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Identification and characterization of an anti-tyrosine kinase factor in cystic gliomas

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In view of the frequent activation of the epidermal growth factor receptor (EGF-R) in gliomas and autocrine hypothesis, we searched for 'EGF-like' factor(s) in cystic fluids (CFs) associated with gliomas. Membranes of A431 cells, which overexpress EGF-R, were used to explore such activity in 20 CFs. In all cases CFs induced inhibition of EGF-R phosphorylation. Biochemical analysis revealed an anti-tyrosine kinase activity which was identified as a 18 kDa proteic factor. Effectiveness at high dilution and anti-proliferative effect on living cells in culture suggest that this factor may be involved in the negative regulation of glial oncogenesis.

EGF-R; Gliomas; Cystic fluid; Proto-oncogene; Tyrosine kinase; Inhibitory effect

1. INTRODUCTION

Brain tumors of which 60% are gliomas, account for almost 8% of human malignancies. These tumors are characterized by their poor and almost always fatal prognosis [1]. During the last decade, the new concept of oncogenes, and more recently of anti-oncogenes, has brought new perspectives for a better understanding of the mechanisms of the disease and therefore for new therapeutic weapons [2].

The proto-oncogene c-erbB encoding the epidermal growth factor receptor (EGF-R), appears to be involved in at least 40% of gliomas [3–7]. EGF-R is a 170 kDa membrane glycoprotein which contains 3 domains including an intracellular one with tyrosine kinase activity. EGF and TGF-α are the principle related polypeptide growth factors. Intrinsic tyrosine kinase activity is essential in the signal transduction of EGF, resulting in cellular proliferation (for review see [8 and 9]).

The interest in cystic fluid (CF) came from its privileged and direct relationship with the tumor, the biology of which could be specifically reflected in the fluid formed from their tissues. In view of the frequent activation of EGF-R in gliomas and autocrine hypothesis [10], we searched for 'EGF-like' factor(s) in CF.

Contrary to the expected result, we observed in CFs an inhibitory activity of EGF-R autophosphorylation. Identification and characterization of the related factor are reported.

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2. MATERIALS AND METHODS

2.1. *Cystic fluids (CFs)*

CFs were obtained from patients admitted to the Neurosurgery department of the University Hospital of Grenoble. Most of them were obtained by aspiration during stereotaxic investigation for biopsy sampling. Each sample was immediately frozen in liquid nitrogen. Neuropathological diagnosis of the associated tumor was assessed in paraffin-embedded tissues obtained by stereotaxic biopsy. 20 CFs were studied: 10 associated with grade I–II, 7 with grade III and 3 with grade IV astrocytomas.

2.2. *EGF receptor phosphorylation assay*

EGF-R phosphorylation was analyzed using membranes from A431 cells which are known to overexpress this receptor [11]. After purification A431 membranes were homogenized in buffer containing 20 mM HEPES (pH 7.4), 1.5 mM $MgCl_2$, 1 mM EGTA, 10 μg/ml leupeptine, 1 mM PMSF and 1% Triton X-100. Phosphorylation was performed with 15 mM $MgCl_2$ and 20 μg/ml leupeptine in the presence or absence of EGF (2 μM) and CF for 10 min at 4°C. Self-phosphorylation was allowed to proceed for 10 min at 4°C with 10 mM $MgCl_2$ and 2 μCi [γ-^{32}P]ATP. The phosphorylation reaction was stopped by adding 30 μl of Laemmli buffer [12]. The effects of heat denaturation, trypsin digestion, influence of vanadate (inhibitor of tyrosine phosphatase) and previous immunoabsorption with an antibody against the extracellular domain of EGF-R (Amersham) were analyzed. Samples were electrophoresed on 7.5% SDS-PAGE, dried and exposed to AGFA XRP1 film. EGF-R phosphorylation was quantified by Cerenkov effect on the radioactive band detected at 170 kDa by autoradiography.

2.3. *Phosphoamino acid analysis*

EGF-R phosphorylation was performed as described above in the absence of EGF, in the presence of EGF (2 μM) and in the presence of EGF and CF. The gel fragment containing phosphorylated EGF-R was cut out, washed overnight in 20% methanol at 37°C and incubated with 100 μg/ml of trypsin for 24 h. Eluted peptides were hydrolysed 2 h in 6 N HCl at 110°C. Resultant phosphoamino acids were electrophoresed on a thin-layer cellulose plate and subjected to autoradiography. Unlabeled standards added to the phosphoamino acids were visualized with ninhydrin spray.

2.4. *In vivo phosphorylation assay*

The biochemical effect obtained on A431 cells membranes was analyzed in whole cells in culture on A 431 cell line, a human vulva carcinoma cell line, and in the GHD cell line, a human glioma cell line, in which EGF-R mRNA is hyperexpressed and the related protein hyperphosphorylated. Cells were cultured in 50% DMEM and 50% F12 supplemented with 10% fetal calf serum or 10% CF. In vivo proteic patterns were analyzed by the incorporation of [^{32}P]orthophosphate in the presence or absence of CF. Labelling was performed for 12 h. After two washes, cells were homogenized in buffer containing 9.5 M urea, 2% Nonidet 40 (v/v), 5% β-mercaptoethanol (v/v), 2% Ampholines 3.5–9.5 (Pharmacia) (v/v). Samples were then subjected to bidimensional electrophoresis.

2.5. *Bidimensional electrophoresis*

Bidimensional electrophoresis was performed according to O'Farrel [12] with minor modifications using Ampholites 3.5–9.5 (Pharmacia). After equilibration in Laemmli buffer [13] the second SDS-PAGE dimension was performed in a 7.5% acrylamide gel using a mini-gel-apparatus (Bio-Rad).

2 6. *Chromatography*

Crude cystic fluid was separated on Superose 12 into 8 fractions corresponding to proteic peaks detected by absorbance at 280 nm. An aliquot (50 μl) of each fraction was then analyzed for its inhibitory effect on EGF-R phosphorylation without concentration.

2.7 *Protein purification by preparative protein blotting*

Cystic fluid were absorbed on a Hitrap blue (Pharmacia) column to eliminate albumin and apply more protein on the polyacrylamide gel. Resultant proteins were separated by SDS-PAGE on 15% (w/v) polyacrylamide gels. After running, proteins were transferred to Immobilon P (Millipore) in a transfer buffer of 20 mM Tris, 150 mM glycine, and 20% (v/v) methanol. Protein bands immobilized on Immobilon were visualised by Ponceau red staining. Then blot slices were made and eluted by incubation for 3 h in 40% acetonitril and subsequently centrifuged and lyophilized to remove the volatile solvent.

2.8. *The effects of purified factor on cells in culture*

The effects of the protein blotting purified factor were studied on GHD cells. Proliferation was quantified with the MTT test which is a colorimetric proliferation assay [14] (Sigma) for 12 h. A control using albumin purified identically was assessed.

3. RESULTS

In A431 cell membranes, EGF induces autophosphorylation of its receptor which is reflected in SDS-PAGE by an increase in the phosphorylation detected at 170 kDa. Unexpectedly, all CFs decreased phosphorylation detected at 170 kDa (Fig. 1A). Basal as well as EGF induced autophosphorylation of the receptor were inhibited. We quantified this inhibitory effect and compared the inhibitory effect observed in benign tumors (low grade astrocytomas: grade I and II) with that observed in malignant tumors (high grade astrocytomas: grade III and IV). The mean inhibition for basal phosphorylation of EGF-R was 90% in grade I–II and 50% for Grade III–IV (Significantly different $P < 0.018$). Previous treatment with trypsin and heat denaturation abolished the observed effect of CF on EGF-R autophosphorylation (data not shown). Vanadate as well as previous absorption with an anti-EGF-R antibody were without effect (data not shown).

In some of the A431 cell membrane samples analyzed, two molecular forms of EGF-R are observed at 170 and 150 kDa. Previous studies have demonstrated that the 150 kDa form is a proteolytic product of the former [15] via a calcium-dependent protease. The 150 kDa proteolytic product is known to be phosphorylated

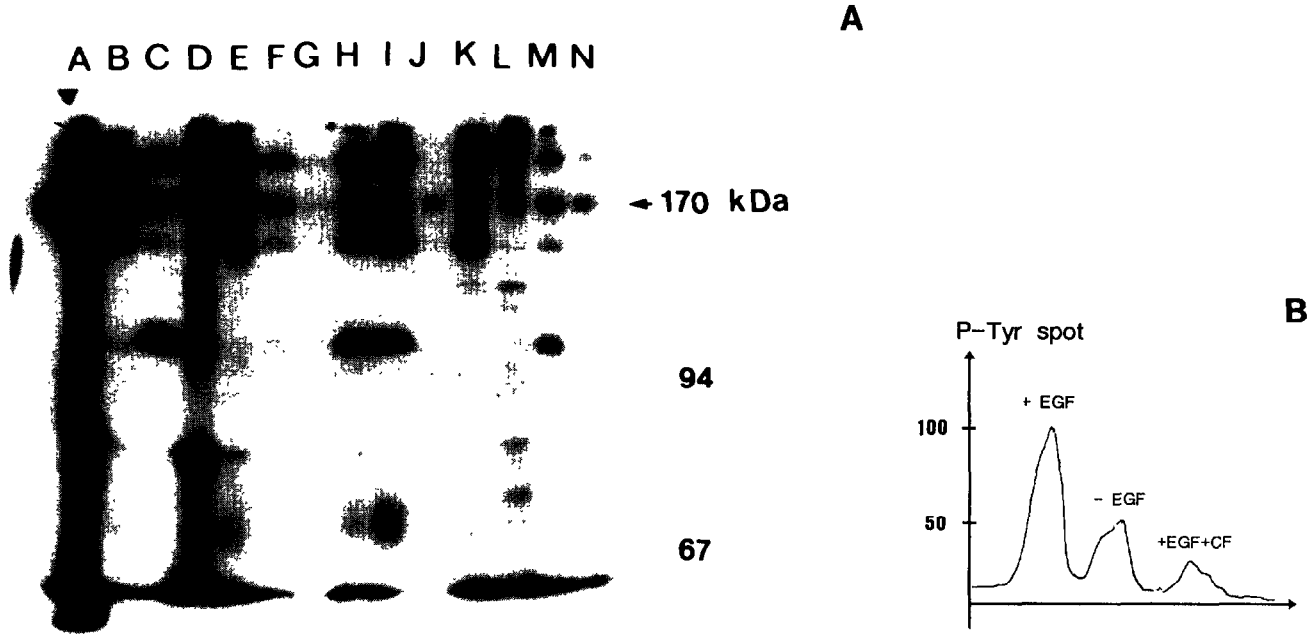

Fig. 1. A. Inhibition of EGF-R phosphorylation in A431 membranes. Lane A (control), A431 membranes with EGF; lanes B to N, A431 membranes with EGF and CF associated with low grade astrocytomas in F, G, J, L, N and with high grade astrocytoma in B, C, D, E, H, I, K, M. Phosphorylation at 170 kDa is inhibited (arrow). B. Phosphotyrosine phosphorylation at 170 kDa in A 431 membrane with (+ EGF), without (– EGF) and in the presence of EGF and Cystic fluid (+ EGF + CF).

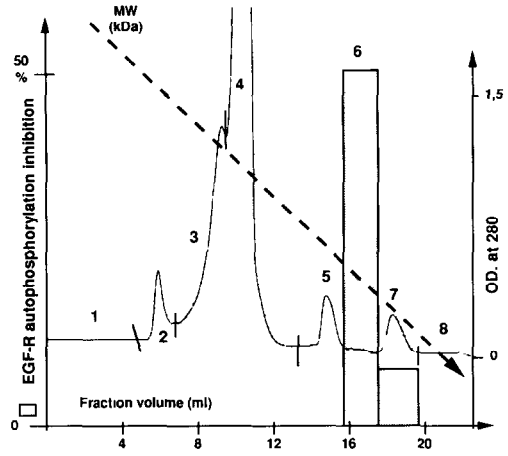

Fig. 2. Analysis of the inhibitory effect of EGF-R phosphorylation in A 431 membranes in the presence of 8 fractions obtained after HPLC separation of crude CF on Superose 12. O.D. at 280 nm are indicated on the right, inhibition of EGF-R phosphorylation on the left, fractions volumes on the X axis.

but lacks phosphotyrosine residues. When the CF fluid inhibitory effect was analysed by bidimensional electrophoresis, only the 170 kDa form was inhibited (data not shown). Phosphoamino acid analysis was performed to investigate which phosphorylated residue was inhibited. Addition of CF to the A431 membrane phosphorylated with EGF decreased the phosphotyrosine spot fourfold (Fig. 1B). Experiments were done on A431 and GHD cell lines in culture. A phosphorylation assay on whole cells in vitro demonstrated an inhibition of EGF-R phosphorylation with CF when compared to fetal calf serum, as was also observed on A431 isolated membranes.

Eight fractions, eluted from a Superose 12 column (Fig. 2), were tested. An aliquot of the unconcentrated fraction was tested for its inhibitory activity on EGF-R phosphorylation. Dilution of the CF after HPLC separation was 1/150. A peak of inhibitory activity compared with control in the absence of CF was identified in fraction 6 corresponding to a molecular weight of 20–30 kDa. Inhibition of basal phosphorylation in the absence of EGF was 50%; in the presence of EGF inhibition was quantified as 75%. We also detected, in fraction 4 and 8 an 'EGF-like' activity. Fraction 1 corresponds to albumine one and fraction 8 is the fraction below 10 kDa. Purification was completed by preparative protein blotting on 15% acrylamide gels according to the MW range identified by HPLC. Albumin, the main proteic component of CF was absorbed on a Hitrap blue column to avoid electrophoretic streaking caused by to much protein. Proteic bands identified with Ponceau red were eluted and tested for their biological effect on EGF-R phosphorylation. An inhib-

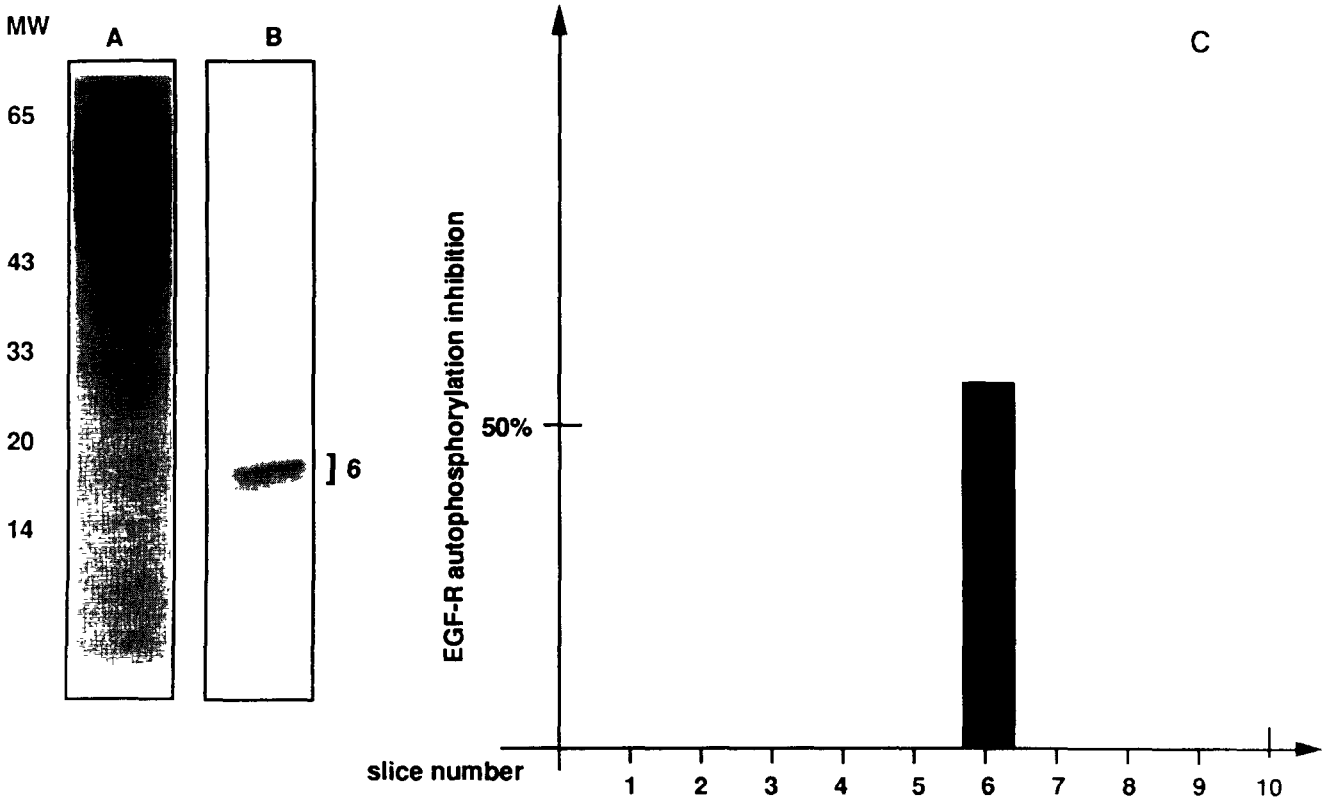

Fig. 3. Electro-elution of one CF after SDS-PAGE on 15% acrylamide gel. 10 slices were tested for the inhibitory effect. A. Crude CF. B. Silver staining of the inhibitory band detected at 18 kDa, purified after absorption of CF on blue Hitrap column and protein blotting (0.5 μg) on slice 6. C. Inhibitory effect of EGF-R in A 431 membranes with each electro-eluate expressed as a percentage of the control without eluted protein.

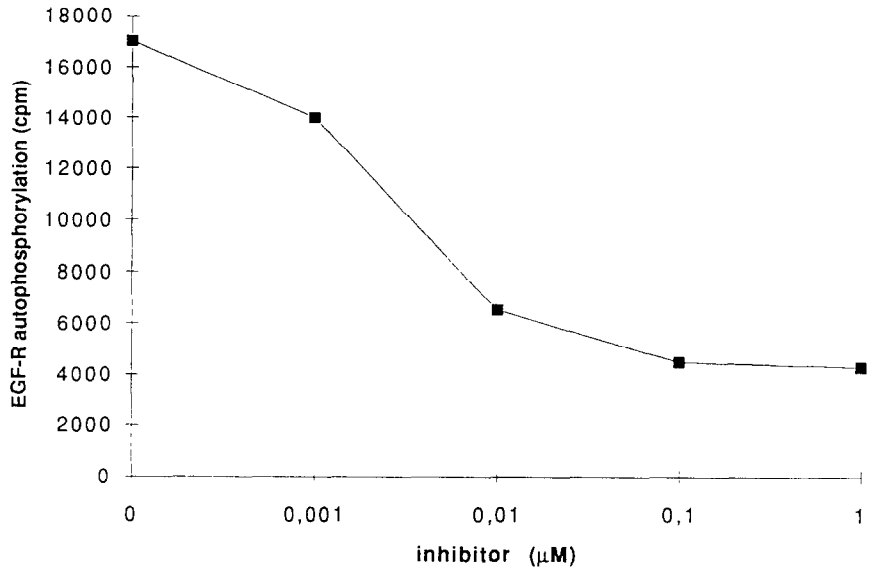

Fig. 4. Inhibition of EGF-R basal autophosphorylation in A431 membranes in the presence of different concentrations of inhibitor.

itory activity was identified at 18 kDa (Fig. 3). A dose effect in inhibition was evaluated from 80 to 20% in the concentration range from 1 μM to 0.001 μM (Fig. 4). The purified factor was also tested in vivo on GHD cells. Proliferation was analyzed with the MTT test. Inhibition was observed from 100 μM to 0.1 μM in the range 90% to 30% (Fig. 5). Albumin did not induce any inhibition of proliferation.

4. DISCUSSION

Many transforming growth factors have been identified in malignant effusions. Stimulation of the growth of cultured gliomas with cystic fluid has been reported [16] and 'insulin growth factor like' activity identified in CFs [17]. The A431 cell line provides a good system for the identification of an 'EGF-like' activity. In view of such an autocrine growth regulation we tested A431 cell membranes with CFs. Paradoxically we found an inhibitory activity of EGF-R autophosphorylation. CFs induced an inhibitory effect on both basal and EGF-induced EGF-R autophosphorylation in A431 cell membranes. This effect was observed in the 20 CFs analyzed. What is the nature of this inhibitory activity? Absence of the inhibition of phosphorylation of the 150 kDa proteolytic product of the EGF-R argues for the specificity of this inhibitory effect. The effect on basal EGF-R autophosphorylation suggests the absence of competition or sequestration of EGF by any CF component. This point is important to discuss because A431 cells are

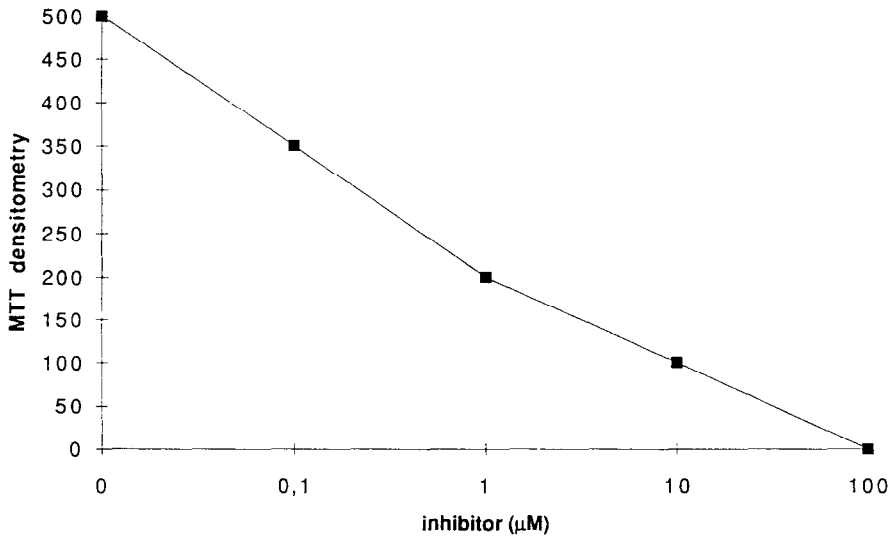

Fig. 5. GHD proliferation in the presence of different concentrations of inhibitor after a 12-h incubation. Proliferation is quantified by the MTT test.

known to secrete truncated forms of the EGF-R with EGF binding activity. Moreover an EGF binding activity has been identified in brain tissues [18]. The absence of the modification of CF inhibitory effect by previous immunoabsorption with an anti-EGF-R antibody supports the absence of such an inhibitory factor. Phosphoamino acid analysis as well as the absence of inhibition of the 150 kDa proteolytic form indicate that the inhibitory effect acts through phosphotyrosine residues. This could explain an effect on basal phosphorylation. Vanadate did not influence the inhibitory effect which suggests that the related factor is not a tyrosine phosphatase. Tryptic and heat denaturation suggest that our anti-tyrosine kinase factor is of a proteic nature. Characterization by HPLC and preparative protein blotting purification identified a 18 kDa MW factor. It is interesting to observe that separation of the different components of crude CF by HPLC individualizes 'EGF-like' activities which were blotted out by the strong inhibitory activity in crude CF. To our knowledge Müllerian inhibiting substance (MIS) is the only known physiological factor isolated in vivo with an anti-tyrosine kinase activity. It is responsible for the regression of Müller ducts in males during embryogenesis. This is probably the only known situation where a substance inhibiting a proto-oncogene function generates tissular regression. MIS has been shown to inhibit tumoral proliferation in vitro, especially growth of the A431 cell line [19]. Synthetic substances with anti-tyrosine kinase function have been isolated and have recently been tested in vitro and in vivo with success as an anti-neoplastic agent [20]. However the molecular weight of MIS is different from that of the factor identified in this study suggesting that it may be a new anti-tyrosine kinase factor. Further purification and microsequencing procedures are necessary to clarify these issues. Persistance of this inhibitory effect at high dilutions shows its effectiveness and suggests its biological role. Moreover, the strongest effect is observed with non-diluted samples of CF which represents the pathological 'in vivo' situation. Its persistence on whole cells in culture argues for possible in vivo involvement. The finding of a more important inhibitory effect in low grade astrocytoma compared to the more malignant ones suggests that this factor could be involved in the tumoral progression of astrocytoma. Furthermore, EGF-responsive stem cells have recently been isolated from adult mammalian central nervous systems [21] which focused on the regulatory factors involved in the local inhibition of these cells. The factor identified in our study may be a candidate for inhibition of local proliferation of these cells in the non-tumoral adult central nervous system.

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