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Structural studies of the carbohydrate chains of human γ-interferon

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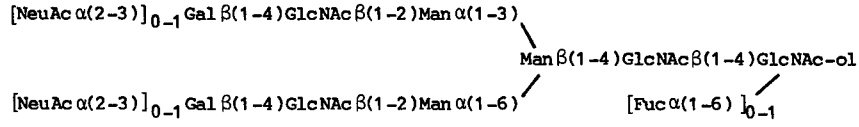
Human γ-interferon (IFN-γ) was prepared biotechnologically using Chinese hamster ovary cells. These cells were shown to be able to produce glycosylated IFN-γ. Sugar analysis revealed the presence of Man, Gal, GlcNAc, NeuAc and Fuc residues in a molar ratio of 3.8:2.0:3.5:0.6:0.4 suggesting the occurrence of *N*-glycosidically linked *N*-acetyllactosamine type of carbohydrate chains. For structure determination of these chains, the glycoprotein was subjected to the hydrazinolysis procedure, yielding oligosaccharide-alditols. The latter compounds were analysed by 500-MHz ^{1}H-NMR spectroscopy. The carbohydrate material was found to consist of biantennary structures, exhibiting microheterogeneity as to the terminal sialic acids and the core Fuc residue:



As similar carbohydrates are present on several human secreted proteins, this glycosyl group is not expected to be immunogenic in man.

It remains to be established to what extent the carbohydrate chains of this biotechnologically produced IFN-γ are identical to those of naturally occurring human IFN-γ.

Human IFN-γ is a highly interesting glycoprotein because of its potential therapeutic application against some types of cancer, in addition to its antiviral activity. It has been purified from different sources such as human spleen or tonsillar-derived cell cultures and from mitogen-stimulated human peripheral blood lymphocyte cultures [1, 2]. For naturally occurring IFN-γ it was established that a 20-kDa form is glycosylated at Asn-28, whereas a 25-kDa form is glycosylated at Asn-28 and Asn-100 [3]. The carbohydrate chains are exclusively *N*-linked and they are believed to be heterogeneous with respect to their sialic acid content [3, 4].

In order to obtain larger amounts of IFN-γ, the human IFN-γ cDNA gene has been expressed in *Escherichia coli* [5, 6] as well as in CHO cells [7, 8]. The amino acid sequence indicates the existence of two possible sites of *N*-glycosylation (Asn-Xaa-Ser/Thr sequences) in IFN-γ [5, 8]. For human IFN-γ obtained from CHO cells, three forms with different molecular masses have been identified, representing non-glycosylated (M_r 17000; a very minor component) and glycosylated (M_r 21000 and 25000) species of interferon [7]. The relative proportions of the latter two species, as well as their mobility upon electrophoresis in denaturing gels, is nearly identical to that of natural IFN-γ [2, 3].

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Abbreviations. IFN-γ, γ-interferon; CHO, Chinese hamster ovary; ConA, concanavalin A; SDS, sodium dodecyl sulfate; GlcNAc-ol, *N*-acetylglucosaminitol; NeuAc, *N*-acetylneuraminic acid; Fuc, fucose; Man, mannose; Gal, galactose; GlcNAc, *N*-acetylglucosamine.

For clinical use of glycoproteins, obtained by recombinant DNA techniques, detailed knowledge of the structure of the carbohydrate chains is essential, because the latter can influence the biological properties of the protein, such as pharmacokinetics and antigenicity. Here we report on the carbohydrate structures of human IFN-γ obtained from CHO cells, employing sugar analysis and 500-MHz ^{1}H-NMR spectroscopy.

MATERIALS AND METHODS

Preparation of recombinant human IFN-γ expressed in CHO cells

Human IFN-γ was produced in cultures of a Chinese hamster ovary cell line, transformed with a combination of plasmids encoding human IFN-γ cDNA and mouse dihydrofolate reductase cDNA. Subsequently the cells were selected for growth in the presence of methotrexate [7]. Human IFN-γ, secreted into the medium, was purified successively by ion-exchange chromatography on phosphocellulose (Whatman P11 cellulose), affinity chromatography on ConA-Sepharose (Pharmacia) and by ion-exchange chromatography on DEAE-Sephacel (Pharmacia) as described [4]. For storage IFN-γ was kept in a 10 mM sodium phosphate buffer, pH 7, containing 20% ethylene glycol, 0.3 M NaCl and 0.001% NaN_3. Since buffer components, especially ethylene glycol, interfere in sugar analysis and hydrazinolysis, they needed to be removed. To this end the IFN-γ preparation (1.6 mg in 2.5 ml buffer) was mixed with 300 µl 20% SDS, dialysed at

room temperature for 24 h against 200 ml bidistilled water with two intermediate changes and finally lyophilized.

Sugar analysis

The IFN-γ/SDS mixture was subjected to methanolysis (1.0 M methanolic HCl, 24 h, 85°C) followed by gas-liquid chromatography on a capillary CPsil-5 WCOT fused silica column (0.34 mm × 25 m, Chrompack) of the trimethylsilylated (re-*N*-acetylated) methyl glycosides [9].

Hydrazinolysis

The IFN-γ/SDS mixture, dried over P_2O_5, was suspended in 0.5 ml anhydrous hydrazine and heated for 15 h at 103°C. After evaporation of hydrazine, the material was *N*-acetylated and reduced with $NaBH_4$ as described [10]. The oligosaccharide alditols were purified by gel filtration on a Bio-Gel P-2 column (1.5 × 20 cm, Bio-Rad), eluted with bidistilled water. Fractions positive in the orcinol/H_2SO_4 assay, were pooled and lyophilized.

For reference purposes, human serotransferrin was also subjected to the hydrazinolysis procedure.

Desialylation

The oligosaccharide-alditols obtained from human serotransferrin were desialylated with 0.1 M trifluoroacetic acid for 2 h at 80°C. The sample was evaporated to dryness and the liberated sialic acid was removed by filtration over Dowex 1X2 (200–400 mesh, $HCOO^-$ form). The sample was re-*N*-acetylated as described [10] and finally purified by gel filtration on a Bio-Gel P-2 column (1.5 × 20 cm Bio-Rad), eluted with bidistilled water. Fractions positive in the orcinol/H_2SO_4 assay, were pooled and lyophilized.

500-MHz ^{1}H-NMR spectroscopy

Deuterium-exchanged oligosaccharide-alditols were obtained by threefold lyophilization of the ^2H_2O solutions. Finally, the samples were dissolved in 0.4 ml ^2H_2O (99.96 atom % ^{2}H, Aldrich). 500-MHz ^{1}H-NMR spectra were recorded on a Bruker WM 500 instrument (SON hf-NMR facility, Department of Biophysical Chemistry, University of Nijmegen, The Netherlands) operating in the pulsed Fourier-transform mode at a probe temperature of 27°C. Chemical shifts (δ) were expressed in ppm downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate, but were actually measured by reference to internal acetone (δ 2.225 ppm) with an accuracy of 0.002 ppm [11].

Ion-exchange chromatography

Anion-exchange chromatography of the oligosaccharide-alditols obtained from IFN-γ was performed on analytical scale on a Mono Q column (Pharmacia) eluted with a gradient of 0–100 mM NaCl (Van Pelt, J., Kamerling, J. P. and Vliegenthart, J. F. G., unpublished results).

RESULTS

IFN-γ is a basic protein (pI 8.5–9) [2] which, in the absence of glycerol or ethylene glycol, tends to stick to glass or to dialysis tubing. To keep the glycoprotein in solution in the absence of ethylene glycol, SDS was added. Sugar analysis of IFN-γ revealed the presence of Man, Gal, GlcNAc, NeuAc and Fuc in a molar ratio of 3.8:2.0:3.5:0.6:0.4 (Gal is taken as 2.0, GlcNAc is corrected for the amount of Asn-linked GlcNAc that is not cleaved under the applied conditions of methanolysis). In sugar analysis of samples containing SDS, the main mannose peak (pertrimethylsilyl methyl α-mannopyranoside) in the gas chromatogram has a retention time very close to that of an intense peak derived from SDS. Therefore, the value calculated for Man is somewhat too high. This result suggests the presence of *N*-glycosidically linked *N*-acetyllactosamine type of chains.

The carbohydrate chains were released from the protein backbone by hydrazinolysis and, after *N*-acetylation and reduction, investigated by 500-MHz ^{1}H-NMR spectroscopy. The relevant chemical shift data are compiled in Table 1. The ^{1}H-NMR spectrum of the mixture of oligosaccharide-alditols

Table 1. *Chemical shifts of the structural-reporter-group protons of the constituent monosaccharides for the oligosaccharide-alditols derived from IFN-γ (2) and a reference compound derived from human serotransferrin (1)*

Chemical shifts are expressed downfield from sodium 4,4-dimethyl-4-silapentane-1-sulfonate in ^2H_2O at 27°C. For complete structures of the compounds see text: N = NeuAc, F = Fuc. n.d. = not determined

Reporter group	Residue	Chemical shift in (1) 6-5-4 / 3-2-1-ol / 6′-5′-4′	(2) (N)-6-5-4 / 3-2-1-ol / (N)-6′-5′-4′ (F) asialo-, afuco-compound	sialo-compound	fuco-compound
		ppm			
H-1	2	4.637	4.641		n.d.
	3	4.773	n.d.		
	4	5.120	5.120		
	4′	4.925	4.922		
	5	4.580	4.581	4.572	
	5′	4.580	4.581	4.572	
	6	4.466	4.470	4.544	
	6′	4.471	4.470	4.544	
	Fuc	–			4.888
H-2	1-ol	4.244	4.237		4.224
	3	4.248	4.250		
	4	4.190	4.190		
	4′	4.110	4.109		
NAc	1-ol	2.056	2.056		
	2	2.080	2.080		2.089
	5	2.053	2.052	2.048[a]	
	5′	2.046	2.047[a]	2.042	
	NeuAc			2.031	
H-3*ax*	NeuAc	–		1.799	
H-3*eq*	NeuAc	–		2.758	
H-5	Fuc	–			4.072
CH_3	Fuc	–			1.225

[a] These values may have to be interchanged.

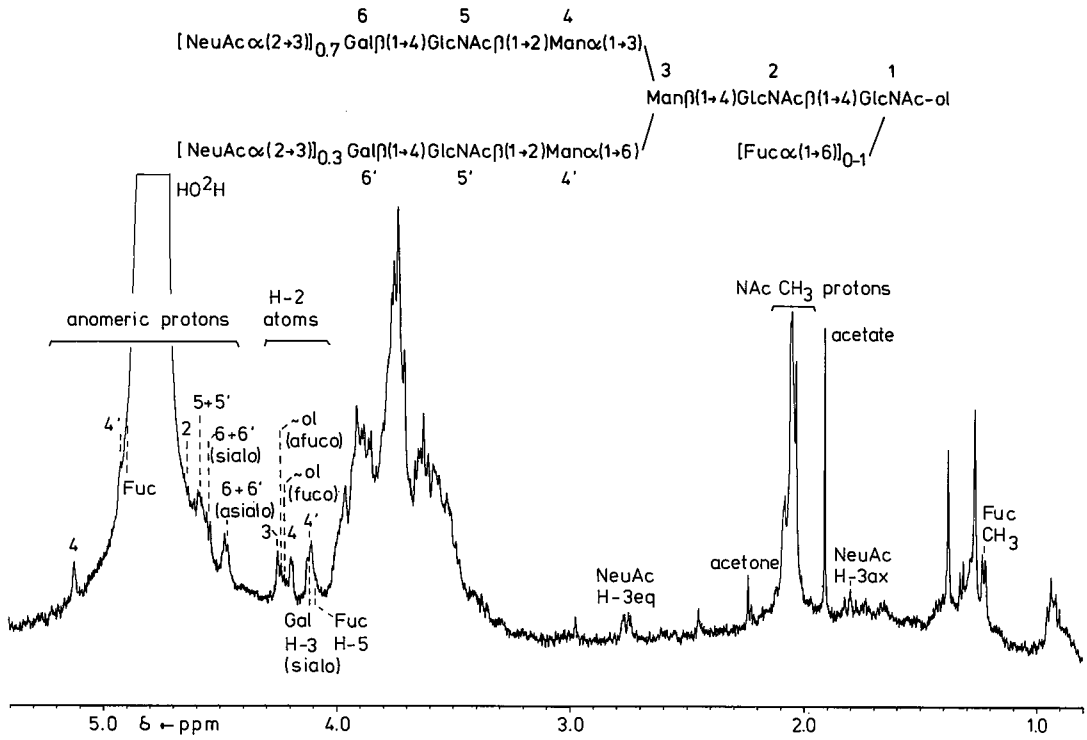
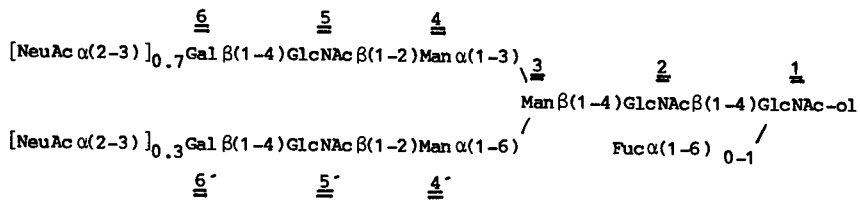


Fig. 1. *500-MHz ^{1}H-NMR spectrum of the mixture of oligosaccharide-alditols derived from human IFN-γ in ^2H_2O at 27°C*

obtained from IFN-γ, as shown in Fig. 1, reveals the characteristic features of partially sialylated, biantennary, *N*-glycosidic carbohydrate chains. The reduced *N,N'*-diacetylchitobiose core unit is characterized by a set of chemical shifts for H-2 and NAc of GlcNAc-1-ol and H-1 and NAc of GlcNAc-2 at $\delta = 4.237$, 2.056, 4.641 and 2.080 ppm respectively. Furthermore, a set is present at $\delta = 4.224$, 2.056, 4.7 and 2.089 ppm respectively, which corresponds to a core extended with a Fuc α(1−6)-linked to GlcNAc-1-ol. This Fuc is characterized by its H-1, H-5 and CH_3 at $\delta = 4.888$, 4.072 and 1.225 ppm, respectively [12]. The biantennary type of structure is evident from the set of chemical shifts of H-2 atoms of Man-3, Man-4 and Man-4' (see Table 1) [11]. Each of the *N*-acetyllactosamine branches can be terminated by NeuAc in α(2−3) linkage to Gal. The α(2−3) linkage can be derived from the set of chemical shifts of H-3*ax* and H-3*eq* of NeuAc being 1.799 and 2.758 ppm, respectively, and from the resonance position of H-3 of (2−3)-sialylated Gal, downfield of the bulk of skeleton protons, at $\delta = 4.116$ ppm [11]. Sialylated Gal-6 gives rise to an NAc signal of GlcNAc-5 at $\delta = 2.048$ ppm, the asialo analogue to $\delta = 2.052$ ppm. From the relative intensities of these NAc signals, it was estimated that 70% of the Gal-6 residues are sialylated. The chemical shift value for the NAc signal of GlcNAc-5' is 2.042 ppm when Gal-6' is sialylated and 2.047 ppm for the asialo analogue. Gal-6' is sialylated to about 30%. In conclusion, the structures of the carbohydrate chains of human IFN-γ can be summarized as follows:



An analytical run of the sample on a Mono Q column revealed the presence of neutral, monosialo and bisialo compound in the ratio of approximately 1:1:1, which is in accordance with the sugar analysis and the NMR data.

DISCUSSION

The carbohydrate chains of human IFN-γ obtained from CHO cells were found to be biantennary type of structures, exhibiting heterogeneity with respect to α(2−3)-linked NeuAc and α(1−6)-linked Fuc. It is not clear whether different types of cells produce differently glycosylated IFN-γ, so it remains to be established whether the carbohydrate chains elucidated here for CHO-derived IFN-γ are identical to those of the naturally occurring glycoprotein. It is known that the carbohydrate chains of the latter are *N*-glycosidically linked and that they are heterogeneous in sialic acid content [3, 4]. The linkage type of sialic acid is not known. The mobility in denaturing gels and the affinity towards ConA-Sepharose of CHO-produced IFN-γ is comparable to that of naturally occurring IFN-γ [2, 4] suggesting that the latter may also bear a biantennary type of glycan chain.

The carbohydrate moiety of IFN-γ is not required for the expression of antiviral or antiproliferative activity. For therapeutic application of IFN-γ the carbohydrate chains may be important because they possibly protect the IFN-γ from proteolytic breakdown and may thereby increase the lifetime

of the drug. On the other hand, IFN-γ can be removed from the blood circulation by the reaction of the carbohydrate chains with specific receptors. For instance the Gal residue in terminal position in a fraction of the CHO-produced IFN-γ may interact with the well-known hepatic receptors. If this was the case, then the quality of the IFN-γ can be improved by sialylating all Gal residues present in IFN-γ with the aid of a sialyltransferase.

As the chemical structure of the glycosyl group, established here for CHO-produced human IFN-γ, is identical to that found in secreted human proteins (e.g. human chorionic gonadotropin [13, 14], it is reasonable to expect that this bioengineered glycoprotein will not be antigenic for man. This is an important issue, considering that cloned human genes, especially those for complex glycoproteins, are often expressed in CHO cells for production purposes and some of these proteins may be developed for clinical use.

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