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Antiviral Research, 2 (1982) 177–190
Elsevier Biomedical Press

Invited paper

VIRAL GLYCOPROTEIN METABOLISM AS A TARGET FOR ANTIVIRAL SUBSTANCES

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(Received 17 April 1982; accepted 17 April 1982)

glycoproteins inhibition of glycosylation tunicamycin deoxyglucose glucosamine dolichol pathway of glycosylation

INTRODUCTION

Many viruses, pathogenic for man and animals, possess a surface membrane, the viral envelope. Although significant differences in number and arrangement of envelope constituents occur, there are several features of membrane structure shared by all of these viruses. These include the presence of a lipid bilayer and glycosylated proteins exposed on the external surface of the bilayer. The glycoproteins are usually present in the form of spikes about 7–10 nm in length. The glycoproteins are amphipathic molecules. They consist of an external hydrophilic part and of a hydrophobic segment by which the spikes are strongly associated with the lipid bilayer (for review see Compans and Klenk [7]).

It is generally accepted that the virus spikes play important roles in the interaction between viral envelopes and cellular membranes and that their primary biological function is the initiation of infection at the cellular level. This has probably been shown best with the ortho- and paramyxoviruses where the specific roles played by the viral glycoproteins in adsorption and penetration are quite well understood. It has also been demonstrated that the glycoproteins of these viruses are important determinants for the spread of infection and thus for pathogenicity (for review see Klenk et al. [39]). Another important aspect is the function of the viral glycoproteins in the host defense. As the major surface antigens they are usually the targets of neutralizing antibodies.

Most of the viral glycoproteins studied to date contain oligosaccharide side chains that are attached by *N*-glycosidic linkages to the polypeptide. These glycoproteins include among others the hemagglutinin and the neuraminidase of influenza viruses, the hemagglutinin-neuraminidase and the fusion protein of paramyxoviruses, and the glycoproteins of Semliki Forest virus, Sindbis virus, and vesicular stomatitis virus (for review, see Compans and Klenk [7]). Glycoproteins with *O*-glycosidic linkages have so far rarely

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been observed in viruses. Such a glycoprotein has recently been characterized in coronaviruses where it is present together with another glycoprotein of the *N*-glycosidic type [25, 49].

The biosynthesis of viral glycoproteins with *N*-glycosidic linkages has been studied in detail. It involves translation at membrane-bound ribosomes, insertion into the membrane of the rough endoplasmic reticulum, and transport to the site of virus assembly, which is usually the plasma membrane. In the course of transport, the glycoproteins are processed by glycosylation, proteolytic cleavage, and the covalent binding of fatty acids. At the cotranslational level, proteolytic cleavage in many instances removes the signal sequences required for insertion of the nascent polypeptides into the membrane of the rough endoplasmic reticulum. Posttranslational cleavage is involved in the processing of a series of these glycoproteins, such as the influenza hemagglutinin and the glycoproteins of paramyxoviruses, alphaviruses, and oncoviruses. In the case of the myxovirus glycoproteins, posttranslational proteolytic cleavage was found to be a precondition for biological activity (for review, see Klenk and Rott [35]).

The biosynthesis of glycoproteins can be inhibited by interfering with the intracellular transport, with proteolytic cleavage, and with glycosylation. This article will be confined to the inhibition of glycosylation. To date, only glycosylation inhibitors interfering with the biosynthesis of *N*-glycosidically linked oligosaccharides are known. Thus, only observations made on glycoproteins containing this type of side chains will be reviewed.

THE BIOSYNTHESIS AND STRUCTURE OF CARBOHYDRATE SIDE CHAINS LINKED BY *N*-GLYCOSIDIC BONDS TO THE POLYPEPTIDE

The oligosaccharide side chains of the viral glycoproteins are assembled through the biosynthetic machinery of the host cell by the same general principles as the carbohydrates of cellular glycoproteins. Glycosylation of glycoproteins with *N*-glycosidic linkages is initiated in the rough endoplasmic reticulum by the attachment of preformed oligosaccharides to asparagine residues in the polypeptide chain. The attachment sites have the sequence asparagine-*X*-threonine or asparagine-*X*-serine, with *X* being a variable amino acid.

The oligosaccharides transferred to the nascent polypeptide are synthesized via the dolichol pathway of glycosylation which recently has been reviewed in detail elsewhere [26] and can be summarized as follows (Fig. 1). The carbohydrate chains are assembled on the phosphate ester of the isoprenoid alcohol dolichol with UDP-*N*-acetylglucosamine, GDP-mannose, mannosylphosphoryldolichol, and glucosylphosphoryldolichol as sugar donors. After the en bloc transfer of the oligosaccharide to the polypeptide and cleavage of the pyrophosphate linkage dolichol phosphate is available for a new assembly cycle. The oligosaccharide synthesized in this way is usually a tetra-decasaccharide of the structure $(\text{glucosyl})_3-(\text{mannosyl})_9-(N\text{-acetylglucosamine})_2$. Under certain conditions to be described below a decasaccharide of the structure $(\text{glucosyl})_3-(\text{mannosyl})_5-(N\text{-acetylglucosamine})_2$ can be transferred. Glycosylation via the decasaccharide lipid is

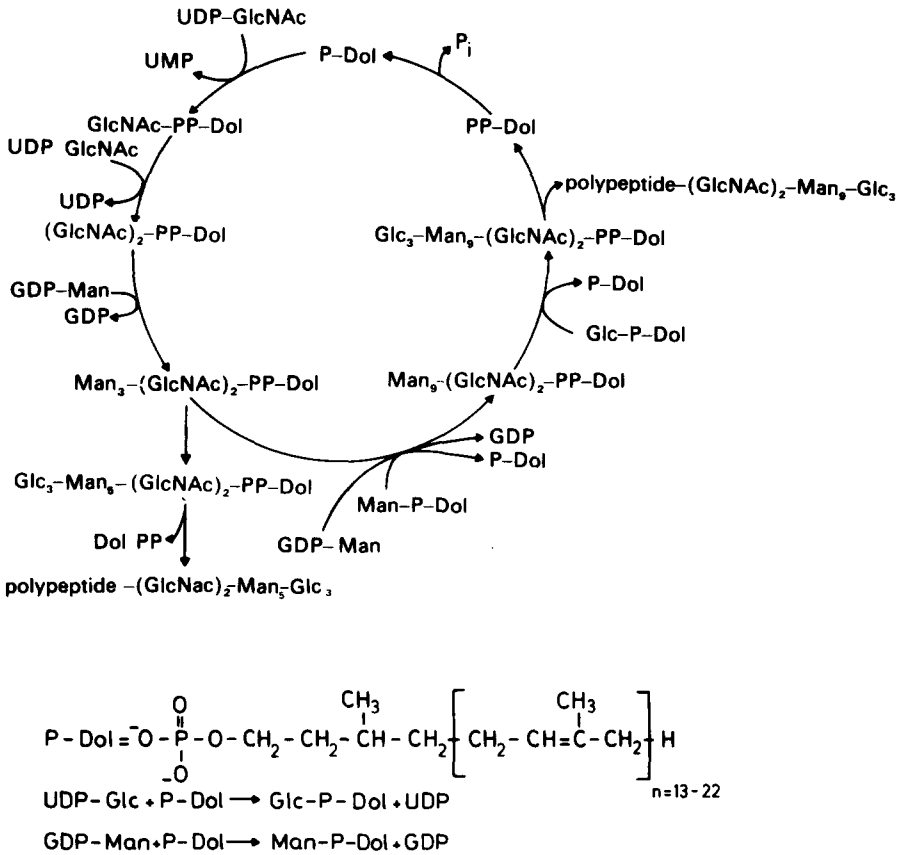

Fig. 1. The biosynthesis of the dolichol-linked oligosaccharide precursors of the asparagine-linked carbohydrate side chains. Dol, dolichol; GlcNAc, *N*-acetylglucosamine; Man, mannose; Glc, glucose.

called the alternate pathway. The enzymes involved in the assembly of the lipid-linked oligosaccharides are membrane-bound and appear to occur mainly in the rough endoplasmic reticulum (for a discussion, see Schwarz and Datema [61].

After the transfer to the polypeptide the oligosaccharide side chains are further processed (Fig. 2). First, the three glucose residues are removed by specific glucosidases to yield a mannose-rich carbohydrate side chain [23, 24]. At this stage, processing of the oligosaccharide may cease. In most instances, however, it continues by a trimming process resulting in the release of all the mannoses but three. By the subsequent addition of *N*-acetylglucosamine, galactose, fucose, and neuraminic acid the complex oligosaccharides are formed [41]. The available evidence indicates that the enzymes involved in this last step in the glycosylation sequence are located in the Golgi apparatus (for review, see Klenk and Rott [35].

From this review on the carbohydrate synthesis it is clear that complex and mannose-rich side chains of the general structures shown in Fig. 2 exist in mature viral glycopro-

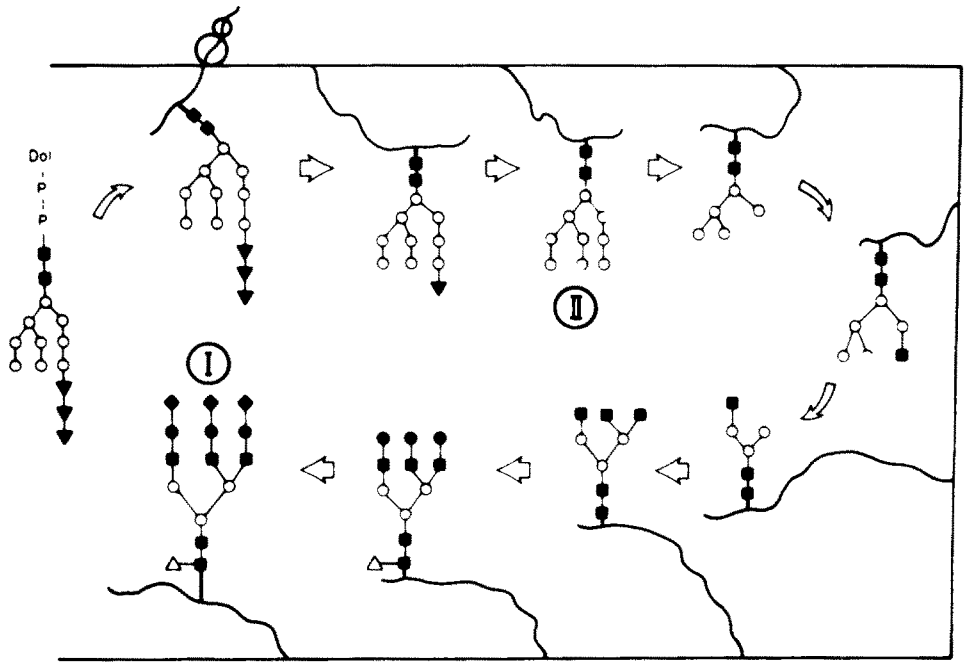

Fig. 2. The processing of the protein-bound oligosaccharides. The structures of the complex (I) and of the mannose-rich (II) side chains found in mature glycoproteins are indicated. Dol, dolichol. *N*-Acetylglucosamine (■); mannose (○); glucose (△); galactose (●); neuraminic acid (◆); fucose (△). (Adapted from Kornfeld et al. [41].)

teins and that both types are derived from a common precursor. It is also evident from what has been said above that the number and the position of the oligosaccharides on the polypeptide is determined by the presence of appropriate amino acid sequences that can act as attachment sites. The number of side chains attached to different glycoproteins varies therefore over a wide range; e.g. the G protein of vesicular stomatitis virus has only two oligosaccharides [17, 53], whereas as many as seven side chains can be observed on the influenza hemagglutinin. The studies on the influenza hemagglutinin revealed also that the many variants of this glycoprotein vary widely in the distribution of the glycosylation sites and the oligosaccharide types attached to them [38, 48, 59, 70]. These results indicate that the structure of the polypeptide plays an important role in determining whether a mannose-rich or a complex oligosaccharide is attached to a given glycosylation site. Despite of the high variability of most glycosylation sites of the hemagglutinin, some of them appear to be conserved. It is interesting to note that according to the three-dimensional model of the hemagglutinin [71] all conserved side chains are located in close vicinity to each other at the base of the spike. This may indicate that these side chains have a special function in maintaining the proper implantation of the glycoprotein in the lipid bilayer [38].

INHIBITORS OF GLYCOSYLATION

In recent years a variety of substances have been discovered which interfere with the biosynthesis of enveloped viruses (Table 1). These include, among others, D-glucosamine

TABLE 1

Viruses susceptible to glycosylation inhibitors

Virus group	Inhibitor	Reference
Arenaviruses	Glucosamine	45
Bunyaviruses	Tunicamycin	4
	Glucosamine	4
Coronaviruses	Deoxyglucose	49
	Fluoroglucose	49
	Glucosamine	49
	Tunicamycin	49
		25
		54
Herpesviruses	Benzhydrazone	3
	Deoxyglucose	8, 18
	Glucosamine	42
	Tunicamycin	66
Oncoviruses	Glucosamine	27
	Tunicamycin	63
Orthomyxoviruses	Deoxyglucose	34
		19
		30
		36
		58
	Fluoroglucose	13, 14
	Glucosamine	19
		30
		36
		58
	Tunicamycin	63
		47
Paramyxoviruses	Deoxyglucose	2
	Glucosamine	2
	Tunicamycin	66
Togaviruses	Deoxyglucose	31
	Fluoroglucose	55
	Glucosamine	15
	Tunicamycin	63

and 2-deoxy-D-glucose [19, 30, 34], 2-deoxy-2-fluoro-D-glucose [55], and tunicamycin [66]. The first virus to demonstrate that these substances specifically interfere with glycosylation was influenza virus, where in the presence of inhibitors the unglycosylated form of the viral hemagglutinin could be identified [36, 58]. Inhibitors of glycosylation became therefore valuable tools in studying the role of the carbohydrate moiety in the biosynthesis and function of viral glycoproteins. Most of these substances interfere with the assembly of dolichol-linked oligosaccharides. Little is known on inhibitors interfering with the transfer to the polypeptide or with the processing of the protein-bound oligosaccharide. Because the different steps in glycosylation occur in different cell compartments, inhibitors of intracellular transport (for review, see Tartakoff [68] will indirectly interfere with processing. In the following the mode of action of the glycosylation inhibitors interfering with virus multiplication will be briefly discussed. For further information the reader is referred to more comprehensive reviews [57, 60–62]. The latter two deal also with other glycosylation inhibitors, such as bacitracin, amphomycin, showdomycin, and diumycin which so far have not been found to be active in intact animal cells.

Deoxyglucose (2-deoxy-D-glucose, 2-deoxy-D-arabinohexose)

This sugar analogue is readily metabolized in the cell, and some of these metabolites, namely UDP-deoxyglucose, GDP-deoxyglucose, and deoxyglucosylphosphoryldolichol, are the actual inhibitory agents [11, 12, 64]. The reaction of the dolichol cycle inhibited by UDP-deoxyglucose is the formation of glucosylphosphoryldolichol (Fig. 1). GDP-deoxyglucose, which appears to be the most important inhibitory metabolite, interferes 1) with the formation of dolichol-linked monosaccharides by trapping phosphoryldolichol as deoxyglucosylphosphoryldolichol, and 2) with the assembly of the lipid-linked oligosaccharide by incorporation of deoxyglucose instead of mannose. Deoxyglucosylphosphoryldolichol interferes also with the formation of the lipid-bound oligosaccharide by replacing glucose with deoxyglucose. Studies in cell-free systems have shown that lipid-linked oligosaccharides containing deoxyglucose are not transferred to the polypeptide [11]. On the other hand, it has also been shown that under non-inhibitory conditions deoxyglucose can be incorporated into glycoproteins [31]. This apparent discrepancy is not understood. It is obvious from these findings that deoxyglucose acts primarily as an analogue to mannose. It is therefore not surprising that its inhibitory action can be reversed by mannose [31].

Fluoroglucose (2-deoxy-2-fluoro-D-glucose)

Fluoroglucose interferes with mannosylation reactions. It inhibits the formation of mannosylphosphoryldolichol and, therefore, blocks the assembly of the lipid-bound tetra-decasaccharide [13]. The inhibition of this assembly can be reversed or prevented by mannose [10]. It is interesting to note that in the presence of fluoroglucose the

alternative pathway of glycosylation is not inhibited. Thus, the decasaccharide shown in Fig. 1 can still be transferred to the protein [14].

CCCP (m-chlorocarbonylcyanide-phenylhydrazone)

Studies on the effects on protein glycosylation of this uncoupler of oxidative phosphorylation have shown that glycosylation via the alternate pathway is not a unique feature of fluoroglucose. When cells infected with influenza virus are treated with CCCP, endoglucosaminidase H-resistant mannose-rich intermediates of the alternate pathway are found in the viral glycoproteins. These oligosaccharides are not processed to complex forms because energy depletion by CCCP decreases the migration rate of the glycoproteins to the Golgi system [11].

Glucosamine (2-deoxy-2-amino-D-glucose)

The investigation of the metabolism of glucosamine under conditions that inhibit protein glycosylation did not reveal any unusual metabolites, but intracellular concentration of glucosamine was strongly increased. Incubation with glucosamine-free medium rapidly reverses the inhibition of glycosylation. During this reversion only the intracellular concentration of glucosamine and not of its metabolites decreases. Therefore, it was deduced that glucosamine itself is the inhibitor of protein glycosylation [40]. Subsequent studies have shown that glucosamine inhibits an early step in the assembly of the lipid-linked oligosaccharide [10], lipid-bound oligosaccharides with a reduced sugar content have been detected [50] (Datema and Schwarz, unpublished results). However, the exact mechanism of action is not yet clear.

Tunicamycin

This nucleoside antibiotic from *Streptomyces lysosuperificus* is a tight-binding, competitive inhibitor of the enzyme transferring phosphoryl-*N*-acetylglucosamine from UDP-*N*-acetylglucosamine to phosphoryldolichol. It therefore inhibits the formation of *N*-acetylglucosaminylpyrophosphoryldolichol [44, 66, 69]. However, if relatively high concentrations of tunicamycin are needed to achieve inhibition of glycosylation, for example in human fibroblasts [6], other effects of tunicamycin, such as inhibition of glucosylphosphoryldolichol formation [16] and inhibition of protein synthesis, may become important.

Benzhydrazone (1H-benz(f)indene-1,3(2H)dione-bis-aminidinohydrazone)

This compound has been found to inhibit glycosylation of herpes virus glycoproteins at an early step. Its exact mode of action is unknown [3].

As has been pointed out already, all of these substances interfere only with the glyco-

sylation of glycoproteins containing *N*-glycosidic linkages. They can therefore be used to differentiate these glycoproteins from glycoproteins with side chains synthesized via other pathways. Using this approach it has been possible to demonstrate, for example, that coronavirus glycoprotein E_1 has *O*-glycosidic linkages, whereas glycoprotein E_2 has *N*-glycosidic linkages [25, 49, 54].

BIOLOGICAL EFFECTS OF GLYCOSYLATION INHIBITORS

It is generally assumed that the carbohydrate side chains influence the biological properties of a glycoprotein either by determing the conformation of the molecule or, less likely, by directly participating in the reactions. For example, all oligosaccharide side chains of the influenza hemagglutinin are located on the surface of the spike [71]. Removal of the carbohydrate might therefore factilitate the access of proteolytic enzymes. As will be shown below this is indeed the case. It has already been pointed out that a substantial portion of this carbohydrate is close to the membrane and may be necessary for the proper implantation of the spike in the lipid. Removal of the carbohydrate may therefore disturb the intracellular transport.

The other part of the carbohydrate is located at the distal end of the hemagglutinin close to its antigenic sites and to the neuraminic acid binding site. It may therefore contribute to the interaction of the hemagglutinin with antibodies and with the cell receptor. In the following a brief review is given on the biological effects observed after inhibition of glycosylation in different virus systems.

Effects on the susceptibility of viral glycoproteins to proteolytic cleavage

As pointed out above, by masking potential cleavage sites the oligosaccharides may contribute to the specificity of the cleavage pattern of a glycoprotein. It is therefore not surprising that in the presence of deoxyglucose or glucosamine cleavage of the non-glycosylated precursor HA_0 of the influenza virus hemagglutinin gave heterogeneous products [58]. It was not expected, however, that in chick embryo cells that had been infected with the same virus and treated with tunicamycin neither HA_0 nor its cleavage products could be detected unless a protease inhibitor was added to prevent degradation of HA_0 [63]. This difference between the sugar analogue and tunicamycin in the effect on the proteolytic stability of the non-glycosylated hemagglutinin is not understood. It has to be pointed out that it is difficult to make generalizations. Nakamura and Compans [47] found that the non-glycosylated form of the hemagglutinin that had been synthesized in the presence of tunicamycin without protease inhibitors could be detected in smooth membranes, when a different strain of influenza virus was studied in different host cells. The unglycosylated glycoprotein of Semliki Forest virus [28, 29, 63], Sindbis virus, and vesicular stomatitis virus [43] also appear to be stable in the cell.

Effects on the intracellular transport of viral glycoproteins

It seems quite clear that cotranslational glycosylation is not required for membrane insertion of the glycoproteins. This is indicated by the observation that in the presence of glycosylation inhibitors the influenza virus hemagglutinin is incorporated into the rough endoplasmic reticulum and not found in the cytoplasm or on free ribosomes [37, 47]. Studies in an in vitro system have also shown that the glycoproteins of Semliki Forest virus can be incorporated into membranes in the presence of tunicamycin [20]. The unglycosylated influenza virus hemagglutinin is transported from the rough endoplasmic reticulum to the plasma membrane in the same manner as the glycosylated compound [37, 47]. Studies on the effect of tunicamycin on the G protein of the Indiana strain of vesicular stomatitis virus have shown that here the molecule has the correct conformation for intracellular transport only when the carbohydrate side chains are present. However, the carbohydrate-free G protein of the Orsay strain of the same virus was transported under certain conditions. Thus, in this case the carbohydrate-free glycoprotein can acquire the correct conformation for transport [5, 21, 22]. These observations taken together indicate that the carbohydrate may or may not be involved in the intracellular transport of a viral glycoprotein. Furthermore, the data suggest that it depends on the amino acid sequence of a glycoprotein whether or not glycosylation is required.

Effects on virus assembly

It is clear that effects of glycosylation inhibitors on the stability and on the intracellular transport of viral glycoproteins as described in the previous sections have consequences for virus assembly. Thus, the assembly of Semliki Forest and Sindbis virions does not take place in the presence of tunicamycin or deoxyglucose, because transport of the glycoproteins is blocked under these conditions [15, 31, 43, 63]. In the case of fowl plaque virus, proteolytic degradation of the glycoproteins synthesized in the presence of tunicamycin appears to be the reason for the failure of virus maturation [63]. However, with the WSN strain of influenza virus, particles lacking the hemagglutinin were assembled, when glycosylation was inhibited by tunicamycin [47]. Similarly, Rous sarcoma virions without spikes were produced in the presence of tunicamycin [63].

Effects on the antigenicity of viral glycoproteins

Our knowledge on the role in antigenicity played by the carbohydrate of viral glycoproteins is quite fragmentary. It is conceivable that the side chains themselves act as antigenic sites, but it is more commonly assumed that they have an indirect effect on antigenicity by their influence on the conformation of the whole glycoprotein molecule. The latter concept is supported by studies carried out with Semliki Forest virus showing that antigenic sites are exposed on unglycosylated glycoproteins synthesized in the pre-

sence of glycosylation inhibitors and that these sites are cryptic on the glycosylated analogues [32].

Effects on the function of viral glycoproteins

In the presence of glycosylation inhibitors, hemagglutinating and neuraminidase activities are drastically reduced in infected cells [30], even though unglycosylated glycoproteins are made [36]. Fusion activity exerted by paramyxoviruses and herpes simplex virus also depends on the glycosylation of the viral glycoproteins [2, 18, 46]. It is not surprising that virus particles that contain glycoproteins devoid of carbohydrates or lack glycoproteins completely after synthesis in the presence of glycosylation inhibitors have a reduced infectivity. This has been shown for influenza virus [47], herpes simplex virus [8], and Rous sarcoma virus [63]. Different results, however, have been obtained with vesicular stomatitis virus. Particles containing unglycosylated glycoprotein had a specific infectivity similar to that of normal particles [21]. Thus, in this instance the lack of carbohydrates appears to have little effect on the biological activity of the glycoprotein.

GLYCOSYLATION INHIBITORS AS THERAPEUTIC AGENTS

Despite of the fact that several of the glycosylation inhibitors presented here have been known for many years, little is known on the clinical use of these substances. To our knowledge only two such studies exist. Both are concerned with herpes virus infections, and in both instances deoxyglucose has been used.

In the first study, herpes simplex virus infections of the rabbit eye were treated with the sugar analogue that was found to be effective in preventing or reducing the severity of herpetic keratitis [52]. Both topical and subconjunctival administration of deoxyglucose were efficacious and toxic effects were not observed. Topically applied [^{3}H]-deoxyglucose penetrated the cornea, aqueous compartment, ciliary body, vitreous and retina in the infected eyes. At least a five-fold increase in the specific activity of the sugar analogue was obtained after herpes simplex virus infection compared with uninfected but scarified controls.

The second study was concerned with the treatment of human genital herpes infections [1]. Thirty-six women were treated in a double-blind placebo-controlled study over a period of 3 weeks. Medication was applied topically. In initial cases, 90% were cured with two recurrences after 24 months. In the case of recurrent or secondary infections, 90% had a notable improvement. In initial infections, discomfort cleared within 12–72 h of therapy. It was concluded that the use of deoxyglucose provides a simple and unique approach to the treatment of genital herpes virus infections.

CONCLUSIONS

Glycosylation of viral glycoproteins with *N*-glycosidic linkages can be inhibited by a

number of substances of different chemical nature. In general, these compounds interfere with the synthesis of the dolichol-bound precursors of the carbohydrate side chains. In molecular virology the glycosylation inhibitors became a standard tool for the analysis of the structure, biosynthesis, and function of viral glycoproteins. Although it is difficult to establish a unifying concept on the role of the carbohydrate in viral glycoproteins, evidence has been obtained in experiments with glycosylation inhibitors that the oligosaccharides may be necessary for the resistance of the glycoproteins to proteolytic degradation, for the expression of their biological functions, for the formation of antigenic sites, for the intracellular transport of the glycoproteins, and for the formation of infectious virus particles. Glycosylation inhibitors proved also to be useful agents to differentiate between viral glycoproteins with *N*-glycosidic and *O*-glycosidic linkages.

It should be emphasized that glycosylation of viral and of cellular glycoproteins follow the same metabolic pathways and that inhibitors of glycosylation are not specifically antiviral drugs. Their use as therapeutic agents appears therefore restricted to viral infections where the drug can be applied topically, as has been demonstrated by the successful treatment of ocular and genital herpes simplex virus infections with deoxyglucose.

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