

'Brain-type' *N*-glycosylation of asialo-transferrin from human cerebrospinal fluid

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Abstract Asialo-transferrin from human cerebrospinal fluid was purified to homogeneity. Investigation of the structural characteristics of its oligosaccharides support our hypothesis of 'brain-type' glycosylation of intrathecally synthesized cerebrospinal fluid proteins. For carbohydrate structural analysis, high-pH anion-exchange chromatography, methylation analysis, liquid secondary ion- and matrix-assisted laser desorption/ionization mass spectrometry of the permethylated derivatives were used. The major structure turned out to be a complex-type agalactodiantennary oligosaccharide with bisecting *N*-acetylglucosamine and proximal fucose. Analysis of a second transferrin preparation containing both asialo- and sialo-transferrin revealed another major glycan species derived from the sialylated transferrin variant which is galactosylated and lacks bisecting *N*-acetylglucosamine and fucose.

Key words: *N*-Glycosylation ('brain-type'); Cerebrospinal fluid; Human transferrin; Human β -trace protein

1. Introduction

We have recently shown that β -trace protein isolated from human cerebrospinal fluid (CSF) is modified by *N*-linked complex-type diantennary oligosaccharides which are characterized by large proportions of asialo- and agalacto-chains typically containing proximal fucose (Fuc) and bisecting *N*-acetylglucosamine (GlcNAc) [1]. As discussed previously, such glycoforms cannot persist in blood due to the existence of specific hepatic clearance mechanisms (see, e.g. [2]).

In order to further substantiate our hypothesis on 'brain-type' glycosylation of intrathecally synthesized secretory polypeptides [1] we have structurally characterized the *N*-glycans of CSF-transferrin.

Human transferrin is a 79,570 Da glycoprotein with two *N*-glycosylation sites at Asn-413 and Asn-611 [3]. The protein isolated from human serum bears complex-type diantennary (85%) and triantennary (15%) oligosaccharides with α 2,6-linked *N*-acetylneuraminic acid (NeuNAc) and α 2,3-linked

NeuNAc on the additional branch of the triantennary oligosaccharides [4–6]. As both glycosylation sites may be occupied by two di-, one di- and one tri- or two triantennary chains, the maximum number of NeuNAc-residues varies from 4–6 (82%/17%/<1% of the respective glycoforms in serum [7]); only low amounts of asialo- and monosialo-variants have been reported [8]. Di- and trisialo-transferrin also constitute minor species, the disialoform being elevated in cases of alcoholism [3]. Sero-transferrin isolated from the human hepatocarcinoma cell line Hep G2 has been shown to contain large amounts of higher antennary oligosaccharides with proximal fucose as well as peripheral fucose [9]. Altered glycosylation of serum transferrin has been reported to be associated with certain diseases [10–12] and has also been detected during pregnancy [7].

Transferrin is also an important protein constituent of human CSF, some 30% being present in an unsialylated form [13]. This asialo-transferrin, also named β_2 -transferrin or τ -globulin, can be distinguished from the sialylated variant by its higher mobility e.g. in agarose electrophoresis [13]. The origin of CSF-asialo-transferrin has not yet been conclusively elucidated. Suggestions include either intrathecal synthesis by a neuraminidase acting on serum-derived transferrin in the brain compartment or de-novo-synthesis of transferrin [14–17]. Here we present for the first time a characterization of human asialo- and sialotransferrin from cerebrospinal fluid providing evidence for local synthesis of the asialo-fraction.

2. Experimental

2.1. Isolation of transferrin from human cerebrospinal fluid (CSF)

Lumbar CSF was obtained from patients of the Neurological Clinic after informed consent as part of routine diagnostic procedures. For the analyses reported here, only non-pathological samples were pooled. After 35-fold concentration using a YM 3-membrane (Amicon, Witten, Germany) and dialysis against 20 mM Tris-HCl (pH 8.0) a sample containing 40 mg protein was subjected to anion exchange chromatography with DEAE MemSep 1010 (Millipore, Eschborn, Germany). The unbound material (containing asialo-transferrin) and the fronting part of a double-peak eluted with 25 mM NaCl in 0.02 M Tris pH 8.0 (residual asialo-transferrin and a part of the sialo-transferrin) were fractionated separately by gel filtration on an AcA 54 column as described [18]. The transferrin-containing fractions were either pooled for analysis of whole CSF-transferrin or analyzed separately in the case of the asialo-variant after removal of contaminating IgG by binding to protein A-CPG-gel (Bioprocessing, Durham, UK).

2.2. Sodium-dodecylsulfate polyacrylamide gel electrophoresis

Protein separation was analyzed as detailed [18] using 10% polyacrylamide gels.

2.3. Generation of tryptic glycopeptides and isolation of *N*-linked glycans

Since native transferrin is not accessible to digestion with polypeptide-*N*⁴-(*N*-acetyl-glucosaminyl)asparagine amidase F (PNGase

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Abbreviations: CSF, cerebrospinal fluid; deoxyHex, deoxyhexose; Fuc, fucose; Gal, galactose; GlcNAc, *N*-acetylglucosamine; Hex, hexose; HexNAc, *N*-acetylhexosamine; HexNAc-ol, *N*-acetylhexosaminol; HPAEC, high-pH anion-exchange chromatography; HPLC, high-performance liquid chromatography; MALDI/TOF-MS, matrix-assisted laser desorption/ionization time of flight mass spectrometry; NeuNAc, *N*-acetylneuraminic acid; PNGase F, polypeptide-*N*⁴-(*N*-acetyl-glucosaminyl)asparagine amidase F.

F), tryptic digestion of the protein and separation of the peptides by reversed-phase HPLC on a Vydac C₁₈-column were performed essentially as detailed [18] using gradient elution with solvent A: 0.1% (v/v) trifluoroacetic acid and solvent B: 80% (v/v) acetonitrile in 0.1% trifluoroacetic acid. Glycopeptides were identified by carbohydrate compositional analysis of aliquots of the HPLC-fractions. N-linked glycans were recovered from the pooled glycopeptide material by digestion with PNGase F and oligosaccharides were isolated by reversed-phase HPLC.

2.4. Oligosaccharide structural analysis

Liberation of N-linked glycans by PNGase F, desalting of oligosaccharides, anion exchange chromatography of N-linked oligosaccharides at neutral pH on a MonoQ-column, enzymatic removal of neuraminic acid, and carbohydrate compositional and methylation analysis have been described in detail [1,19].

2.5. High-pH anion exchange chromatography (HPAEC) of oligosaccharides

Analysis method and standard oligosaccharides were detailed previously [1,20]. Desialylated, desalted oligosaccharides were separated by the following gradient (solvent A = 0.2 M NaOH, solvent B = 0.6 M sodium acetate in A): after 2 min isocratic run at 100% A, a 38-min linear run to 90% A and then a 10-min linear run to 80% A were applied. After 10 min at 80% A, the gradient was completed by a 1-min run to 0% A.

2.6. Matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI/TOF-MS)

2,5-Dihydroxybenzoic acid was used as UV-absorbing matrix. 10 mg/ml 2,5-dihydroxybenzoic acid were dissolved in 10% ethanol in water. For analysis by MALDI/TOF-MS the solutions of the reduced and permethylated oligosaccharides were mixed with the same volume of matrix. 1 µl of the sample was spotted onto a stainless steel tip and dried at room temperature. The concentrations of the analyte mixtures were approximately 10 pmol/µl.

Measurements were performed on a Bruker REFLEX MALDI/TOF mass spectrometer using a N₂ laser (337 nm) with 3 ns pulse width and 107 to 108 W/cm² irradiance at the surface (0.2 mm² spot). Spectra were recorded at an acceleration voltage of 20 kV using the reflectron for enhanced resolution.

3. Results

3.1. Isolation of N-linked oligosaccharides from CSF-transferrin

About 100 µg of asialo-transferrin or 560 µg of a mixture of asialo-transferrin and sialo-transferrin were obtained from 100 ml of pooled human CSF as described in section 2. Amino acid sequencing yielded the single N-terminal sequence VPDKTVRW thus demonstrating the purity of both protein preparations.

Since oligosaccharides could not be detached by PNGase F-digestion from the native protein, the transferrin preparations were subjected to reduction, carboxamidomethylation and tryptic digestion as described [18]. Oligosaccharides were then liberated enzymatically from the tryptic glycopeptides and were isolated in the flow-through of a second reversed-phase HPLC-run.

3.2. Characterization of transferrin glycans

Preliminary characterization of the oligosaccharides from the asialo-transferrin preparation yielded one major peak, I, upon HPAEC (see Fig. 1a) which had an identical retention time as an agalacto-diantennary reference oligosaccharide with bisecting GlcNAc and proximal fucose which represents the predominant N-glycan in human β-trace protein isolated from CSF (cf. Fig. 1c and Table 2) [1]. MALDI-MS which yielded a prominent molecular ion signal at 2097 Da [Hex₃ HexNAc₄

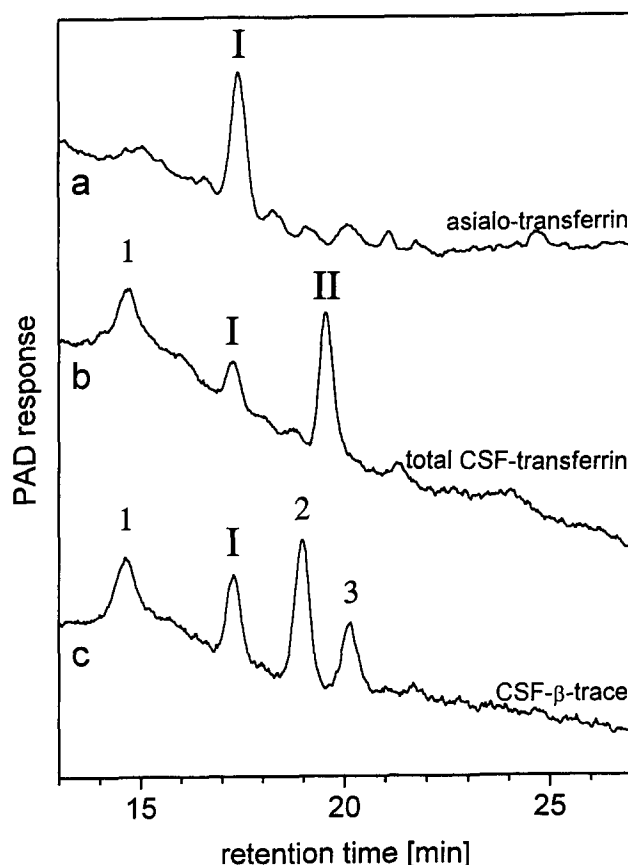


Fig. 1. High-pH anion-exchange profile of human CSF-transferrin oligosaccharides obtained as described in section 2. Panel a shows the elution profile of oligosaccharides from asialo-transferrin, (b) from asialo- plus sialo-transferrin, (c) of total oligosaccharides from CSF-β-trace protein. 1 = NeuNAc, 2 = diantennary complex-type glycan with proximal fucose plus diantennary complex-type with proximal Fuc and bisecting GlcNAc lacking one Gal, 3 = diantennary complex-type oligosaccharide with bisecting GlcNAc and proximal Fuc (1–3 were described in [1]). I and II represent a diantennary complex-type glycan with bisecting GlcNAc and proximal Fuc lacking two Gal and a diantennary complex-type glycan, respectively (cf. Table 2).

deoxyHex HexNAc-ol] + Na⁺, Fig. 2a, and methylation analysis (data not shown) confirmed this interpretation. Based on the monosaccharide derivatives detected, the presence of partially galactosylated as well as higher antennary chains and those lacking proximal fucose could also be inferred. Detection of several minor molecular ion signals as explained in the legend to Fig. 2 supported these findings.

Due to the low amounts of oligosaccharide material available from the pure asialo-transferrin, the structural analyses were repeated and extended using a mixture of asialo- and sialo-transferrin obtained as described under Experimental. The total N-linked oligosaccharides from this preparation were subjected to HPAEC after desialylation (Fig. 1b), MALDI-MS (Fig. 2b), and methylation analysis (Table 1). As shown in Fig. 1b, HPAEC revealed two major structures, I and II, in a ratio of about 1:2 (the third peak, 1, is caused by NeuNAc liberated during the digestion process which was not removed prior to HPAEC). Peak I had the same elution time as the prominent peak in Fig. 1a and thus clearly derived from asialo-transferrin whereas peak II eluted at a position identical with a diantennary

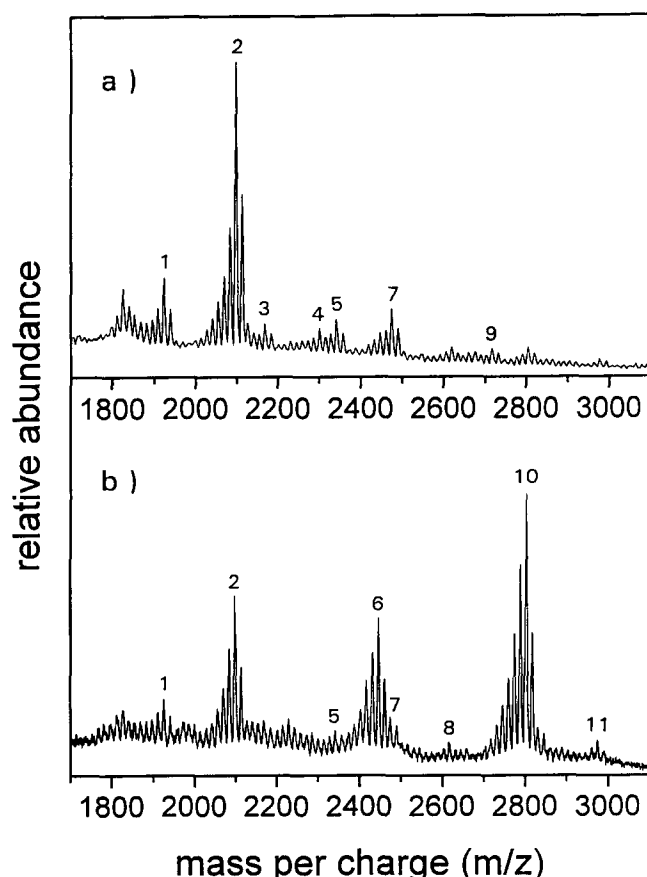


Fig. 2. MALDI-MS of the reduced and permethylated oligosaccharides of human transferrin isolated from cerebrospinal fluid. Panel a shows the complex-type oligosaccharides of CSF-asialo-transferrin, panel b the oligosaccharides of total CSF-transferrin. Based on the data presented in Fig. 1 and Table 1 as well as upon liquid secondary ion mass spectrometry we propose the following structures to be present: (1) 1923 Da [$\text{Hex}_3 \text{HexNAc}_4 \text{HexNAc-ol}] + \text{Na}^+$ is compatible with a diantennary glycan bearing bisecting GlcNAc and lacking two Gal, (2) 2097 Da [$\text{Hex}_3 \text{HexNAc}_4 \text{deoxyHex HexNAc-ol}] + \text{Na}^+$: diantennary with proximal Fuc and bisecting GlcNAc lacking two Gal, (3) 2168 Da [$\text{Hex}_3 \text{HexNAc}_5 \text{HexNAc-ol}] + \text{Na}^+$: triantennary with bisecting GlcNAc lacking three Gal or tetraantennary lacking four Gal, (4) 2302 Da [$\text{Hex}_4 \text{HexNAc}_4 \text{deoxyHex HexNAc-ol}] + \text{Na}^+$: diantennary with proximal Fuc and bisecting GlcNAc lacking one Gal, (5) 2343 Da [$\text{Hex}_3 \text{HexNAc}_5 \text{deoxyHex HexNAc-ol}] + \text{Na}^+$: triantennary with proximal Fuc and bisecting GlcNAc lacking three Gal or tetraantennary with proximal Fuc lacking four Gal, (6) 2447 Da [$\text{NeuNAc Hex}_5 \text{HexNAc}_3 \text{HexNAc-ol}] + \text{Na}^+$: diantennary, monosialylated, (7) 2476 Da [$\text{Hex}_4 \text{HexNAc}_4 \text{deoxyHex}_2 \text{HexNAc-ol}] + \text{Na}^+$: diantennary with proximal and one peripheral Fuc and bisecting GlcNAc lacking one Gal, (8) 2620 Da [$\text{NeuNAc Hex}_5 \text{HexNAc}_3 \text{deoxyHex HexNAc-ol}] + \text{Na}^+$: biantennary with proximal Fuc, monosialylated, (9) 2721 Da [$\text{Hex}_4 \text{HexNAc}_5 \text{deoxyHex}_2 \text{HexNAc-ol}] + \text{Na}^+$: triantennary with two Fuc and bisecting GlcNAc lacking two Gal or tetraantennary with two Fuc lacking three Gal, (10) 2809 Da [$\text{NeuNAc}_2 \text{Hex}_5 \text{HexNAc}_3 \text{HexNAc-ol}] + \text{Na}^+$: diantennary, disialylated, (11) 2983 Da [$\text{NeuNAc}_2 \text{Hex}_5 \text{HexNAc}_3 \text{deoxyHex HexNAc-ol}] + \text{Na}^+$: diantennary with one Fuc, disialylated. In addition, molecular ion signals resulting from K^+ -adducts of the respective structures ($[\text{M}+16]^+$) as well as from undermethylated structures ($[\text{M}-14]^+$) can be distinguished.

nary complex-type standard oligosaccharide lacking proximal fucose and bisecting GlcNAc (cf. Table 2 and [1]) and was released from the sialylated transferrin variant. Corresponding with this interpretation, MALDI-MS analysis (Fig. 2b) of the

reduced and permethylated oligosaccharide mixture revealed three predominant molecular ion signals. The signal at $m/z = 2097$ Da (peak no. 2: [$\text{Hex}_3 \text{HexNAc}_4 \text{deoxyHex HexNAc-ol}] + \text{Na}^+$) was already seen with asialo-transferrin (cf. Fig. 2a), the signals at $m/z = 2447$ and 2809 Da (no. 6 and 10, [$\text{NeuNAc Hex}_5 \text{HexNAc}_3 \text{HexNAc-ol}] + \text{Na}^+$ and [$\text{NeuNAc}_2 \text{Hex}_5 \text{HexNAc}_3 \text{HexNAc-ol}] + \text{Na}^+$) are caused by diantennary complex-type glycans bearing one or two NeuNAc-residues, respectively. Minor molecular ion signals are explained in the legend to Fig. 2. The methylation data obtained (Table 1) further confirmed and expanded these results. Thus, detection of about 0.3 mol 2-mono-*O*-methylmannitol argues for the presence of about 30% of an oligosaccharide with bisecting GlcNAc. This glycan also bears Fuc in a proximal position (30% 4,6-disubstituted *N*-acetylglucosaminitol-derivative) but lacks terminal Gal-residues on both antennae (high values of non-substituted *N*-acetylglucosaminitol) as evidenced by the MS data (structure A, Table 2). Oligosaccharide B, the desialylated structure of which is shown in Table 2, constitutes about 70% of the total sugar material. It is completely galactosylated, bearing NeuNAc almost exclusively in $\alpha 2,6$ -linkage (1.5 mol 6-substituted galactose) but lacks bisecting GlcNAc (0.7 mol 3,6-disubstituted mannitol) and Fuc (0.7 mol 1,3,5,6-tetra-*O*-methyl-*N*-acetylglucosaminitol). This glycan is identical to the major transferrin oligosaccharide found in human serum.

4. Discussion

To our knowledge, pure asialo-transferrin from human CSF was analyzed for the first time in this report. This transferrin variant turned out to contain one major type of N-linked diantennary complex-type oligosaccharides with two agalacto-antennae, bisecting GlcNAc and proximal fucose. Thus, the so-called asialo-transferrin turned out to be in fact an asialo-agalacto-transferrin. This oligosaccharide structure has never

Table 1
Methylation analysis of total CSF-transferrin oligosaccharides

Peracetylated derivative of	Transferrin	Substitution in position
Fucitol		
2,3,4-Tri- <i>O</i> -methyl-	0.3	—
Galacitol		
2,3,4,6-Tetra- <i>O</i> -methyl-	0.3	—
2,4,6-Tri- <i>O</i> -methyl-	0.1	3
2,3,4-Tri- <i>O</i> -methyl-	1.5	6
Mannitol		
3,4,6-Tri- <i>O</i> -methyl-*	2.0	2
3,6-Di- <i>O</i> -methyl-	<0.1	2; 4
3,4-Di- <i>O</i> -methyl-	0.1	2; 6
2,4-Di- <i>O</i> -methyl-	0.7	3; 6
2-Mono- <i>O</i> -methyl-	0.3	3; 4; 6
2- <i>N</i> -Methylacetamido-2-deoxyglucitol		
1,3,5,6-Tetra- <i>O</i> -methyl•-	0.7	4
3,4,6-Tri- <i>O</i> -methyl-	0.8	—
1,3,5-Tri- <i>O</i> -methyl•-	0.3	4; 6
3,6-Di- <i>O</i> -methyl-	2.7	4
6- <i>O</i> -Methyl-	0.2	3; 4

*Values are based on 3,4,6-tri-*O*-methylmannitol = 2.0.

•, derivatives of the proximal *N*-acetylglucosaminitol.

Table 2
Major oligosaccharide structures of CSF-transferrin (desialylated)

[illegible]

before been reported in natural human transferrin preparations but has previously been identified as the major glycan constituent of human CSF- β -trace protein [1]. The striking structural characteristics of β -trace and asialo-transferrin oligosaccharides, high amounts of terminal galactose and/or terminal GlcNAc including bisecting GlcNAc and complete proximal fucosylation as well as significant amounts of peripheral fucosylation, have now led us to establish the hypothesis of 'brain-type' glycosylation of secretory proteins produced by human brain tissue. These structural motifs seem to be common for glycoproteins synthesized by brain tissue of different mammalian species since they have also been reported for rat [21] and mouse [22] brain membrane glycoproteins as well as human myelin-associated glycoprotein and P₀ [23] and ribonucleases from human CSF [24]. In the case of secreted glycoproteins in human serum such structural features would lead to rapid clearance from the circulation due to the carbohydrate specificity of the hepatic receptor system [2] whereas in the central nervous system such a clearance mechanism is apparently not operating.

The truncated oligosaccharide structures in glycoproteins synthesized from mammalian brain tissue presumably result from low levels of terminal glycosyltransferase activities (GlcNAc β 1 \rightarrow 4: galactosyltransferase and Gal β 1 \rightarrow 4GlcNAc: sialyltransferase as well as high levels of Gal β 1 \rightarrow 4GlcNAc: α 1 \rightarrow 6 fucosyltransferase and GlcNAc- transferase III activities. By contrast, the degradation of protein-bound oligosaccharides within the CSF by glycosidases is highly unlikely since only negligible activities of e.g. fucosidase, β -galactosidase, and neuraminidase have been measured in this body fluid (M. Gawlitsek et al., unpublished results, [14,25])

The site of biosynthesis of CSF-proteins is often unclear. According to Wiederkehr [26] some 20% of the CSF-protein is of local origin. Transferrin mRNA and/or transferrin have been detected in epithelial cells of the choroid plexus, oligodendrocytes, and many other parts of the rat brain [15–17]. No transferrin mRNA was detected in choroid plexus of humans and some selected animals [27], however, transferrin immunoreactivity has been detected in human brain cell types [28,29], and transferrin mRNA was detected in cultured astrocytes [30]. Only for a limited number of proteins present in CSF of different animal species has local synthesis in the central nervous system been conclusively demonstrated whereas others (amongst them albumin) could be shown to derive from serum only. Our results obtained for the asialo-agalacto-transferrin in

human CSF which turned out to be 'brain-type' glycosylated based on the criteria described above thus clearly argue for a local origin of this special transferrin variant.

The sialylated form of transferrin in human CSF which contains preponderantly $\alpha 2,6$ -linked NeuNAc and lacks proximal fucose as well as bisecting GlcNAc is identical to the major glycoform present in human serum [4]. Its glycosylation features thus differ clearly from those of 'brain-type' *N*-glycosylated polypeptides. Therefore, it is probably derived from plasma via transfer across the blood-brain barrier by yet unidentified mechanisms which is in accordance with the notion that 80% of the protein constituents of human CSF are derived from serum [26]. The transferrin molecule may be a useful tool for studying the transport of glycoproteins across the blood-brain barrier. This situation, intra- and extrathecal origin of CSF-proteins, probably holds true for many CSF-proteins which are not exclusively 'brain-specific' as are myelin basic protein, neuron-specific enolase, glial fibrillary acidic protein, or β -trace.

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