acquired resistance to Endo H generally indicates that proteins have been processed through the Golgi apparatus and can serve as a marker for determining the sequence of events leading to development of mature viral glycoproteins. Digestion of Hantaan virus proteins with Endo H and Endo F indicated that most, but not all, oligosaccharides attached to G1 and G2 were high-mannose residues, and may suggest that this virus is not completely processed through the Golgi complex. The greater Endo H resistance detected on the more highly glycosylated Hantaan virus G1 protein may indicate the presence of complex sugars or structures similar to the unique intermediate oligosaccharides described for other members of the Bunyaviridae (Kuismanen et al., 1982; Madoff & Lenard, 1982; Pesonen et al., 1982; Kuismanen, 1984).

In summary, our data indicate that glycosylation processes are necessary for production of mature, infectious Hantaan virus particles. Disruption of either the transfer of core sugars to the polypeptide chain or transport through the Golgi apparatus interferes with virus replication. More detailed examination of the exact nature of Hantaan oligosaccharides, including terminal sugar analysis, should help to define which cell compartments are utilized during various stages of Hantaan virus maturation.

REFERENCES


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