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Development of Antibody to Human GM3 Synthase and Immunodetection of the Enzyme in Human Tissues

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Abstract—Polyclonal antibody was raised to a cloned fragment of human GM3 synthase. Affinity purified R27C1 antibody to the tagged recombinant protein inhibited GM3 synthase activity in human liver and HL-60 cells in a dose-dependent manner. However, the R27C1 antibody did not affect liver sialyltransferase activity towards asialofetuin. We are the first to measure GM3 synthase activity in human liver (194 ± 60 pmol NeuAc/h per mg protein), which was about 10-fold lower than in phorbol myristate acetate-stimulated HL-60 cells (1353 ± 573 pmol NeuAc/h per mg protein). On immunoblotting the R27C1 antibody recognized a common protein band in a number of human tissues (liver, brain, atherosclerotic aortic intima, HL-60 cells) with molecular mass of about 60 kD, which is similar to that of the purified GM3 synthase from rat liver. In human liver and aortic intima, the 60-kD band was almost a single band, which makes possible the use of the R27C1 antibody for immunohistochemical studies in these tissues.

Key words: human GM3 synthase activity, antibody to human GM3 synthase, human liver, HL-60 cells

Gangliosides are ubiquitous membrane components of all eucaryotic cells. It is known that the ganglioside composition and level in cells and tissues undergo significant changes during the cell cycle and differentiation [1]. Increased cell levels of certain gangliosides were established to be associated with their specific functions. Notably, a significant increase in GM3 content and activation of GM3 synthase (CMP-NeuAc:lactosylceramide α2,3-sialyltransferase) were demonstrated during monocyte/macrophage-like differentiation of many human myelocytic and monocytic leukemia cell lines (HL-60, K562, ML-1, etc.). In contrast to monocytic/macrophage-like differentiation, a remarkable decrease in GM3 and increase in other gangliosides were demonstrated during granulocytic differentiation of HL-60 cells. Furthermore, it was found that GM3 itself was potent to induce monocytic/macrophage-like differentiation [2].

We recently demonstrated that GM3 levels were dramatically enhanced in atherosclerotic lesions of human aorta in contrast to uninvolved tissue [3]. The study of GM3 localization in sections of atherosclerotic plaques with anti-GM3 antibody showed that accumulation of excessive amounts of GM3 occurred in different cell types transforming into foam cells [4]. It is known that cell differentiation to monocytic/macrophage type plays a significant role in foam cell formation during atherosclerotic lesion development in arterial intima [5-7]. We suggested that accumulation of ganglioside GM3 in atherosclerotic aortic intima through activation of GM3 biosynthesis favored monocytic/macrophage type of cell differentiation. The study of GM3 synthase activity in human normal and atherosclerotic aortic intima was needed to support our hypothesis. We tried to measure GM3 synthase activity by traditional radioligand technique but failed, probably because of low tissue levels of the enzyme, though we were able to detect an increase in total sialyltransferase activity on protein substrate (asialofetuin) in membrane fraction of atherosclerotic aortic intima and in blood plasma of patients with atherosclerosis [8].

In the present study, we cloned a fragment of human GM3 synthase, raised polyclonal antibody to the fragment, and revealed with it in some human tissues (brain, liver, atherosclerotic aortic intima, and HL-60 cells) a protein with molecular mass corresponding to GM3 synthase [9].

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MATERIALS AND METHODS

Materials. Chemicals of analytical grade from Sigma (USA), Serva (Germany), Ferak (Germany), BioRad (USA), Gibco (USA), as well as of domestic production; reagents for cloning from New England Biolabs (USA), Promega (USA), Novagen (USA), MBI Fermentas (Lithuania), and Qiagen (USA); cytidine 5′-monophospho[^{14}C]-N-acetylneuraminic acid (CMP-[^{14}C]NeuAc, 287 mCi/mmol) from Amersham (Great Britain); high-performance thin-layer chromatographic (HPTLC) plates from Merck (Germany) were used in the present work. Oligonucleotide primers were synthesized by Syntol (Russia). Human liver ganglioside GM3 was isolated according to the method described earlier [10]. Human GM3 synthase cDNA was kindly donated by M. Saito (Japan).

Cloning of a fragment of human GM3 synthase cDNA. The cDNA fragment encoding amino acids 191-245 of human GM3 synthase was prepared by PCR using two oligonucleotide primers with incorporated into their 5′-termini *Nco*I and *Xho*I restriction sites to enable subsequent cloning of the amplified fragment into the expression vector. The primers were as follows: forward (5′-TATACCATGGGCGCACCACTGTCTGAC-3′) and reverse (5′-ATACTCGAGTGGGATTTTTTCTGCCACC-3′). As a template for PCR, a pBluescript II KS(+)-derived plasmid containing human GM3 synthase cDNA (GenBank Accession Number AB018048) was used. PCR reaction mixture (final volume 30 μl) contained 10 mM Tris-HCl buffer, pH 8.8, 50 mM KCl, 0.5% Tween-20, 5% formamide, 200 μM of each dNTP, 1.7 mM $MgCl_2$, 167 nM of each primer, 1 U of Taq DNA polymerase, and 10 ng of plasmid DNA. The mixture was subjected to 30 cycles of amplification (40 sec at 95°C, 40 sec at 60°C, and 1 min at 72°C). After digestion with *Nco*I and *Xho*I restrictases, PCR product was cloned into *Nco*I and *Xho*I sites of the pET28a(+) vector producing the recombinant plasmid pST that encodes a fragment of human GM3 synthase corresponding to amino acids 191-245 and containing a C-terminal 6×His affinity purification tag. *Escherichia coli* BL21(DE3) competent cells were transformed with the recombinant plasmid using a standard protocol [11]. For production of fusion protein, the cells harboring the plasmid pST were grown at 37°C until the optical density at 600 nm reached 0.8. Then to induce expression isopropyl-1-thio-β-D-galactoside was added to give a final concentration of 1 mM and the culture was incubated for an additional period of 3 h. The cells were harvested by centrifugation, washed with 20 mM Tris-HCl buffer (pH 7.5), containing 200 mM NaCl, and resuspended in 35 ml buffer A (20 mM Tris-HCl buffer, pH 7.8, 1 mM phenylmethylsulfonyl fluoride, 0.1% (w/v) Nonidet P-40, and 200 mM NaCl). After sonication (4 × 30 sec) with an MSE sonicator (England), the pellet containing the recombinant protein in the form of insoluble inclusion bodies was collected by centrifugation at 20,000*g* for 20 min, washed with 30 ml buffer A, and dissolved in 35 ml buffer B (20 mM Tris-HCl buffer, pH 7.9, 8 M urea, 0.5 M NaCl, and 5 mM imidazole). The recombinant protein was purified on Ni-NTA resin (Qiagen, USA) according to the manufacturer's instructions. The purified protein was recovered in 20 mM Tris-HCl buffer, pH 7.9, containing 8 M urea, 0.5 M NaCl, and 0.5 M imidazole at 2.35 mg/ml. The recombinant protein was analyzed at different steps of isolation and purification procedure by 15% SDS-PAGE according to the procedure of Laemmli [12].

GM3 synthase antibody production. An antibody to GM3 synthase was raised in rabbits against hGM3 synthase(m,191-245,e)6×h fusion protein (100 μg per 1 kg animal weight). The protocol included one preimmune 30-ml bleed. The rabbits were initially injected with antigen preparation in complete Freund's adjuvant and were subsequently boosted with antigen preparation in incomplete Freund's adjuvant every 3 weeks. For antibody isolation, blood (20-30 ml) was taken starting with the third antigen injection. The harvested serum was purified by $(NH_4)_2SO_4$ precipitation (to salt concentration 60%) with subsequent ion-exchange chromatography on DEAE-52 Servacel (Reanal, Hungary). Serum protein was applied on the column in 40 mM sodium phosphate buffer, pH 7.4. The antibody eluted in the volume of proteins not bound to the carrier. The antibody preparation was dialyzed against 20 mM sodium phosphate buffer, pH 7.4, and then portions were affinity purified on CNBr-activated-Sepharose 4B (Pharmacia, Sweden) coupled with the recombinant protein. The affinity-purified antibody was tested by ELISA on modified microtiter plates (Biomedical, Russia) as follows. The microtiter wells were coated with the recombinant protein as an antigen in 40 mM sodium phosphate buffer, pH 7.4. As a diagnostic antibody, anti-rabbit HRP-coupled second antibody was used at dilution 1 : 5000. Staining was performed in 0.15 M citrate-phosphate buffer, pH 5.4, containing 2.5 mM *o*-phenylenediamine and 0.006% hydrogen peroxide. The reaction was stopped by addition of 2 M sulfuric acid. Optical density of solution in microtiter wells was measured at 492 nm. The optical density produced by 1 mg protein of serum or antibody preparation was taken as a measure of antibody activity. The activity of the purified antibody was 1000-fold higher than that of the initial serum.

Preparation of Golgi-enriched microsomal fractions of human tissues and HL-60 cells. Aortic, brain, or liver tissue from 25-65-year-old men and women was obtained aseptically within 12 h after death due to an accident, frozen in liquid nitrogen, and stored at −70°C until use. HL-60 cells (from the SPBIC collection) were cultivated in RPMI-1640 supplemented with 10% fetal calf serum, glutamine, and penicillin-streptomycin. Cell density was monitored and retained not over $0.5 \cdot 10^6$ cells/ml. For

PMA (phorbol myristate acetate) treatment, HL-60 cells were collected, washed once in fresh culture medium, and resuspended in the growth medium at $0.3 \cdot 10^6$ cells/ml density. After stimulation with 10 ng/ml PMA for 48 h, the cells were used for the investigation.

Golgi-enriched microsomal fractions of tissues and HL-60 cells were prepared as follows. Tissue pieces (aortic intima, brain, liver) were ground to powder in liquid nitrogen and then suspended in 0.32 M sucrose (1 g tissue in 20 ml). The tissue or HL-60 cells suspension was homogenized with a Potter homogenizer (Teflon–glass) and centrifuged at 1000*g* for 5 min. The supernatant was separated from the pellet and centrifuged at 27,000*g* for 10 min. The resulting pellet was resuspended in 3 volumes of 0.32 M sucrose. This suspension was layered on top of 1.3 M sucrose and centrifuged for 60 min at 100,000*g*. Interface material was collected, diluted with distilled water, and centrifuged at 27,000*g* for 30 min. The pellet was resuspended in minimal volume of distilled water. Golgi-enriched microsomal fractions were stored at –70°C.

Protein was assayed by a modification of the procedure of Lowry [13] using BSA as a standard.

GM3 synthase activity assay. Sialyltransferase activity was assayed according to the modified procedure [14]. All experiments were carried out in duplicate. Assay mixtures (final volume 50 μl) contained 0.2 mM lactosylceramide, 0.4 mM CMP-[^{14}C]NeuAc (total activity 3.2 mCi), 0.75% Triton X-100, 1 mM $MnCl_2$, 150 mM cacodylate buffer, pH 6.2, and the microsomal fraction (80-350 μg protein). Blanks were obtained without lactosylceramide. After incubation of the mixtures at 37°C for 1.5 h, the reaction was stopped by addition of 0.5 ml 0.05 M acetate buffer, pH 4.4. Then the mixtures were put onto 5 × 10 mm Sep-Pak C_{18} cartridges (Waters, USA). Free CMP-NeuAc and other soluble in water components of the reaction mixture were removed by washing the column subsequently with 1 ml 0.05 M acetate buffer, pH 4.4, 6 ml water, 4 ml mixture of methanol–water (1 : 4). Gangliosides were eluted with 1 ml methanol and 4 ml mixtures of chloroform–methanol (1 : 2 and 2 : 1). The combined eluates with added GM3 as an internal standard, were dried and separated on HPTLC silica gel plates (Merck, Germany) using solvent system chloroform–methanol–0.25% $CaCl_2$ (50 : 40 : 10 v/v). The plates were visualized with resorcinol reagent. Zones corresponding to chromatographic mobility of authentic GM3 were scraped into scintillation vials. Radioactivity was measured on liquid scintillation counter 1215 Rackbeta II (LKB, Sweden). In parallel with this, one replicate chromatogram of each experiment was exposed at –70°C for 7-14 days to an X-ray Retina film (Fotochemische Werke GmbH, Germany). Sialyltransferase activity was calculated as pmol NeuAc transferred to lactosylceramide in 1 h by 1 mg protein of tissue microsomal fraction.

Immunoblotting of human tissues. Golgi-enriched microsomal fractions of human liver, brain, atherosclerotic aortic intima, and HL-60 cells (10-40 μg protein) were separated by 10% SDS-PAGE according to the method of Laemmli [12] at 120 V and transferred to nitrocellulose membrane at 350 mA for 1 h. Nitrocellulose blots were blocked in 5% low-fat milk on TBS-T buffer (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.2% Tween-20) for 1 h. Membrane strips were washed in TBS-T buffer and incubated with affinity purified anti-human GM3 synthase antibody (dilution 1 : 10) in TBS-T buffer, containing 1% low-fat milk, for 1.5 h. Then the strips were washed in TBS-T buffer and incubated for 1 h with HRP (horse-rabbit peroxidase)-coupled anti-rabbit secondary antibody (dilution 1 : 10,000). Antibody binding was detected by non-radioactive ECL reagent (Amersham Pharmacia Biotech, England) according to the manufacturer's recommendations.

RESULTS

The choice of human GM3 synthase fragment for cloning and its subsequent use as an antigen was guided by the data of Stern et al. [15]. A multiple alignment of flanked by sialyl motifs amino acid sequences of 39 human sialyltransferases available from GenBank was constructed using BioEdit software [16]. As a result, amino acid sequence 191-245 of GM3 synthase sharing short identical amino acid stretches (actually consisting of no more than three amino acids) with as few other sialyltransferase peptide sequences as possible was selected for cloning. The fragment of GM3 synthase cDNA was prepared by PCR and subcloned into *Nco*I and *Xho*I sites of pET28-a(+) vector. The produced recombinant plasmid pST that encodes the fragment of human GM3 synthase corresponding to amino acids 191-245 and containing a C-terminal 6×His affinity purification tag was used for transformation of *E. coli* BL21(DE3). The 63-amino acid recombinant protein (hGM3 synthase(m,191-245,e)6×h) was homogeneous on SDS-PAGE, and its mobility agreed with the predicted value.

The antibody to GM3 synthase was raised in rabbits by injections with the recombinant protein. The antibody activity was determined by ELISA using the recombinant protein as an antigen. Consecutive purification of immune sera by chromatography on DEAE-cellulose and by affinity chromatography on Sepharose coupled with the recombinant protein increased antibody activity 1000-fold in comparison with the initial sera. Affinity purified antibody was designated as R27C1. Rabbit preimmune sera did not react with the recombinant protein.

The R27C1 antibody inhibited GM3 synthase activity in human liver and HL-60 cells (Fig. 1). The inhibition was dose-dependent on antibody concentration. At

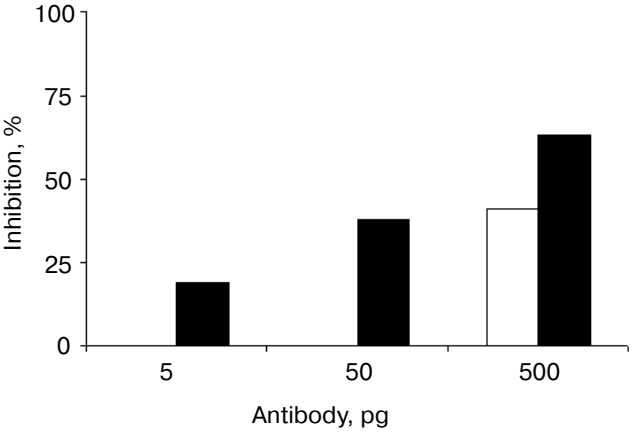

Fig. 1. Inhibition of GM3 synthase activity by the R27C1 antibody. Golgi-enriched microsomal fractions of stimulated HL-60 cells (80-105 μg protein, light column) or human liver (134-350 μg protein, dark column) were preincubated with the R27C1 antibody (5-500 pg protein) for 30 min. GM3 synthase activity was assayed as described in "Materials and Methods".

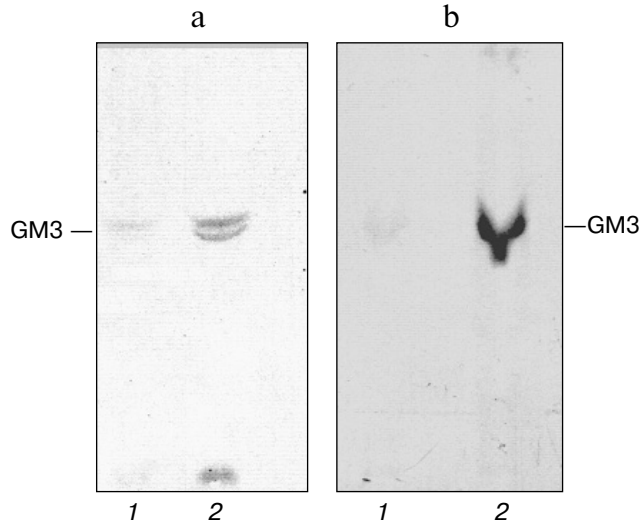

Fig. 2. Autoradiography of total glycolipids isolated from the reaction mixture after GM3 synthase reaction in human liver (a) and HL-60 cells (b). The reaction products were separated on HPTLC silica gel plates using chloroform–methanol–0.25% $CaCl_2$ (50 : 40 : 10 v/v) solvent system. Cold GM3 was added as an internal standard: *1*) without lactosylceramide; *2*) with lactosylceramide. On the right and left, the position of GM3 visualized by resorcinol reagent.

the same time, the R27C1 antibody did not affect liver sialyltransferase activity on asialofetuin (data not shown).

GM3 synthase activity was 194 ± 60 (n = 4) and 1353 ± 573 (n = 4) pmol NeuAc/h per mg protein in human liver and PMA-stimulated HL-60 cells, respectively. As shown in Fig. 2, radioactive products other than GM3 were not found in the reaction mixtures. Formation of GM3 in the absence of lactosylceramide was low (Fig. 2, lanes *1*).

The results on interaction of the R27C1 antibody with human tissues (liver, atherosclerotic intima, brain, HL-60 cells) assessed by immunoblotting are shown in Fig. 3. The R27C1 antibody recognized a common protein band with molecular mass about 60 kD in all tissues studied. The 60-kD band was nearly a single band in human liver and atherosclerotic intima. In human brain, the R27C1 antibody revealed additionally three bands with molecular masses of 55, 50, and 20 kD comparable to the 60-kD band in intensity. In contrast to other tissues, numerous bands with molecular masses above or below 60 kD (82, 55, 50, 33, 28, 25, 20 kD) were revealed in HL-60 cells. A weak 76-kD band could be detected in all tissues studied.

DISCUSSION

Using GM3 synthase cDNA of human leukemia cell line HL-60 [17], we cloned a gene fragment corresponding to amino acids 191-245 of the enzyme. The produced recombinant protein was used to raise polyclonal antibody to enable the immunological detection of GM3 synthase in human tissues.

About 20 sialyltransferases catalyzing the attachment of NeuAc to carbohydrate chains of proteins and lipids are present in Golgi apparatus of eucaryotic cells. Although highly specific to carbohydrate substrate, these enzymes have two conserved regions called L and S sialyl motifs consisting of 45 and 23 amino acids, respectively [18, 19]. To produce antibody capable of recognizing GM3 synthase among other sialyltransferases, it was necessary to select the most specific region of its peptide sequence. As reported earlier by Stern et al. [15], a 46-amino acid-long fragment of intervening peptide sequence flanked by L and S sialyl motifs was successfully used to raise antibodies against mouse embryonic brain GM3 synthase. We performed a multiple alignment of flanked by L and S sialyl motifs amino acid sequences for 39 sialyltransferases cloned from various human tissues and found a sequence specific for GM3 synthase. The recombinant protein corresponding to this sequence proved to be sufficiently immunogenic to raise an antibody in rabbits. The 1000-fold affinity purified R27C1 antibody specifically inhibited GM3 synthase activity in human liver and HL-60 cells. It should be noted that to date there are no data on GM3 synthase activity in human tissues, most likely because of the low tissue levels of the enzyme. The main object for study of human GM3 synthase activity has been continuous cell cultures such as fibroblasts [20], HL-60 cells [2], and some others. In the present work, GM3 synthase activity was determined in human liver for the first time, with GM3 synthase activi-

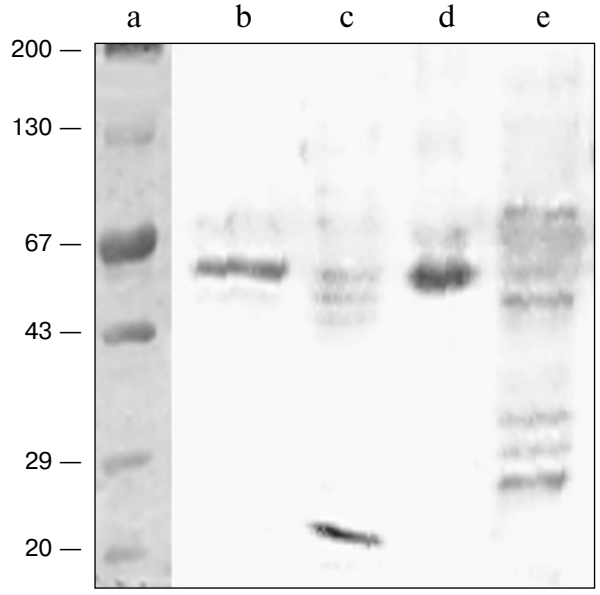

Fig. 3. Immunoblotting of human tissues with the R27C1 antibody. Golgi-enriched microsomal fractions of human tissues (30 μg protein) were separated by 10% SDS-PAGE and transferred to nitrocellulose membrane. The R27C1 antibody recognized a common band with molecular mass of about 60 kD in all tissues studied. a) Protein markers stained by Ponceau S; on the left, their molecular masses in kD; b) liver; c) brain; d) atherosclerotic aortic intima; e) HL-60 cells.

ty in human liver being one order lower than in stimulated HL-60 cells. We showed that in the presence of lactosylceramide as a substrate the only reaction product, GM3, is formed in human liver and HL-60 cells. Thus inhibition of CMP-[^{14}C]NeuAc incorporation by the R27C1 antibody provides evidence for antibody specificity to the GM3 synthase molecule.

On immunoblotting, almost a single band with molecular mass of 60 kD was recognized by the R27C1 antibody in human liver and atherosclerotic aortic intima. This band is present also in human brain and HL-60 cells. A very weak band with molecular mass of about 76 kD could be detected in all tissues studied. In human brain the R27C1 antibody revealed three additional bands: a band with low molecular mass (20 kD) was very intense. In HL-60 cells, the R27C1 antibody also revealed bands with low molecular masses (33, 28, 25, 20 kD). The molecular mass of GM3 synthase is unknown. There are data for GM3 synthase isolated from rat brain (76 kD) [21] and rat liver (60 kD) [9]. In a more recent paper [21], the authors explain this discrepancy either by tissue specificity of protein post-translational modification or partial proteolysis of the protein during isolation and purification. Sialyltransferases are known to be highly glycosylated. Sometimes shares of glycosyl residues and protein part in molecular mass of mature sialyltransferase molecule are equal [18]. Thus, immature forms can be present in the tissues or glycosylation degree may depend on tissue type of the same species. Another property of sialyltransferases that should be kept in mind is cleavage of the catalytic domain of the enzyme by proteolysis with formation of soluble form with lower molecular mass [22]. Kapitonov et al. [23] showed multiple alternatively spliced forms of GM3 synthase that may be characteristic for cells of tumor origin. All the above-mentioned may be responsible for multiple bands for GM3 synthase revealed in human brain and HL-60 cells by immunoblotting. Further valuable information about molecular mass of GM3 synthase was obtained by using the CS2 antibody to the fragment of mouse GM3 synthase. This antibody recognized only a protein of 45 kD in mouse brain, liver, and testes [15]. As reported by the authors [15], cDNA of mouse brain GM3 synthase is identical to cDNA of HL-60 cells encoding a protein with a predicted molecular mass of 41.7 kD [17].

The values for molecular masses of GM3 synthase of human liver and aortic intima obtained in the present work correspond closely with the values for GM3 synthase from rat liver [9]. It is significant that the R27C1 antibody recognizes almost a single band in these tissues and hence may be used for immunohistochemical studies of GM3 synthase in aortic sections.

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