Glycobiology vol. 2 no. 4 pp. 299–312, 1992

Carbohydrate structure of Marburg virus glycoprotein

Hildegard Geyer[1], Christiane Will[2], Heinz Feldmann[2], Hans-Dieter Klenk[2] and Rudolf Geyer[1,3]

[1]Biochemisches Institut am Klinikum der Universität, Friedrichstraße 24, D-6300 Giessen and [2]Institut für Virologie, Philipps-Universität, Robert-Koch-Straße 17, D-3550 Marburg, FRG

[3]To whom correspondence should be addressed

Marburg virus was propagated in E6 cells, a cloned cell line of Vero cells, in the presence of [6-^{3}H]glucosamine. Radiolabelled viral glycoprotein was digested with trypsin, and oligosaccharides were liberated by sequential treatment with endo-β-*N*-acetylglucosaminidase H, peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase F and *O*-glycosidase, by β-elimination, and by alkaline hydrolysis. After fractionation by HPLC and gel filtration, glycans were characterized chromatographically, by digestion with exoglycosidases and, in part, by methylation analysis and liquid secondary ion mass spectrometry. The oligosaccharide structures thus established include oligomannosidic and hybrid-type *N*-glycans, as well as neutral fucosylated bi-, tri- and tetraantennary species, most of which carry an additional bisecting *N*-acetylglucosamine. In addition, high amounts of neutral mucin-type *O*-glycans with type-1 and type-2 core structures were detected. None of the glycans present in this viral glycoprotein carried sialic acid residues.

Key words: Filoviridae/glycoprotein/Marburg virus/oligosaccharides/structure analysis

Introduction

Marburg virus, as well as the morphologically related Ebola virus, are the prototype members of a new virus family, called Filoviridae (Kiley *et al.*, 1982), which constitutes the third family of non-segmented negative-strand RNA viruses within the newly proposed order Mononegavirales besides the Paramyxoviridae and the Rhabdoviridae (ICTV, 1990). Recently, a third member of this virus family, which is antigenically closely related to Ebola virus, was isolated from cynomolgus monkeys (*Macaca fascicularis*) imported to the USA from the Philippines (Centres for Disease Control, 1989; Jahrling *et al.*, 1990). This agent is called Reston virus.

Marburg virus and Ebola virus are extremely pathogenic for humans, causing a severe haemorrhagic disease with mortality rates of 35% for Marburg virus (Martini and Siegert, 1971) and up to 88% for Ebola virus (subtype Zaire) (Johnson *et al.*, 1977). Filovirus infections were unknown until 1967, when Marburg virus was first isolated in outbreaks among laboratory workers in Germany and Yugoslavia. The source of these simultaneous outbreaks of viral haemorrhagic disease has been attributed to virus-infected African green monkeys (*Cercopithecus aethiops*) imported from Uganda (Martini and Siegert, 1971).

Filoviruses are composed of a helical nucleocapsid surrounded by a lipid envelope. Virions contain at least seven structural proteins. For Marburg virus, these proteins are an RNA-dependent RNA polymerase (L protein; 267 kDa), a glycoprotein (GP; 170 kDa), a nucleoprotein (NP; 96 kDa), and the viral proteins VP40 (38 kDa), VP35 (32 kDa), VP30 (28 kDa) and VP24 (24 kDa) (Kiley *et al.*, 1988; Feldmann *et al.*, 1991; Mühlberger *et al.*, 1992; Sanchez *et al.*, 1992).

The glycoprotein of Marburg virus is highly glycosylated and inserted in the viral membrane as a homotrimer (Feldmann *et al.*, 1991). Lectin binding and endoglycosidase studies demonstrated the presence of two types of carbohydrate sidechains: *N*-glycosidically linked complex and high-mannose type glycans as well as the *O*-linked disaccharide Galβ3GalNAc (Feldmann *et al.*, 1991).

Membrane glycoproteins of enveloped viruses play a key role in infection by mediating receptor recognition, binding of the virion, and subsequent fusion of viral and cellular membranes. Possibly, the glycoprotein of Marburg virus has similar functions. Since it is the only integral membrane protein of the virus, the glycoprotein is probably also the major target for the immune response of the infected host. As a prerequisite for studies on the role of this molecule in Marburg virus infection, as well as its antigenicity, we are analysing the primary structure of this viral glycoprotein. In this report, a detailed structural analysis of its carbohydrate substituents is presented.

Results

Isolation and characterization of radiolabelled viral glycoprotein

For metabolic labelling, Marburg virus was propagated in E6 cells, a cloned line of Vero cells. The radioactive nutrients were added 5 days post-infection for 24 h. Virus particles were isolated from the supernatant, purified by gradient centrifugation and sedimentation steps, and the radiolabelled viral proteins were analysed by SDS gel electrophoresis followed by fluorography (Figure 1A). *In vivo* labelling with [^{35}S]methionine marked all viral structural proteins (Figure 1A, lane 1), whereas only the glycoprotein was radiolabelled when D-[6-^{3}H]glucosamine was used (Figure 1, lane 2). Starting from tissue culture supernatant of $\sim 2.5 \times 10^8$ infected cells, metabolically labelled with 2 mCi D-[6-^{3}H]glucosamine, viral glycoprotein comprising 2×10^6 c.p.m. was obtained. For determination of the radioactive sugar constituents, aliquots were hydrolysed and the monosaccharides released were identified by co-chromatography with monosaccharide standards in high-pH anion-exchange (HPAE)-HPLC. The results demonstrated that the [^{3}H]glucosamine-labelled viral glycoprotein contained radiolabelled *N*-acetylglucosamine, *N*-acetylgalactosamine and galactose in a ratio of ~9.8:1.35:1, indicating that small amounts of [6-^{3}H]glucosamine have been metabolized

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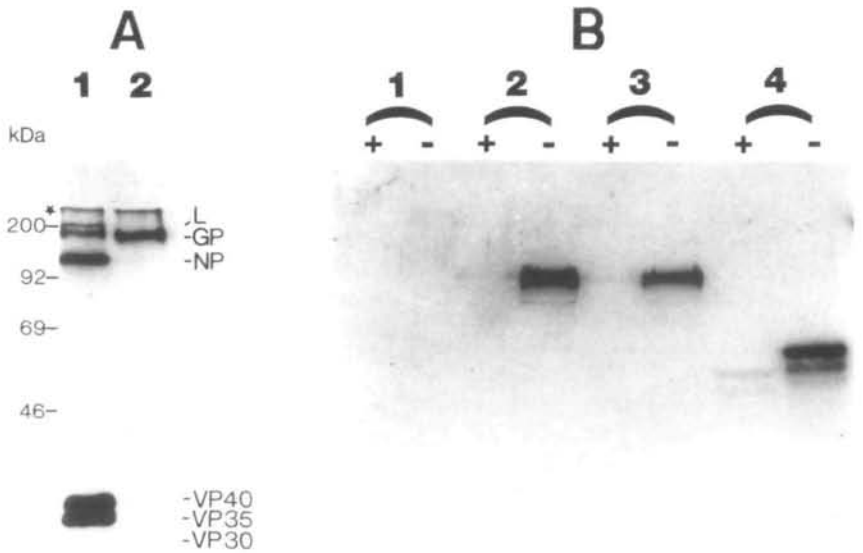


Fig. 1. Characterization of Marburg virus glycoprotein. **(A)** *In vivo* labelling of Marburg virus structural proteins. E6 cells were infected with Marburg virus at a multiplicity of infection of 10^{-2} plaque-forming units/cell. Viral structural proteins were labelled 5 days post-infection in the specific nutrient-deficient Dulbecco's medium containing radioactive nutrients for 24 h at 37°C. Proteins were separated by SDS gel electrophoresis in a 20% gel and visualized by fluorography. Lane 1, Marburg virus labelled with 20 μCi/ml of [^{35}S]methionine; lane 2, Marburg virus labelled with 20 μCi/ml of D-[6-^{3}H]glucosamine. L, RNA polymerase; GP, glycoprotein; NP, nucleoprotein; VP40, VP35 and VP30, viral structural proteins (Kiley *et al.*, 1988). Owing to the experimental conditions, the viral polypeptide VP24 is not visible. **(B)** Reactivity of sialic acid-specific MAA agglutinin to filovirus glycoproteins. Viral proteins were subjected to SDS gel electrophoresis before (−) and after (+) treatment with sialidase from *V.cholerae*, blotted onto nitrocellulose, incubated with digoxigenin-conjugated MAA and visualized using an anti-digoxigenin antibody. Lane 1, Marburg virus; lane 2, Ebola virus; lane 3, Reston virus; lane 4, fetuin (control). The beginning of the running gel is marked by an asterisk.

into *N*-acetylgalactosamine and galactose during metabolic labelling of the glycoprotein. Radiolabelled sialic acid residues were not detected (data not shown).

Characterization of Marburg virus glycoprotein with sialic acid-specific lectins

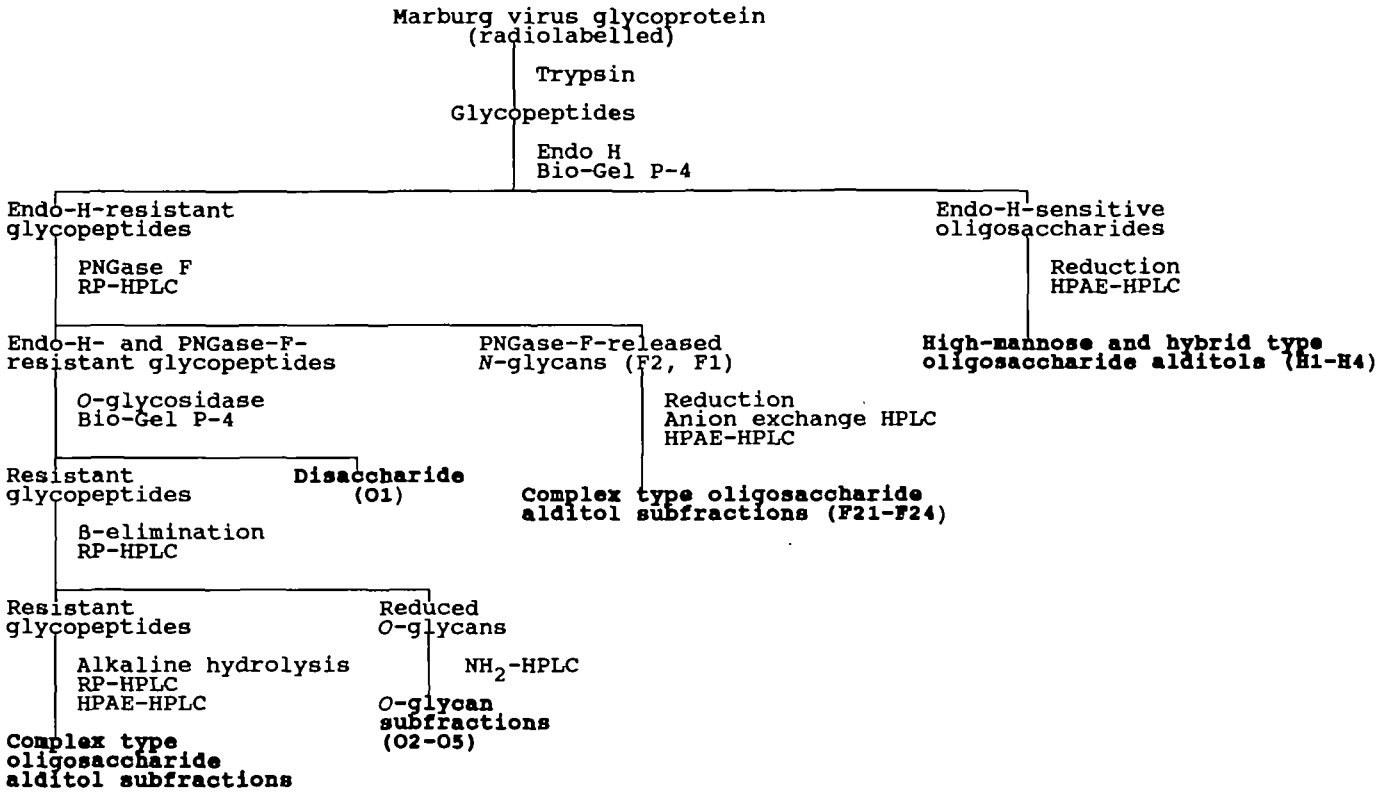
The affinity of carbohydrate-specific lectins for Marburg virus glycoprotein has already been described (Feldmann *et al.*, 1991). In order to analyse for the presence of sialic acid substituents, the same assay was performed using *Maackia amurensis* agglutinin (MAA) and *Sambucus nigra* agglutinin (SNA). MAA recognizes sialic acid linked (α2-3) to galactose in *N*- and *O*-glycans, whereas SNA recognizes sialic acid linked (α2-6) to galactose. For comparison, the respective glycoproteins of two related filoviruses (Ebola and Reston virus), propagated in the same cell line, were also tested. In contrast to SNA, which did not react with any of the three glycoproteins (data not shown), MAA bound to Ebola and Reston virus, but not to Marburg virus glycoprotein (Figure 1B). Binding of the lectin was abolished after treatment of the glycoproteins with sialidase, demonstrating the carbohydrate-specific reaction of MAA. Thus, Ebola and Reston virus glycoproteins obviously comprise (α2-3)-linked sialic acid, whereas Marburg virus glycoprotein seems to lack sialic acid substituents.

Liberation and fractionation of glycans

Following digestion with trypsin (see Scheme 1), glycopeptides obtained were treated with endo-β-*N*-acetylglucosaminidase H (endo H) and the oligosaccharides released (4.27% of total radioactivity) were separated from residual glycopeptides by Bio-Gel P-4 chromatography. After reduction, the oligosaccharide alditols obtained were separated by HPAE-HPLC, yielding four fractions (H1−H4) which represented 0.6, 1.7, 1.3 and 0.6% of total radioactivity (Figure 2A).

Endo H-resistant glycopeptides were treated with peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase F (PNGase F) and subjected to reversed-phase HPLC (RP-HPLC). Two glycan fractions (F1 and F2) comprising 3.23 and 40.9% of total radioactivity were thus isolated (see Scheme 1). The remaining 51.6% of radioactivity still co-eluted with peptides (data not shown). No changes in the elution profile were observed when treatment with PNGase F was repeated once. The minor glycan fraction F1 was not analysed further, whereas fraction F2 glycans were reduced and subjected to anion-exchange HPLC. The result revealed that the vast majority of radioactivity (94.2%) eluted at the position of neutral sugar chains (Figure 3A). Treatment of the trace amounts of charged material with sialidase from *Vibrio cholerae* did not change the elution position of these compounds, excluding the presence of sialylated oligosaccharides. Neutral species were freed from the charged material by preparative anion-exchange HPLC and separated by HPAE-HPLC, yielding four main subfractions (F21−24, see Figure 2B) which comprised 4, 17, 3.5 and 6.4% of total radioactivity, respectively.

Endo H- and PNGase F-resistant glycopeptides were treated with *O*-glycosidase and subjected to gel filtration. About 2.4% of total radioactivity was liberated and eluted at ~3 glucose units, i.e. the position of a Gal-GalNAc disaccharide (fraction O1 in Scheme 1). An aliquot of the remaining glycopeptide fraction was then sequentially treated with sialidase and *O*-glycosidase without any further release of radioactivity. Following reductive β-elimination and RP-HPLC, however,



Scheme 1. Liberation and fractionation of carbohydrate moieties from Marburg virus glycoprotein. NH_2-HPLC, HPLC using a LiChrosorb-NH_2 column.

32% of total radioactivity was recovered in the oligosaccharide fraction, whereas 17% still remained bound to peptides. The reduced *O*-glycans obtained were analysed for the presence of negatively charged components by anion-exchange HPLC. The results demonstrated that this glycan fraction contained exclusively neutral oligosaccharides (see Figure 3B). *O*-Glycans were fractionated by HPLC on a LiChrosorb-NH_2 column, yielding four major subfractions (O2–O5) which comprised 2.8, 1.3, 24.6 and 2.1% of total radioactivity (Figure 4).

Glycans still bound to peptides (cf. Scheme 1) were liberated by alkaline hydrolysis and separated from remaining peptides by RP-HPLC. Oligosaccharides obtained were fractionated by HPAE-HPLC, leading to an oligosaccharide pattern which was almost identical to that of F2 glycans (data not shown). Starting from the glycoprotein preparation described above, ~25–500 pmol of mostly individual glycans were thus obtained.

Characterization of glycans

Oligosaccharide alditol fractions H2 and H3, F21–F24 and O2–O4 (cf. Figures 2 and 4, and Scheme 1) were subjected to methylation analysis (see, for example, Figure 5). The results are summarized in Table I. Owing to the small amounts of material, fractions were only qualitatively characterized. The presence or absence of the corresponding alditol derivatives, however, was clearly significant in all cases. In addition, major fractions F22 and O4 were studied by liquid secondary ion mass spectrometry (LSIMS) (Figure 6).

For determination of the anomeric configurations of monosaccharide constituents, as well as for enzymic sequencing of the major glycan fractions, oligosaccharide alditols H2, H3, F21–F24 and O1–O5 were degraded by successive treatment with the exoglycosidases listed in Table II. Untreated glycans and reaction products were analysed by Bio-Gel P-4 chromatography and their sizes were determined by co-chromatography with glucose oligomers (Yamashita *et al.*, 1982; Kobata *et al.*, 1987; see, for example, Figures 7 and 8). For chromatographic characterization, the main oligosaccharide fractions were further subjected to LiChrosorb-Diol- (endo H-sensitive glycans) or LiChrosorb-NH_2-HPLC (PNGase F-released species) and their chromatographic behaviour was compared to that of standard oligosaccharides (data not shown).

Structural conclusions

In conjunction with the chromatographic data obtained in HPAE-, LiChrosorb-Diol- and LiChrosorb-NH_2-HPLC, analytical results permit the structural conclusions summarized in Table III. Assuming that all *N*-acetylglucosamine and *N*-acetylgalactosamine residues are equally labelled, and disregarding the fact that galactose residues are labelled to a small extent too, the relative amount of each glycan was roughly estimated from the distribution of ^3H radioactivity in the different structures. Given values include corresponding glycans obtained after alkaline hydrolysis (see Scheme 1).

Endo H-sensitive glycans (~9 mol%) comprise high-mannose and hybrid-type glycans. Methylation analysis (cf. Table I) of fraction H2 (3.3 mol%) revealed the presence of terminal galactose (2,3,4,6-GalOH) and 4-substituted *N*-acetylglucosamine (3,6-GlcN[Me]AcOH), in addition to terminal (2,3,4,6-ManOH), 2-substituted (3,4,6-ManOH) and 3,6-disubstituted (2,4-ManOH) mannose residues and 4-substituted *N*-acetylglucosaminitol (1,3,5,6-GlcN[Me]AcOH). In gel filtration, two radioactive peaks could be detected eluting at 10.4

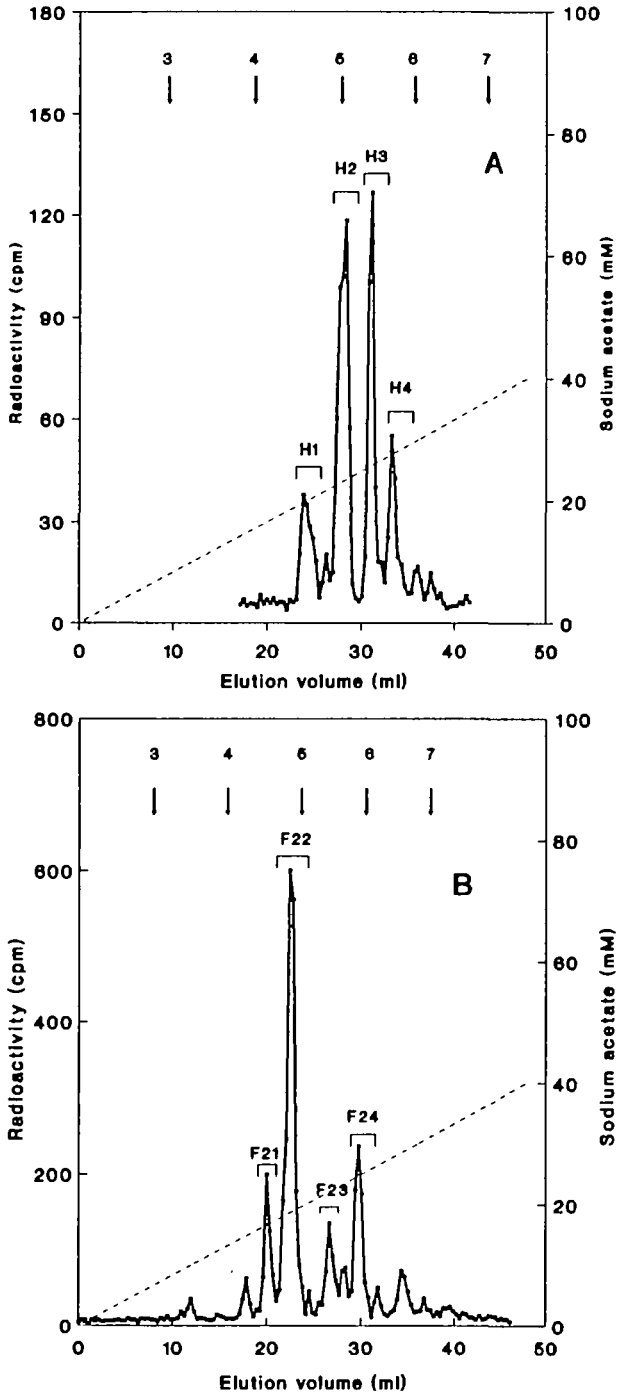


Fig. 2. Separation of *N*-glycans from Marburg virus glycoprotein by HPAE-HPLC. Oligosaccharide alditols obtained after treatment of tryptic glycopeptides with endo H (**A**) or PNGase F (**B**) and reduction (see Scheme 1) were separated by HPAE-HPLC on a CarboPac PA1 column (4 × 250 mm) using a gradient of sodium acetate in 0.1 M NaOH as indicated. Fractions (0.35 ml) were collected at 1 ml/min and monitored for radioactivity by liquid scintillation counting. Fractions subjected to structural analysis were pooled as indicated by horizontal brackets. Numbers (3–7) with arrows are elution volumes of isomaltosyl oligosaccharide alditols with 3–7 glucose units.

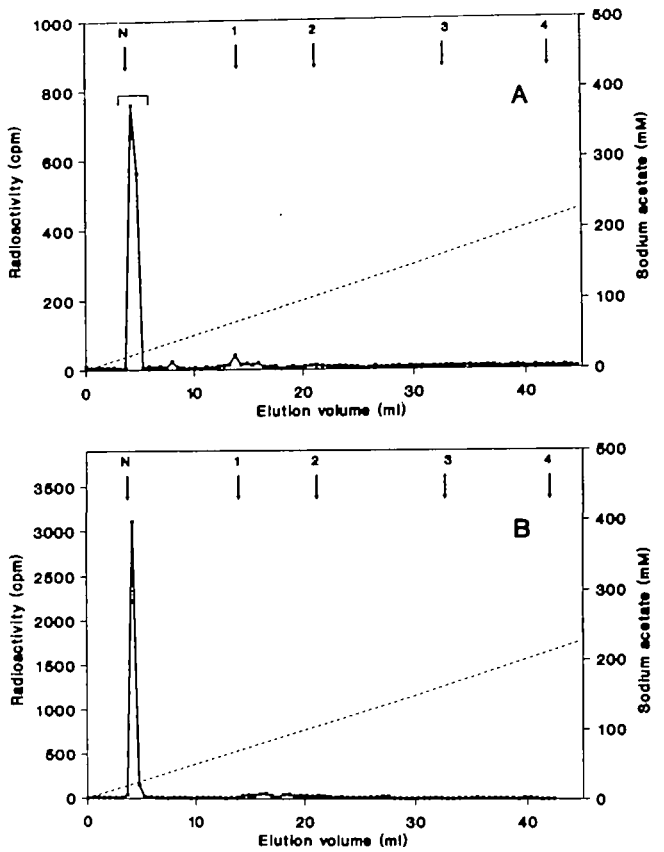


Fig. 3. Anion-exchange HPLC of glycan fractions obtained from Marburg virus glycoprotein. Oligosaccharide alditols obtained after treatment of tryptic peptides with PNGase F and reduction (**A**) or reductive β-elimination (**B**; see Scheme 1) were analysed by anion-exchange HPLC using a Carbopak PA1 column (4×250 mm) and a linear gradient of sodium acetate in bidistilled water as indicated. Fractions (0.5 ml) were collected at 1 ml/min and monitored for radioactivity. N and numbers (1–4) with arrows are elution volumes of neutral glycans and sialylated oligosaccharide standards with 1–4 sialic acid residues.

and 8.6 glucose units (Table II). Sequential treatment with β-galactosidase, α-mannosidase and β-*N*-acetylhexosaminidase resulted in the removal of one or zero galactose, three or six mannose and one or zero GlcNAc residues, respectively.

Incubation with α-1,2-specific mannosidase from *Aspergillus oryzae* and subsequent gel filtration demonstrated that the glycan originally eluting at 8.6 glucose units lost two mannose residues, whereas the elution position of species eluting at 10.4 glucose units was not changed (data not shown). Thus, it can be concluded that fraction H2 contained the two glycans depicted in Table III.

Fraction H3 (3.7 mol%) contained solely $Man_8GlcNAcOH$ species. This can be concluded from methylation data (presence of 2,3,4,6-ManOH, 3,4,6-ManOH, 2,4-ManOH and 1,3,5,6-GlcN[Me]AcOH and absence of other derivatives; see Table I) as well as from results obtained after degradation with α-mannosidase (see Table II) and α-1,2-specific mannosidase (data not shown). The isomeric structure of this species is proposed on the basis of its co-chromatography with the corresponding $Man_8GlcNAcOH$ standard in LiChrosorb-Diol-HPLC (data not shown), which can be separated from other isomers under the conditions used (Geyer *et al.*, 1984a).

Minor fractions H1 and H4 (~1 mol% each) contained hybrid-type species and traces of $Man_6GlcNAcOH$, as evidenced by LiChrosorb-Diol-HPLC, gel filtration and digestion with α-mannosidase and α-1,2-mannosidase (data not shown). Owing to the small amounts of material, these fractions were not analysed further.

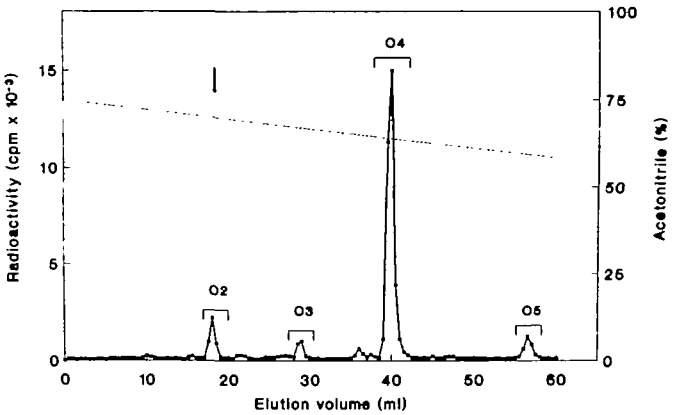


Fig. 4. Separation of *O*-glycans from Marburg virus glycoprotein. Reduced *O*-glycans obtained from endo H- and PNGase F-resistant glycopeptides by β-elimination were fractionated by HPLC on a LiChrosorb-NH_2 column (5 μm, 4.6 × 250 mm) using a gradient of acetonitrile in bidistilled water as indicated. Radiolabelled glycans were monitored by a continuous-flow radioactivity detector. Fractions (0.5 ml) were collected at 1 ml/min and pooled as indicated by horizontal brackets. The elution position of fraction O1 disaccharide is shown by an arrow.

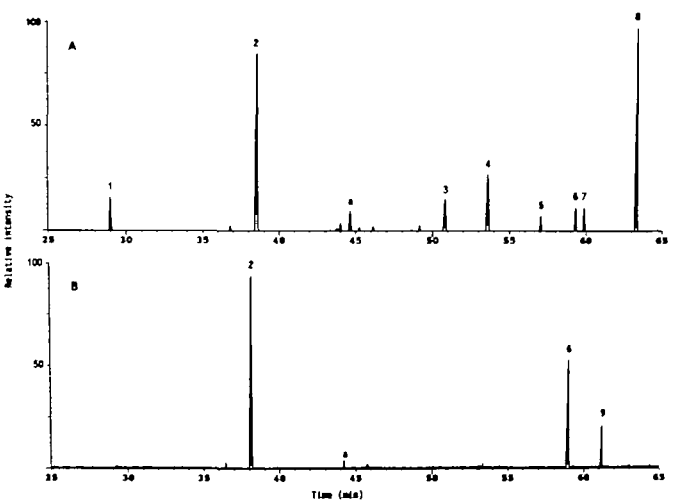


Fig. 5. Methylation analysis of fraction F24 (**A**) and O3 (**B**) glycans from Marburg virus glycoprotein. Partially methylated alditol acetates obtained after methylation, hydrolysis, reduction and peracetylation were analysed by capillary gas chromatography–mass fragmentography using a fused-silica, bonded-phase DB-1 column (0.25 mm i.d., 60 m length). Detection was achieved by chemical ionization with ammonia and selective monitoring of the ions $(M+NH_4)^+$ (hexitol acetates) or $(M+H)^+$ (aminohexitol acetates). Alditol derivatives were assigned as follows: (1) 2,3,4-FucOH; (2) 2,3,4,6-GalOH; (3) 3,6-ManOH; (4) 3,4-ManOH; (5) 2-ManOH; (6) 3,4,6-GlcN(Me)AcOH; (7) 1,3,5-GlcN(Me)AcOH; (8) 3,6-GlcN(Me)AcOH; (9) 1,4,5-GalN(Me)AcOH (for an explanation of this short-hand notation see the legend to Table I). The peak designated (a) represents 2,3,6-GlcOH, probably derived from cellulose contaminants. Subtle variations in the retention times of (2) and (6) arise from slight changes in the temperature gradients used.

Fraction F21 still comprised a mixture of complex-type glycans eluting at 15.2 (major component; 2.2 mol%) and 17.8 glucose units (minor fraction; 0.9 mol%) in gel filtration (Table II). For exoglycosidase studies, the two constituents were separated prior to digestion with β-galactosidase. Analysis of the reaction products revealed the release of two and three galactose residues, respectively. Subsequent treatment with β-*N*-acetylhexosaminidase using standard amounts of enzyme (see Materials and methods) resulted in the removal of zero or one *N*-acetylglucosamine residue. Only small amounts of the fragment eluting at 14.1 glucose units could be converted to a product co-eluting at 8.2 glucose units with authentic standard $Man_3GlcNAc(Fuc)GlcNAcOH$ (cf. Table II). Using a 10-fold higher concentration of the enzyme, however, both components yielded the fucosylated core fragment eluting at 8.2 glucose units. In agreement with data reported by Yamashita *et al.* (1983a) it may, therefore, be assumed that the majority of fraction F21 glycans carry a bisecting *N*-acetylglucosamine residue (GlcNAc-**9**) at Man-**3**. This assumption is corroborated by methylation data revealing the presence of terminal *N*-acetylglucosamine and 3,4,6-trisubstituted mannose in addition to terminal fucose, terminal galactose, 2-substituted as well as traces of 2,4- and 3,6-disubstituted mannose, 4-substituted *N*-acetylglucosamine and 4,6-disubstituted *N*-acetylglucosaminitol residues (see Table I). Taken together, the data obtained indicate the presence of fucosylated biantennary species carrying a bisecting *N*-acetylglucosamine residue (major component), as well as triantennary glycans (minor components), about half of which are similarly substituted by GlcNAc-**9**. Obviously, the latter species represented a contamination by F22 glycans (see below).

By the same line of evidence, fraction F22 (10.9 mol%) could be similarly deduced to comprise glycans with bisecting *N*-acetylglucosamine (see Figure 7A–D and Table II). Since F22 species represented a major glycan fraction of Marburg virus glycoprotein, these oligosaccharides could be further studied by LSIMS. As shown in Figure 6A–C, the analysis revealed a quasi-molecular ion ($[M+H]^+$) at *m/z* 2932 consistent with a sugar composition of $Hex_6HexNAc_5dHexHexNAcOH$ where Hex is hexose, HexNAc is *N*-acetylhexosamine, HexNAcOH is *N*-acetylhexosaminitol and dHex is deoxyhexose. In addition, characteristic fragment ions were observed at *m/z* 228 ($HexNAc^+$ minus methanol), 260 ($HexNAc^+$), 432 ($HexHexNAc^+$ minus methanol), 464 ($HexHexNAc^+$) and 450/2465 (fission of the chitobiose unit). LSIMS further revealed a significant amount of undermethylated products, which are commonly observed when glycans with bisecting *N*-acetylglucosamine residues are subjected to permethylation (R.Geyer, unpublished observation). Methylation analysis revealed the presence of terminal fucose, terminal galactose, 2-substituted, 2,4-disubstituted and 3,4,6-trisubstituted mannose, terminal and 4-substituted *N*-acetylglucosamine and 4,6-disubstituted *N*-acetylglucosaminitol. Thus, fraction F22 species can be assumed to comprise the triantennary structure proposed in Table III.

Fraction F23 glycans (2.3 mol%) were identified as fucosylated tetraantennary species. Sequential digestion with β-galactosidase and β-*N*-acetylhexosaminidase resulted in the liberation of four galactose and four *N*-acetylglucosamine residues, respectively. As shown below, the resulting fragment eluting at 8.2 glucose units again represented the fucosylated pentasaccharide core structure. Methylation analysis revealed the presence of 2,4-, 2,6- and 3,6-disubstituted mannose residues, and the absence of 3,4,6-trisubstituted mannose and terminal *N*-acetylglucosamine, demonstrating that this fraction represented tetraantennary glycans without bisecting *N*-acetylglucosamine.

Methylation data of fraction F24 (3.5 mol%) revealed the presence of terminal fucose and terminal galactose, 2,4- and

Table I. Methylation analysis of the major oligosaccharides from Marburg virus glycoprotein expressed in E6 cells

Alditol acetate	Oligosaccharide alditol fraction									Linkage
	H2	H3	F21	F22	F23	F24	O2	O3	O4	
2,3,4-FucOH	−	−	+	+	+	+	−	−	−	Fuc(1-
2,3,4,6-ManOH	+	+	−	−	−	−	−	−	−	Man(1-
3,4,6-ManOH	+	+	+	+	−	−	−	−	−	-2)Man(1-
2,4-ManOH	+	+	(+)	−	+	−	−	−	−	-3,6)Man(1-
3,4-ManOH	−	−	−	−	+	+	−	−	−	-2,6)Man(1-
3,6-ManOH	−	−	(+)	+	+	+	−	−	−	-2,4)Man(1-
2-ManOH	−	−	+	+	−	+	−	−	−	-3,4,6)Man(1-
2,3,4,6-GalOH	+	−	+	+	+	+	+	+	+	Gal(1-
1,3,5,6-GlcN(Me)AcOH	+	+	−	−	−	−	−	−	−	-4)GlcNAcOH
1,3,5-GlcN(Me)AcOH	−	−	+	+	+	+	−	−	−	-4,6)GlcNAcOH
3,4,6-GlcN(Me)AcOH	−	−	+	+	−	+	−	+	−	GlcNAc(1-
3,6-GlcN(Me)AcOH	+	−	+	+	+	+	−	−	+	-4)GlcNAc(1-
1,4,5,6-GalN(Me)AcOH	−	−	−	−	−	−	+	−	−	-3)GalNAcOH
1,4,5-GalN(Me)AcOH	−	−	−	−	−	−	−	+	+	-3,6)GalNAcOH

Oligosaccharide alditol fractions (see Figures 2 and 4) isolated from Marburg virus glycoprotein, metabolically labelled with [^{3}H]glucosamine, were permethylated and hydrolysed. The partially methylated alditol acetates obtained after reduction and peracetylation were analysed by capillary GLC/MS. Owing to the small amounts of material, fractions were only qualitatively characterized and the presence or absence of the alditol derivatives is indicated by + or −. 2,3,4-FucOH, 2,3,4-tri-*O*-methylfucitol; 1,3,5,6-GlcN(Me)AcOH, 2-deoxy-2-(*N*-methyl)acetamido-1,3,5,6-tetra-*O*-methylglucitol; etc.; (+), traces.

2,6-disubstituted and 3,4,6-trisubstituted mannose, terminal and 4-substituted *N*-acetylglucosamine, and 4,6-disubstituted *N*-acetylglucosaminitol (see Figure 5A and Table I). This is consistent with data from exoglycosidase studies which demonstrated the presence of four lactosamine antennae and bisecting *N*-acetylglucosamine. Thus, fraction F24 can be concluded to represent the fucosylated tetraantennary species depicted in Table III.

Core fragments (8.2 glucose units in gel filtration) obtained from fractions F21–F24 were pooled and sequentially degraded with α-mannosidase, β-mannosidase, β-*N*-acetylhexosaminidase and α-fucosidase. As shown in Table II, two α-linked and one β-linked mannose, one *N*-acetylglucosamine and one fucose residue could be released, leaving radioactive *N*-acetylglucosaminitol (cf. Figure 7E–H).

O-Glycans constitute more than half of oligosaccharides bound to Marburg virus glycoprotein. Fractions O1 and O2 (7.2 and 8.2 mol%) both originate from the disaccharide Galβ3GalNAc, as evidenced by its sensitivity to *O*-glycosidase (only O1) and β-galactosidase from bovine testes, and resistance to the corresponding β-1,4-specific enzyme from *Diplococcus pneumoniae* (Distler and Jourdian, 1973; Paulson *et al.*, 1978). Consequently, methylation analysis of fraction O2 species revealed the presence of terminal galactose and 3-substituted *N*-acetylgalactosaminitol (cf. Table I). The fact that part of these glycans (i.e. O2 species) were not liberated by *O*-glycosidase may be ascribed to steric hindrance.

Sequential degradation of fraction O3 glycans (1.9 mol%) with β-*N*-acetylhexosaminidase and β-galactosidase (from bovine testes) resulted in the liberation of one HexNAc and one galactose residue, producing a radioactive fragment which co-eluted with HexNAcOH at 2.5 glucose units. The HexNAc residue liberated was collected and identified as *N*-acetylglucosamine by HPAE-HPLC (data not shown). Methylation analysis revealed the presence of terminal galactose, terminal *N*-acetylglucosamine and 3,6-disubstituted *N*-acetylgalactosaminitol (see Figure 5B and Table I), demonstrating that these glycans represented a type 2 core (Carraway and Hull, 1989) with the structure Galβ3[GlcNAcβ6]GalNAcOH. It is noteworthy that terminal galactosyl residues could not be liberated by treatment with high amounts of β-galactosidase from bovine testes prior to digestion with β-*N*-acetylhexosaminidase (cf. Table II). Obviously, this enzyme is sterically hindered by the simultaneous presence of *N*-acetylglucosamine residues linked to C-6 of *N*-acetylgalactosaminitol.

Fraction O4 glycans comprise ~36.2 mol% of the total carbohydrate constituents of Marburg virus glycoprotein. LSIMS analysis revealed a quasimolecular ion ($[M+H]^+$) at *m/z* 961.6 consistent with a sugar composition of $Hex_2HexNAcHexNAcOH$ (Figure 6D). Sequential digestion with β-galactosidase from *D.pneumoniae* and β-galactosidase from bovine testes demonstrated the release of only one β4-linked galactose. Subsequent treatment with β-*N*-acetylhexosaminidase and β-galactosidase liberated one β-linked *N*-acetylglucosamine and again one β-linked galactose residue, leaving a radioactive fragment co-eluting with HexNAcOH (cf. Table II). In contrast, methylation analysis revealed only the presence of terminal galactose, 4-substituted *N*-acetylglucosamine and 3,6-disubstituted *N*-acetylgalactosaminitol, ruling out a linear molecule with a type-1 core. As already discussed in the case of fraction O3 glycans, the terminal galactose residue linked to C-3 of *N*-acetylgalactosaminitol obviously can not be removed by treatment of the tetrasaccharide with β-galactosidase from bovine testes due to steric hindrance. Thus, it can be concluded that fraction O4 glycans comprised the branched tetrasaccharide depicted in Table III.

Sequential enzymic digestion of fraction O5 glycans (2.1 mol%) revealed the presence of two different hexasaccharides (see Figure 8 and Table II). Liberation of one or two terminal Galβ4 residues by treatment with β-galactosidase from *D.pneumoniae* (Figure 8A) and one or two β-linked *N*-acetylglucosaminyl residues by incubation with β-*N*-acetylhexosaminidase (Figure 8B) resulted in a tetra- and a disaccharide fragment eluting at 6.5 and 3.5 glucose units, respectively (Figure 8C), which could be further degraded to HexNAcOH (Figure 8D–F). Although methylation data were not available

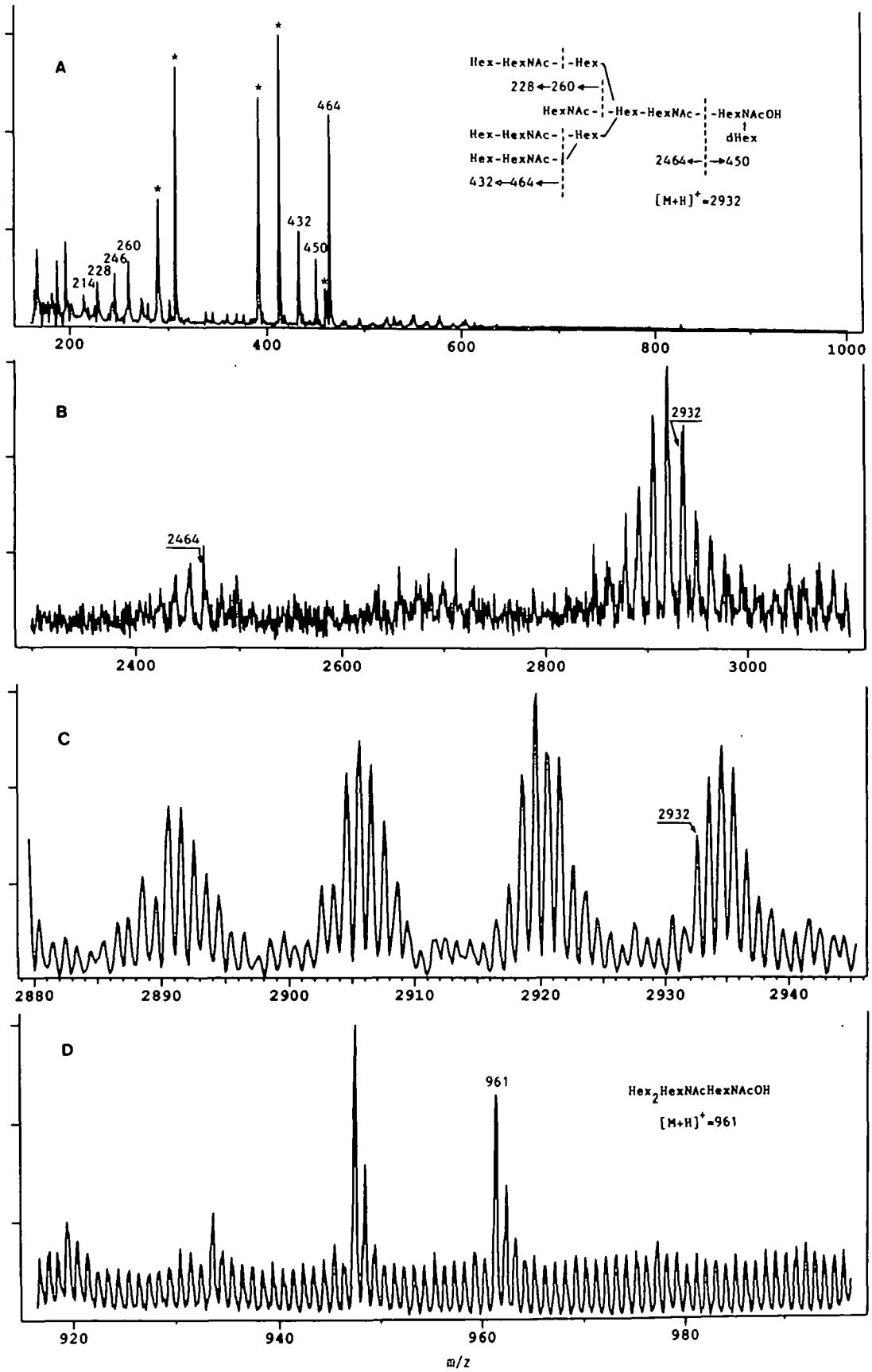


Fig. 6. Positive-ion LSIMS spectra of methylated oligosaccharide alditol fractions F22 and O4 from Marburg virus glycoprotein. Partial LSIMS spectra of fractions (A) F22 (*m/z* 160–1000), (B) F22 (*m/z* 2300–3100), (C) F22 (enlargement of the molecular weight region shown in B) and (D) O4 (*m/z* 920–990) are shown. Fragment and quasimolecular ions are indicated by accurate mass values. Signals with ($n \times 14$) Da lower than the quasimolecular ions, as well as signals at *m/z* 246 and 214 (in A), are due to undermethylation. Ions marked by an asterisk arise from the matrix (3-nitrobenzyl alcohol) and unknown impurities commonly observed.

Table II. Degradation of *N*- and *O*-glycans from Marburg virus glycoprotein with exoglycosidases

Enzyme used	Glucose units of fragments obtained from					
	H2	H3	F21	F22	F23	F24
–	10.4/8.6[a]	9.5	15.2/17.8[a]	18	19.2	20.2
β-Galactosidase[b]	9.3/8.7		12.8/14.1	14.7	15.5	15.8
β-*N*-Acetylhexosaminidase			12.8/8.2;12.8	12.8;14.5	8.2	12.7;14.3
β-*N*-Acetylhexosaminidase (10-fold amount)			8.2/8.2	8.2		8.2
α-Mannosidase	6.1/3.2	3.5		6.3		
β-Mannosidase				5.6		
β-*N*-Acetylhexosaminidase	4.0/3.1			3.6		
α-Fucosidase				2.6		

Enzyme used	Glucose units of fragments obtained from				
	O1	O2	O3	O4	O5
–	2.9[c]	3.6	5.5	6.5	9.6
β-Galactosidase[b]	2.9[c]	3.6		5.3	8.6/7.5
β-Galactosidase[d]	2.5	2.5	5.5	5.4	8.6/7.5
β-*N*-Acetylhexosaminidase			3.5[e]	3.6[e]	6.5[e]/3.5[e]
β-Galactosidase[d]			2.5	2.5	5.5/2.5
β-*N*-Acetylhexosaminidase					3.5[e]
β-Galactosidase[d]					2.5

Neutral oligosaccharide alditol fractions (H2, H3 and F21–F24 in Figure 2; O2–O5 in Figure 4; O1 see Scheme 1) isolated from Marburg virus glycoprotein, metabolically labelled with [^{3}H]glucosamine, were sequentially digested with the enzymes indicated and the products were analysed by gel filtration using a Bio-Gel P-4 column. The results are expressed as the number of glucose units (Yamashita *et al.*, 1982, 1983b; Kobata *et al.*, 1987) of the fragments obtained.

[a]Fractions contained at least two different oligosaccharide species.

[b]β-Galactosidase from *D.pneumoniae*.

[c]After reduction, the corresponding oligosaccharide alditol elutes at 3.5 glucose units.

[d]β-Galactosidase from bovine testes.

[e]Liberated HexNAc eluted at ~2 glucose units and was identified as GlcNAc.

in this case, it may be assumed that O5 glycans represent O4 species elongated by an additional lactosamine unit at either galactose. The linkage positions of the corresponding *N*-acetylglucosamine residues, however, were not determined. Obviously, the galactose linked to C-3 of *N*-acetylgalactosaminitol is again resistant to β-galactosidase from bovine testes as long as *N*-acetylgalactosaminitol is further substituted at C-6.

Discussion

This study describes the first detailed carbohydrate structure analysis of the envelope glycoprotein of a filovirus. Marburg virus, a highly pathogenic member of this family, was propagated in E6 cells which were cultivated in the presence of [6-^{3}H]glucosamine. Starting from the supernatant (100 ml) of five tissue culture flasks only, radiolabelled virus particles were harvested and purified by gradient centrifugation. Since only the viral glycoprotein was metabolically labelled by this procedure (cf. Figure 1), the protein preparation, obtained after disintegration of the virions with detergents, could be subjected to carbohydrate analysis directly without further purification of the glycoprotein.

Oligosaccharide constituents were sequentially released from tryptic glycopeptides by enzymatic as well as chemical cleavage, and fractionated by different HPLC methods. Oligosaccharides obtained were studied by sequential degradation with exoglycosidases, methylation analysis and, in part, by LSIMS. The results reveal that Marburg virus glycoprotein is heterogeneously glycosylated in E6 cells. About 9 mol% of total glycans were of the high-mannose or hybrid type, whereas 19 mol% comprised fucosylated bi-, tri- and tetraantennary complex-type species, most of which carried a bisecting *N*-acetylglucosamine residue. The majority of oligosaccharides (~55 mol%) were found to represent O-linked carbohydrate chains with 2–6 sugar residues. Owing to the small amounts of starting material, the structures of the remaining glycans could not be elucidated.

The results obtained are in agreement with previous data (Feldmann *et al.*, 1991) demonstrating the presence of high-mannose and complex-type *N*-glycans, as well as *O*-glycosidically linked Galβ3GalNAc disaccharides by endoglycosidase treatment of the total glycoprotein and by lectin studies. Furthermore, in that study the reaction of peanut agglutinin with this glycoprotein was not extinguished by treatment with *O*-glycosidase. Similar results were now obtained in the case of tryptic peptides. Only about half the amount of disaccharide present could be released by repeated enzyme treatment, whereas the remaining half had to be liberated by reductive β-elimination. Possibly due to steric hindrance, part of the linkages between *N*-acetylgalactosamine and the corresponding serine (or threonine) residue seem to be inaccessible to this

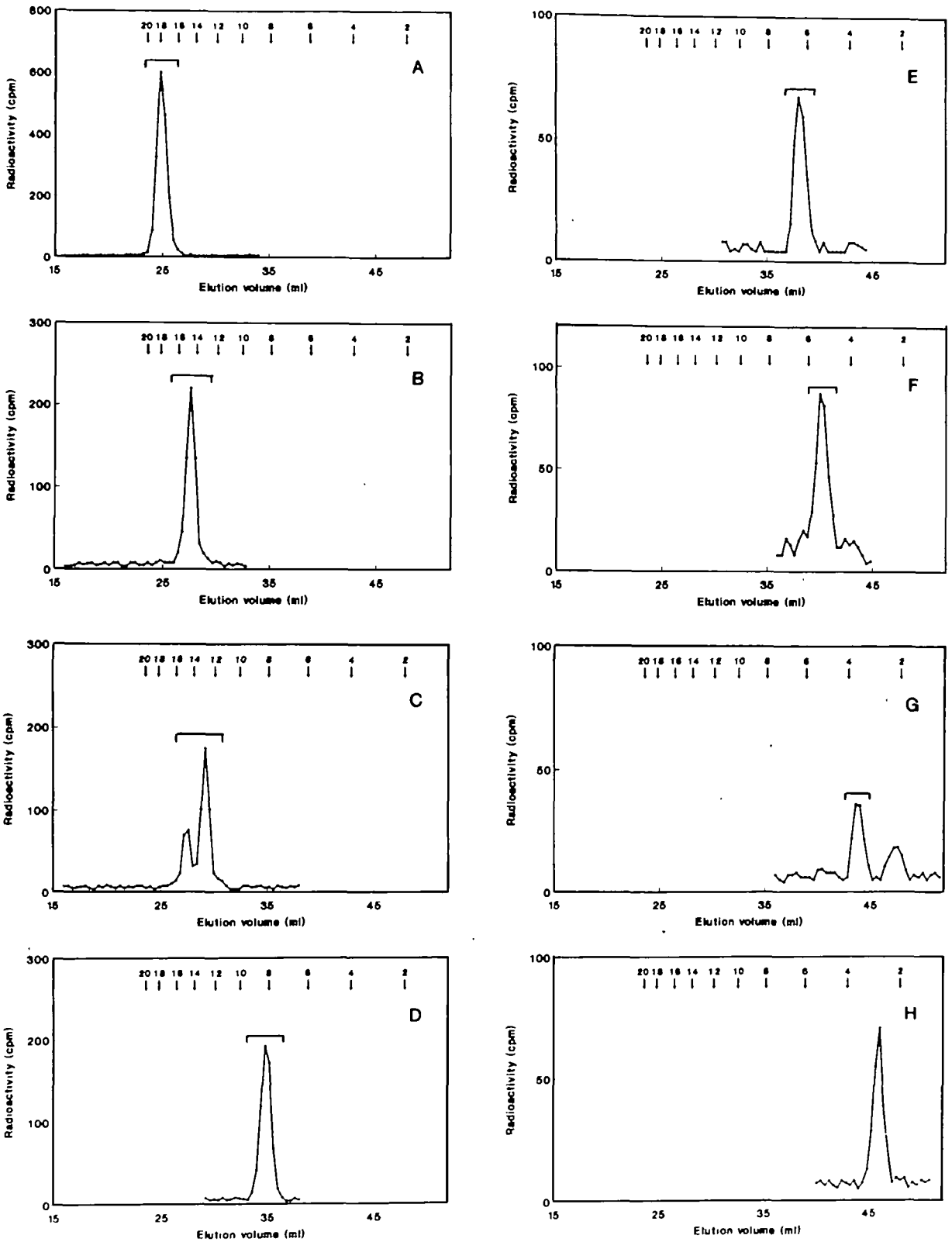


Fig. 7. Sequential degradation of oligosaccharide alditol fraction F22 from Marburg virus glycoprotein with exoglycosidases. Fraction F22 (A) was successively digested with β-galactosidase (B) and β-*N*-acetylhexosaminidase (C, standard conditions; D, 10-fold amount). The core fragment thus obtained was combined with core fragments of fractions F21, F23 and F24, and degraded by α-mannosidase (E), β-mannosidase (F), β-*N*-acetylhexosaminidase (G) and α-fucosidase (H). Starting material and reaction products were chromatographed on a calibrated Bio-Gel P-4 column (−400 mesh; 16 × 600 mm) at 55°C using 0.02% aqueous sodium azide as eluant. Fractions (0.4 ml) were collected at 0.2 ml/min and monitored for radioactivity. Brackets indicate fractions pooled for further degradation. Column calibration with glucose oligomers (2–20 glucose residues) is shown by arrows.

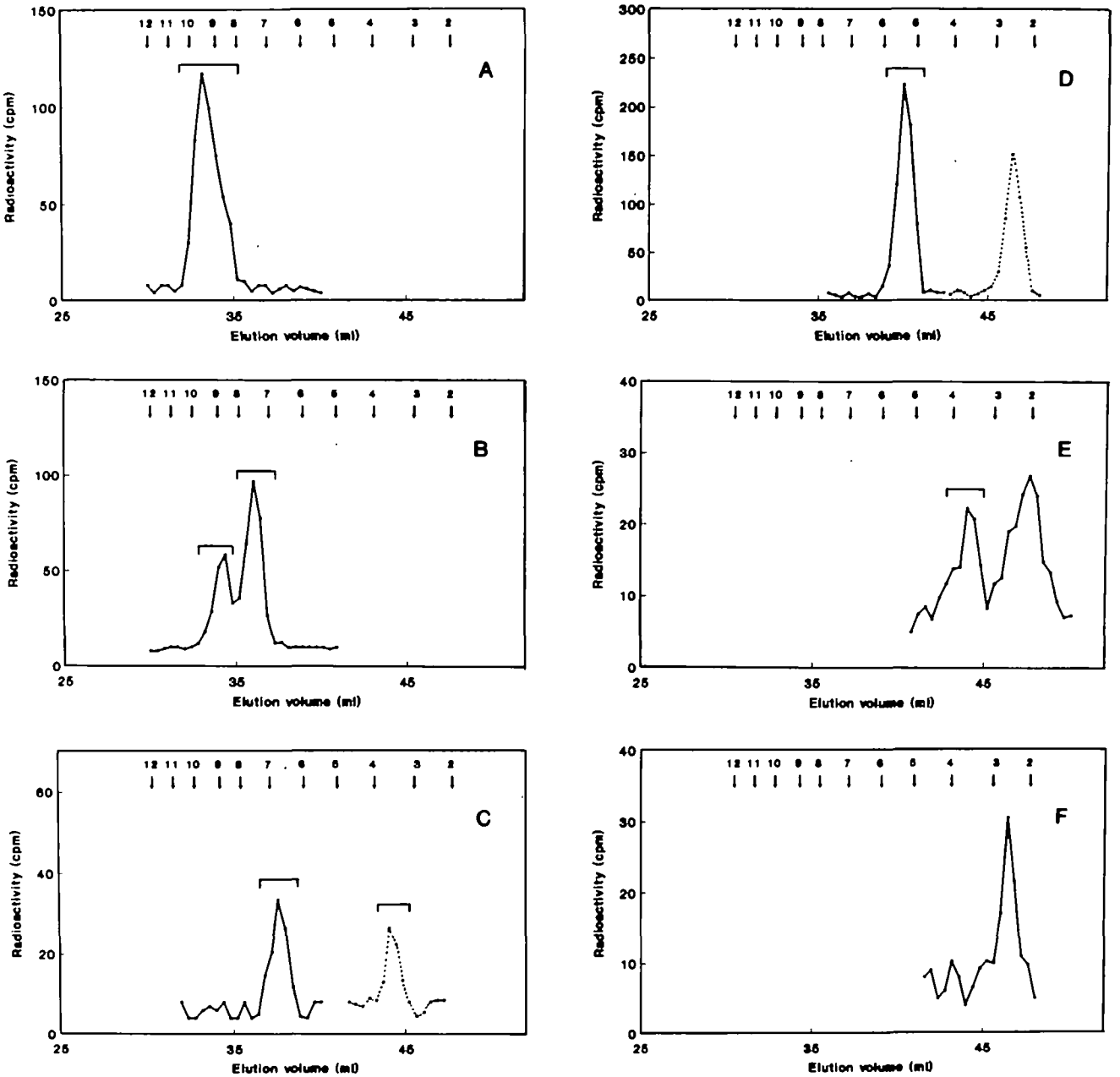


Fig. 8. Enzymic sequencing of oligosaccharide alditol fraction O5 from Marburg virus glycoprotein. Fraction O5 (**A**) was digested with β-galactosidase from *D.pneumoniae* (**B**). The two fragments obtained were pooled separately and digested with β-*N*-acetylhexosaminidase (**C**) and β-galactosidase from bovine testes (**D**). The resulting trisaccharide (5.5 glucose units) was further degraded by β-*N*-acetylhexosaminidase (**E**) and β-galactosidase from bovine testes (**F**). Fragments originating from the product eluting at 7.5 glucose units in (B) are indicated by dotted lines. For chromatographic conditions see Figure 7.

enzyme. The exact positions of *O*-glycosylation sites, however, remain to be elucidated.

Nucleotide sequencing of the corresponding gene revealed that Marburg virus glycoprotein contains several potential *N*-glycosylation sites, of which the α-amino groups of asparagine residues are connected with the carboxyl group of lysine (data not shown). Thus, tryptic digestion leads, in part, to glycopeptides having glycosylated asparagine residues at their N-termini. Since PNGase F does not release asparagine-linked glycans from such glycopeptides (Tarentino *et al.*, 1985), the PNGase F-resistant glycopeptides obtained in this study obviously represent this type of molecule.

Anion-exchange HPLC of fraction F2 revealed the presence of trace amounts of charged material, the chromatographic properties of which, however, were not influenced by treatment with sialidase, indicating that this material did not contain sialic acids. Since Marburg virus glycoprotein can be radiolabelled with neither [^{35}S]sulphate nor [^{32}P]orthophosphate (data not shown), it may be concluded that this charged material represented small GlcNAc-peptides produced by endo H treatment which were not separated off by RP-HPLC.

The fact that Marburg virus glycoprotein glycans completely lack sialic acid residues is corroborated by lectin studies demonstrating that neither SNA nor MAA recognizing

Table III. Structures proposed for the major oligosaccharide alditol fractions obtained from Marburg virus glycoprotein

Oligosaccharide alditol fraction	Structure	Molar ratio (mol/100 mol)
H2	Manα6┐ Manα6┐ Manα3┘ Manβ4GlcNAcOH Galβ4GlcNAcβ2Manα3┘ + Manα6┐ Manα6┐ Manα2 Manα3┘ Manβ4GlcNAcOH Manα2Manα3┘	3.3
H3	Manα2Manα6┐ Manα6┐ Manα3┘ Manβ4GlcNAcOH Manα2Manα2Manα3┘	3.7
F21 (major component)	Galβ4GlcNAcβ2Manα6┐ Fucα6┐ GlcNAcβ4Manβ4GlcNAcβ4GlcNAcOH Galβ4GlcNAcβ2Manα3┘	2.2
F22	Galβ4GlcNAcβ2Manα6┐ Fucα6┐ GlcNAcβ4Manβ4GlcNAcβ4GlcNAcOH Galβ4GlcNAcβ2Manα3┘ Galβ4GlcNAcβ4┘	10.9
F23	Galβ4GlcNAcβ6┐ Galβ4GlcNAcβ2Manα6┐ Fucα6┐ Manβ4GlcNAcβ4GlcNAcOH Galβ4GlcNAcβ2Manα3┘ Galβ4GlcNAcβ4┘	2.3
F24	Galβ4GlcNAcβ6┐ 8′ 7′ │ Galβ4GlcNAcβ2Manα6┐ Fucα6┐ 6′ 5′ 4′ │ │ GlcNAcβ4Manβ4GlcNAcβ4GlcNAcOH 9 │3 2 1 Galβ4GlcNAcβ2Manα3┘ 6 5 │4 Galβ4GlcNAcβ4┘ 8 7	3.5
O1,O2	Galβ3GalNAcOH	15.4
O3	GlcNAcβ6┐ GalNAcOH Galβ3┘	1.9
O4	Galβ4GlcNAcβ6┐ GalNAcOH Galβ3┘	36.2
O5	[Galβ4GlcNAcβ] ┌ Galβ4GlcNAcβ6┐ GalNAcOH └ Galβ3┘	2.1

Structures were deduced from the analytical results described. The molar ratios were roughly estimated from the distribution of ^3H radioactivity incorporated into *N*-acetylglucosamine and *N*-acetylgalactosamine residues. In the structure of F24, the designation of sugar residues in *N*-acetyllactosaminic glycans (Vliegenthart *et al.*, 1983) is indicated.

(α2-6)- and (α2-3)-linked sialic acids, respectively, reacted with this glycoprotein. In contrast, the presence of (α2-3)-linked sialic acid residues could be clearly shown in the case of the glycoproteins from the morphologically and genetically related Ebola and Reston viruses propagated in the same cell line. Thus, the sialylation capacity of E6 cells is not generally abolished by filovirus infection. It might be possible, however, that the expression of sialylated glycoproteins is selectively affected by Marburg virus, but further analysis, e.g., of cellular glycoproteins before and after Marburg virus infection, are

required to clarify this point. Alternatively, one might assume that Marburg virus glycoprotein has intrinsic sialidase activity similar to corresponding envelope glycoproteins of para- and orthomyxoviruses. Up to now, however, such enzymatic activity could not be detected in mature virus particles. Possibly, the glycoprotein does not reach the intracellular compartments involved in the sialylation of glycoconjugates.

Only a limited number of viral glycoproteins have been reported to carry *O*-glycans. While glycoprotein E1 of murine coronavirus carries exclusively *O*-linked sugar chains (Niemann *et al.*, 1984), herpes simplex virus glycoproteins (Oloffson *et al.*, 1981; Dall'Olio *et al.*, 1985; Serafini-Cessi *et al.*, 1988), vaccinia virus haemagglutinin (Shida and Dales, 1981), G protein of respiratory syncytial virus (Gruber and Levine, 1985), glycoprotein 65 encoded by Friend spleen focus-forming virus (Gliniak and Kabat, 1989; Pinter and Honnen, 1989) and glycoprotein 71 of Friend murine leukaemia virus (Geyer *et al.*, 1990) comprise both O- and N-linked oligosacharides, similar to Marburg virus glycoprotein. Since carbohydrate side chains can influence the antigenicity and immunogenicity of viral glycoproteins either by masking or by stabilization of polypeptide epitopes [for review, see Klenk (1990)], the heterogeneous glycosylation pattern of Marburg virus glycoprotein might be an important parameter determining the chances of success of the human immune response during infection. In addition, the substitution by large amounts of *O*- and *N*-glycans could influence the maintenance of the biologically active conformation, and protect the peptide chain from proteolytic attack.

Materials and methods

Materials

The origin and structures of oligosaccharide standards used for column calibration were given in detail previously (Pfeiffer *et al.*, 1989, 1990). α-1,2-Specific mannosidase from *A.oryzae* was generously provided by Dr H.Yamaguchi (University of Osaka, Osaka, Japan).

Metabolic labelling and isolation of Marburg virus glycoprotein

The Musoke strain of Marburg virus isolated in 1980 in Kenya (Smith *et al.*, 1982) has been used. The virus was propagated in E6 cells, a cloned line of Vero cells (ATCC CRL 1586), as follows. E6 cells were infected with Marburg virus, strain Musoke, at a multiplicity of infection of 10^{-2} plaque-forming units/cell. Following an adsorption period of 30 min at 37°C, cells were incubated in Dulbecco's medium containing 2% fetal calf serum. *In vivo* radioactive labelling of viral proteins was performed 5 days post-infection. The maintenance medium was removed and the cell monolayers were washed twice with glucose-deficient Dulbecco's medium containing 1.8 g fructose/l (for labelling with [^{3}H]glucosamine) or methionine-deficient medium (for labelling with [^{35}S]methionine). Cells were incubated in deficiency medium for 2 h. The incubation was continued for 24 h at 37°C in the specific nutrient-deficient Dulbecco's medium to which radioactive nutrients were added as follows: 20 μCi/ml of D-[6-^{3}H]glucosamine or 20 μCi/ml of [^{35}S]methionine (Amersham Buchler, Braunschweig, FRG). Isolation and purification of the virus were as described previously (Feldmann *et al.*, 1991; Mühlberger *et al.*, 1992). Briefly, culture fluid was clarified by centrifugation at 6000 *g* (Minifuge RF, Heraeus, FRG) for 15 min at 4°C and virus particles were isolated by centrifugation in a SW28 rotor through a 20% sucrose cushion in TNE buffer (0.01 M Tris HCl, pH 7.4; 0.15 M NaCl; 2 mM EDTA) at 113 000 *g* for 2 h at 4°C. The pellet was resuspended in TNE buffer and the virus particles were purified by gradient centrifugation in a SW28 rotor using a gradient of 0–40% (w/v) potassium tartrate, 30–0% glycerol in TNE buffer at 160 000 *g* for 16 h at 4°C. The virus band was isolated, diluted in TNE buffer, pelleted by centrifugation in a SW41 rotor at 210 000 *g* for 30 min at 4°C and resuspended in TNE buffer. The virus was disintegrated by adding SDS up to a final concentration of 1%. For carbohydrate structure analysis the glycoprotein, metabolically labelled with [6-^{3}H]glucosamine, was precipitated with acetone in order to remove salts and detergents, and redissolved in buffer for proteolytic digestion.

SDS gel electrophoresis

Electrophoresis of proteins was carried out as described by Laemmli (1970). Metabolically labelled virion proteins were visualized by fluorography as follows. Gels were soaked for 30 min in 30% methanol and 10% glacial acetic acid for protein fixation, treated for 30 min with ENLIGHTNING™ autoradiography enhancer (NEN, Bad Homburg, FRG), dried and exposed to an X-ray film (Kodak). For calibration, rainbow [^{14}C]methylated protein molecular mass markers (Amersham) were used.

Glycoprotein staining with lectins

For lectin staining, the glycan differentiation kit (Boehringer, Mannheim) containing digoxigenin-conjugated lectins was used. Viral extracts were subjected to SDS gel electrophoresis before and after treatment with sialidase, blotted onto nitrocellulose, incubated with MAA or SNA and stained with anti-digoxigenin antibodies conjugated with alkaline phosphatase according to the protocol of the manufacturer, except that the nitrocellulose was incubated overnight at 4°C in the blocking solution.

Identification of radiolabelled monosaccharides

For the determination of labelled monosaccharide components, a sample (2×10^3 c.p.m.) of glycoprotein was hydrolysed in 1 ml of 1 N trifluoroacetic acid at 80°C. After 30 min, an aliquot (500 μl) was taken for the determination of labelled sialic acids, whereas the remaining 500 μl were further incubated at 80°C for 24 h. Both samples were dried by rotary evaporation, repeatedly redissolved in 1 ml of methanol and dried again. The radiolabelled monosaccharides obtained were identified by HPAE-HPLC (see below).

Isolation of oligosaccharides

Marburg virus glycoprotein was digested with trypsin (Serva, Heidelberg, FRG) as described elsewhere (Schlüter *et al.*, 1985). N-Linked oligosaccharides were released from tryptic glycopeptides by sequential treatment with endo H from *Streptomyces griseus* and PNGase F from *Flavobacterium meningosepticum* (both from Boehringer, Mannheim, FRG), separated from residual (glyco)-peptides by gel filtration or RP-HPLC, reduced and desalted as described earlier (Strube *et al.*, 1988; Geyer and Geyer, 1990). *O*-Glycans were liberated by incubation with *O*-glycosidase from *D.pneumoniae* (Boehringer) in 20 mM sodium phosphate buffer (pH 7.2), 50 mM EDTA, 0.02% sodium azide (50 pkat of enzyme; 100 μl buffer, 24 h, 37°C) and isolated by gel filtration. Remaining *O*-glycans were released by β-elimination using 0.8 M sodium borohydride/50 mM sodium hydroxide as described previously (Geyer *et al.*, 1990; Geyer and Geyer, 1992) and separated from peptide material by RP-HPLC. For alkaline hydrolysis of PNGase F- and β-elimination-resistant glycopeptides, the experimental protocol of Geyer *et al.* (1984b) was followed. After re-*N*- and de-*O*-acetylation, residual peptides were removed by binding to mixed-bed ion-exchange resin (Amberlite AG-MB3; Serva, Heidelberg, FRG) (Strube *et al.*, 1988).

Chromatographic procedures

(i) Desalting of glycopeptides and oligosaccharide alditols, (ii) RP-HPLC for separation of oligosaccharides from (glyco)peptides, (iii) separation of glycans by gel filtration, HPAE-HPLC, HPLC using columns of LiChrosorb-Diol or LiChrosorb-NH_2 (Merck, Darmstadt, FRG) and anion-exchange HPLC at neutral pH using a Carbopak PA1 column, and (iv) chromatographic characterization of oligosaccharide alditol fragments by Bio-Gel P-4 chromatography were carried out as described in detail previously (Schlüter *et al.*, 1985; Strube *et al.*, 1988; Holschbach *et al.*, 1990; Pfeiffer *et al.*, 1990; Wendorf *et al.*, 1991; Geyer and Geyer, 1992). For fractionation of *O*-glycans, a LiChrosorb-NH_2 column (250 × 4.6 mm; 5 μm; Merck, Darmstadt, FRG) equilibrated with 75% aqueous acetonitrile was used and the concentration of acetonitrile was reduced to 50% in 90 min (Lamblin *et al.*, 1984).

Identification of radiolabelled monosaccharides by HPAE-HPLC was achieved using 125 mM sodium acetate in 100 mM aqueous sodium hydroxide (sialic acids) or 10 mM sodium hydroxide (neutral and amino sugars) as eluents (Hardy *et al.*, 1988).

Enzymatic digestions

Degradation of oligosaccharide alditols with (i) sialidase from *V.cholerae* (Behringwerke, Marburg, FRG), (ii) β-*N*-acetylhexosaminidase (5 or 50 nkat) and α-mannosidase from jack beans (Sigma, Deisenhofen, FRG), (iii) β-galactosidase from *D.pneumoniae* (Boehringer, Mannheim, FRG), (iv) α-fucosidase from beef kidney (Boehringer), and (v) α-1,2-specific

mannosidase from *A.oryzae* was performed as described elsewhere (Geyer *et al.*, 1982, 1984b; Strube *et al.*, 1988; Holschbach *et al.*, 1990). For treatment with β-galactosidase from bovine testes (Boehringer) or β-mannosidase from *Helix pomatia* (Sigma), oligosaccharide samples were taken up in 50 μl of 100 mM sodium phosphate/citrate buffer, 0.1% (w/v) bovine serum albumin (pH 4.4), or 50 mM sodium acetate buffer (pH 4.0) and incubated at 37°C for 24 h with 1 μkat or 240 nkat of enzyme, respectively.

Methylation analysis

Oligosaccharide alditols were permethylated (Paz Parente *et al.*, 1985) and hydrolysed. Partially methylated alditol acetates obtained after sodium borohydride reduction and peracetylation were analysed by capillary GLC/MS using the instrumentation and microtechniques described earlier (Geyer *et al.*, 1983).

LSIMS

LSIMS was carried out using a Finnigan MAT 900 mass spectrometer (Finnigan MAT, Bremen, FRG) equipped with an array detector and a caesium gun which was operated at 23 kV with an emission current of 2–3 μA. Mass spectra of methylated F22 glycans were recorded in positive ion mode at an accelerating potential of 5 kV and a resolution of ~4000 (high mass range) or 1000 (low mass range) with a linear magnet scan (30 s/1000 mass unit). Within each mass range, 5 (*m/z* 160–1000) or 9 (*m/z* 2000–4000) scans were accumulated. Spectra of methylated O4 glycans were recorded at constant magnetic field solely within the mass range of *m/z* 920–990 (10 s/scan) using the array detector of the instrument. In this case, 20 scans were accumulated. In all experiments, data were acquired with a DEC 2100 data system and 3-nitrobenzyl alcohol (Aldrich, Steinheim, FRG) was used as matrix.

Acknowledgements

We express our gratitude to Werner Mink, Peter Kaese and Siegfried Kühnhardt for methylation, GLC/MS and LSIMS analyses. This project was supported by the Deutsche Forschungsgemeinschaft (Sti 38/21-1; Ge 527/2-1; Sonderforschungsbereich 272, Teilprojekt Z2 and Sonderforschungsbereich 286, Teilprojekt A6).

Abbreviations

dHex, deoxyhexose; endo H, endo-β-*N*-acetylglucosaminidase H; Fuc, L-fucose; GalNAcOH, *N*-acetylgalactosaminitol; GlcNAcOH, *N*-acetylglucosaminitol; GLC/MS, gas–liquid chromatography/mass spectrometry; Hex, hexose; HexNAc, *N*-acetylhexosamine; HexNAcOH, *N*-acetylhexosaminitol; HPAE-HPLC, high-pH anion-exchange HPLC; LSIMS, liquid secondary-ion mass spectrometry; MAA, *Maackia amurensis* agglutinin; PNGase F, peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase F; RP-HPLC, reversed-phase HPLC; SNA, *Sambucus nigra* agglutinin.

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Received on March 18, 1992; accepted on April 23, 1992