Cyclic AMP-independent secretion of mucin by SW1116 human colon carcinoma cells

Differential control by Ca^{2+} ionophore A23187 and arachidonic acid

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The regulation of mucin secretion by SW1116 human colon carcinoma cells has been studied using monoclonal antibody 19-9, which has previously been used to detect mucin in the serum of cancer and cystic fibrosis patients. We found that SW1116 cells constitutively secrete considerable amounts of mucin as the predominant glycoprotein. The secretion of mucin by these cells is independent of cyclic AMP levels, but can be further stimulated by the Ca^{2+} ionophore A23187. However, arachidonic acid and its metabolites inhibit mucin secretion. Electron microscope studies reveal that the mucin is located near the plasma membrane as well as in vesicular and lysosome-like structures. However, the secretion pathway of mucin is different than that of the lysosomal contents, since arachidonic acid, while inhibiting mucin secretion, actually activates the secretion of the lysosomal enzyme β -glucuronidase. We suggest that the mechanism of mucin secretion by SW1116 cells occurs by a pathway different from common exocytosis, and possibly by more than one pathway. The response of mucin secretion by SW1116 cells to common secretagogues resembles that of epithelial cells obtained from cystic fibrosis patients. Thus SW1116 cells are an especially interesting system for studying processes related to pathological states associated with excessive constitutive secretion of mucin.

INTRODUCTION

Mucin secretion by epithelial cells is considered to be a major component of the pathophysiology of a number of disease entities, including colon carcinoma, pancreatic insufficiency, chronic pulmonary obstruction and cystic fibrosis (CF) (Boat *et al.*, 1988). One drawback in the study of mucin secretion has been a paucity of defined cellular systems, since epithelial and mucosal tissues comprise diverse cell types which may differ in their secretory processes and in their responses to different effectors. In recent years several cell systems have been employed for studying mucin secretion and its regulation. These are mainly primary cultures of hamster tracheal epithelial cells (Kim *et al.*, 1987) and the human colon carcinoma cell lines HT-29 (Phillips *et al.*, 1988), T-84 (McCool *et al.*, 1990) and SW1116 (Yedgar *et al.*, 1989).

The main thrust of these studies has been the demonstration that mucin secretion is responsive to various secretagogues. Kim *et al.* (1987), using hamster tracheal cells, have suggested two mechanisms for mucin release: constitutive and regulated secretory processes. Phillips *et al.* (1988) have shown that cholinergic stimulation in HT-29 cells induced depletion of intracellular mucin granules. McCool *et al.* (1990) have shown that mucin secretion in T-84 cells is responsive to secretagogues such as carbachol, Ca^{2+} ionophore A23187, prostaglandin E_1 (PGE_1), phorbol 12-myristate and 13-acetate (PMA) and vaso-active intestinal polypeptide.

Of special interest to us has been the human colon carcinoma cell line SW1116, since these cells secrete mucin as the major glycoprotein. The mucin secreted by these cells is detected by the

antibody 19-9, which has been used previously in our laboratory to detect the aberrantly elevated mucin levels in the serum of cancer and CF patients (Magnani *et al.*, 1983; Roberts *et al.*, 1990).

We have found that these cells constitutively secrete large amounts of mucin. We estimate that SW1116 cells secrete about 10-fold greater amounts of mucin than similarly treated cultures of T-84 or HT-29 cells. Mucin secretion by SW1116 cells does not respond to cyclic AMP mediated stimuli, but can be stimulated by the Ca^{2+} ionophore A23187, while arachidonic acid and its metabolites inhibit the secretion.

EXPERIMENTAL

Materials

The following materials were purchased as indicated: SW1116 cells from American Type Tissue Culture Collection (Rockville, MD, U.S.A.); L-15 medium from Biofluids (Rockville, MD, U.S.A.); Nu Serum IV from Collaborative Research (Waltham, MA, U.S.A.); monoclonal antibody 19-9 from Centocor (Malvern, PA, U.S.A.); arachidonic acid, leukotriene C_4 (LTC_4) LTD_4 , PGE_1 , PGE_2 and 5-hydroxyicosatetraenoic acid (5-HETE) from Calbiochem. (La Jolla, CA, U.S.A.); sheep anti-(mouse Ig)-linked β -galactosidase from Amersham; Ca^{2+} ionophore A23187, 4-methylumbelliferyl (4MU) glucuronide, forskolin, muscarine, dibutyl cyclic AMP, PMA and chondroitine ABC lysase (EC 4.2.2.4) from Sigma (St. Louis, MO, U.S.A.). Human salivary mucin from a Lewis-a donor and mucin from SW1116 cell culture medium were prepared as previously described (Roberts *et al.*, 1990).

Abbreviations used: LT, leukotriene; PG, prostaglandin; HETE, hydroxyicosatetraenoic acid; PKC, protein kinase C; 4MU, 4-methylumbelliferone; DMSO, dimethyl sulphoxide; IBMX, 3-isobutyl-1-methylxanthine; PMA, phorbol 12-myristate 13-acetate; CF, cystic fibrosis.

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Cell culture

SW1116 cells were cultured in L-15 medium supplemented with 20% Nu Serum IV and 20 mM-Hepes at pH 7.4, in a 1% CO₂ incubator.

Determination of mucin secretion

Mucin secretion was measured in serum-free medium collected from confluent cells in 24-well plates. Prior to the assay, the cells were washed once with serum-free L-15 medium, then with medium containing 2 mM-dithiothreitol (to remove protein adsorbed to the cell surfaces) and once again with serum-free medium. To start the assay, the last wash was aspirated from each well and replaced by 1 ml of serum-free medium, and a sample of the medium was taken for the zero time measurement. Subsequent medium samples were taken at the desired times. The whole procedure was carried out at 37 °C. The amount of mucin in the medium samples was determined by solid-phase e.l.i.s.a. (Brockhaus *et al.*, 1985) using the monoclonal antibody 19-9 as the first antibody and β -galactosidase-conjugated anti-mouse Ig, which hydrolyses 4MU galactoside, as a second antibody. The fluorescence of the 4MU produced was measured in a Dynatech e.l.i.s.a. Reader (fluorometer), mucin content was calculated by comparison with mucin standards.

All effectors were provided as a concentrated solution in dimethyl sulphoxide (DMSO), so that the amount of DMSO added to the culture was 10 μ l/ml of medium. The same amounts of DMSO alone were added to the control cells, and such amounts of DMSO were found to have no effect on mucin secretion.

Characterization of secreted glycoconjugates

To characterize the glycoconjugates secreted by SW1116 cells, the cells at confluency were double-labelled with 10 μ Ci of [³H]glucosamine/ml and 100 μ Ci of [³⁵S]sulphate/ml for 48 h. The cells were extracted with 4 M-guanidinium hydrochloride/50 mM-sodium acetate, pH 6.0, buffer containing proteinase inhibitors as previously described (Roberts, 1988). The extract was desalted on Sephadex G50 into 8 M-urea/50 mM-sodium acetate, pH 6.0, applied to DEAE-Sephacel in the same buffer, and eluted with a gradient to 1 M-NaCl in the same buffer (Roberts, 1988). More than 90% of both labels eluted before 0.4 M-NaCl, with only a small amount eluted at 0.6 M-NaCl (which is characteristic for elution of proteoglycans). Only the former material bound the antibody 19-9 by solid phase immunoassay (Roberts *et al.*, 1990). The labelled material was also analysed by density gradient centrifugation in CsCl as described (Roberts *et al.*, 1990), and sedimented at a density of 1.5 g/ml. The labelled material was further subjected to β -elimination using alkaline borohydride degradation (1 M-NaH₄B/50 mM-NaOH, 16 h, 45 °C), and released oligosaccharides were subjected to gel filtration. The results showed that the label was in low-molecular mass O-linked oligosaccharides, typical of mucin, and not in N-linked glycoproteins.

Chondroitin ABC lyase treatment

Application of chondroitin ABC lyase to the medium samples was done according to a modification of a previously described method (Oike *et al.*, 1982). The culture medium at pH 7.4 was supplemented with 10 mM-EDTA (final concentration) as well as 10 mM-N-ethylmaleimide, 5 mM-phenylmethanesulphonyl fluoride and 0.36 mM-pepstatin A (proteinase inhibitors) and 0.5% of the detergent CHAPS. The reaction was initiated by the addition of 0.5 units of chondroitin ABC lyase/ml (final concentration) and maintained for at least 3 h at 37 °C.

Measurement of cyclic AMP

The cellular cyclic AMP content was determined by an automated radioimmunoassay (Gammaflo) as previously described (Eastman & Aurbach, 1982).

RESULTS

Mucin secretion by cultured SW1116 cells

SW1116 cells are known to be slowly growing cells. We therefore first examined their growth under various conditions, and found the optimal conditions to be Hepes-buffered (20 mM) L-15 medium enriched with 20% Nu Serum IV in Falcon dishes incubated in 1% CO₂. Under these conditions the duplication time was 2–4 days, depending on the inoculum density. We found that the cells first grew in colonies until reaching a homogeneous confluent layer of differentiated cells, as determined by the appearance of microvilli and intracellular organelles.

For determination of mucin secretion rate the cells at confluency were washed and incubated in serum-free medium, and the basal secretion rate was determined by measuring the accumulation of mucin in the medium. As shown in Fig. 1, SW1116 cells constitutively secrete a considerable amount of mucin, equalling approx. 6 μ g of mucin standard equivalents/10⁶ cells in 2 h. For comparison, in T-84 cells (McCool *et al.*, 1990) or in HT-29 cells, mucin secretion measured using the 19-9 antibody was an order of magnitude lower (results not shown).

The sialyl-Le^a antigen detected by the 19-9 antibody could also be expressed on other glycoproteins. However, mucin is the predominant macromolecule secreted by SW1116 cells, as more than 90% of the macromolecules secreted by these cells detected by metabolic labelling with glucosamine and sulphate are mucins, on the basis of their size, charge, density and chemical analysis, as described in the Experimental section (see also the experiment of Fig. 5). The immunoreactivity with antibody 19-9 co-purified with the labelled mucin by gel filtration and equilibrium sedimentation (results not shown). Thus the 19-9 antibody reactivity is a specific measure of mucin secretion for these cells.

Cellular localization of mucin in SW1116 cells

To examine the subcellular localization of mucin, the cells were embedded in the hydrophilic resin LR white and labelled with the monoclonal antibody 19-9, using as second antibody

Fig. 1. Mucin secretion from SW1116 colon carcinoma cells

Cultured cells were washed and incubated in serum-free L-15 medium. Aliquots from the culture medium were taken at the desired times and the mucin content was determined by e.l.i.s.a. using monoclonal antibody 19-9 as described in the Experimental section. Results are means $\pm$ S.D. for four measurements.

Fig. 2. Electron micrographs of a SW1116 cell section

(a) Ultra-thin LR white sections of cells collected on formvar-coated Ni grids, labelled with 19-9 antibody and goat anti-(mouse IgG) linked to 10 nm gold particles. (b) Serial sections of cells prepared by cryofixation and molecular distillation ('life-cell' process), embedded in spurr-resin, cut and labelled as in (a). Scale bar = 0.2 μ m.

goat anti-(mouse Ig) linked to 10 nm gold particles. As shown in Fig. 2(a), we observed the antigen in intracellular membrane-enclosed organelles (arrow 1), in a vesicle which seemed to be fused to the plasma membrane (arrow 2) and near the cell surface (arrow 3), as well as in a multivesicular lysosome-like body (Fig. 2a, inset). This labelling was observed in at least 60% of the cells. Non-specific labelling was ruled out by the following control experiments. (1) When the primary antibody was omitted from the labelling procedure, no labelling was obtained. (2) When thin sections were treated with neuraminidase, to hydrolyse sialic acid residues prior to labelling, the extracellular apical label was almost completely obliterated, and the intracellular label was considerably decreased (results not shown).

It should be noted that, although the 19-9 antibody may also detect glycolipids carrying the antigenic sugar NeuAc α 2-3Gal β 1-3 (Fuc α 1-4) GlcNac β 1-3R, this is not likely to be the case in this experiment. This is because, before labelling with the 19-9 antibody, the cells were fixed with glutaraldehyde and osmium, and then washed several times with 70% ethanol, which is known to remove cellular glycolipids. To confirm this,

cells treated in this manner and untreated cells were subjected to lipid extraction with chloroform/methanol/water (1:1:1, by vol.). The extracts were chromatographed on high performance t.l.c. and immunostained with 19-9 antibody. Immunoreactivity was found only in the untreated cells, whereas treated cells were free of residual glycolipids (results not shown). In accordance with this, we found that the label tended to cluster inside the organelles rather than spread over the membrane where glycolipids are predominantly located. Fig. 2(b) depicts serial sections of a Golgi area obtained with a freeze-dried (Life-Cell) technique (Linner *et al.*, 1986) showing a labelled Golgi vesicle (arrow 4) and a labelled large Golgi-associated organelle. These data show that SW1116 cells are capable of packaging mucin, which could then be available for exocytosis.

Effect of secretagogues on mucin secretion

Cyclic AMP. Cyclic AMP is a common secretagogue, and cyclic AMP-mediated stimuli have been shown to induce mucin secretion from colon carcinoma cells (McCool *et al.*, 1990) and from submandibular acinar cells (Bradbury & McPherson, 1988).

Table 1. Effect of forskolin and IBMX on cellular cyclic AMP content in SW1116 cells

The peak cyclic AMP level was usually reached about 5 min after stimulation. However, since mucin secretion was measured up to 40 min after stimulation, the cellular cyclic AMP content was determined at both of these time points. At the desired time the cells were washed and frozen, and cyclic AMP was determined as described in the Experimental section. In the same experiment mucin secretion in control and in stimulated cells was measured up to 40 min after stimulation, but none of the treatments significantly affected the level of secreted mucin. Results are means $\pm$ S.D. ($n = 4$). n.d., not determined.

Treatment	Cellular cyclic AMP content (pmol/ μ g of cell protein)	
	5 min	40 min
Control	0.056 ± 0.019	0.025 ± 0.005
Forskolin ($5 \mu\text{M}$)	0.872 ± 0.091	0.591 ± 0.037
IBMX ($20 \mu\text{M}$)	n.d.	0.073 ± 0.001
Forskolin ($5 \mu\text{M}$) + IBMX ($20 \mu\text{M}$)	n.d.	3.394 ± 0.277

Fig. 3. Effect of arachidonic acid on mucin secretion

The same procedure as in the experiment of Fig. 1 was applied. Samples of culture medium were taken for mucin determination at 10 (●), 20 (▲) and 40 (■) min. Results are means $\pm$ S.D. for four measurements.

We examined the response of mucin secretion in SW1116 cells to dibutyryl cyclic AMP, forskolin (which induces cyclic AMP production), 3-isobutyl-1-methylxanthine (IBMX; an inhibitor of phosphodiesterase) and a combination of forskolin with IBMX. The secretion was measured up to 40 min after stimulation, but none of these treatments significantly affected the level of secreted mucin.

In order to verify that forskolin and IBMX indeed had an effect on the cellular level of cyclic AMP in SW1116 cells, the cellular cyclic AMP content was determined following the introduction of forskolin and IBMX to the cell culture. As shown in Table 1, the cyclic AMP level was markedly enhanced by these effectors, especially by forskolin, which induced an order of magnitude increase. Moreover, the combination of forskolin and IBMX enhanced the cyclic AMP level by two orders of magnitude. It is thus clear that mucin secretion by SW1116 cells is not cyclic AMP-dependent.

In another experiment we found that the phorbol ester PMA, an activator of protein kinase C (PKC), also did not affect mucin secretion by SW1116 cells.

Fig. 4. Effect of ionophore A23187 on mucin secretion

The same procedure as in the experiment of Fig. 1 was applied. The dependence of mucin secretion on A23187 concentration is shown for incubations of 10 (■), 20 (▲) and 40 (●) min. Results are means $\pm$ S.D. for four measurements.

Arachidonic acid. It has been reported that various arachidonic acid metabolites (eicosanoids) increase glycoprotein secretion from normal human airway explants (Marom *et al.*, 1981; Coles *et al.*, 1983). PGE₁ has been shown to stimulate mucin secretion in T-84 colon carcinoma cells (McCool *et al.*, 1990). We therefore examined the effect of various eicosanoids, including leukotrienes LTC₄ and LTD₄, prostaglandin PGE₁ and PGE₂, and 5-HETE, on mucin secretion by SW1116 cells. We found that none of these compounds enhanced mucin secretion, and some clearly inhibited the basal secretion, particularly LTC₄, which at 10 ng/ml inhibited secretion by 50%. Furthermore, we examined the effect of arachidonic acid, the eicosanoid precursor, on mucin secretion and found that it clearly inhibited secretion over a wide range of concentrations (Fig. 3).

Activation of mucin secretion by Ca²⁺ ionophore A23187

Mobilization of Ca²⁺ is a general stimulus for many secretory processes. We therefore examined the effect of the Ca²⁺ ionophore A23187 on mucin secretion by SW1116 cells. As shown in Fig. 4, the secretion was markedly enhanced by the Ca²⁺ ionophore in a concentration-dependent manner. The secretion was measured also at 5 μM -A23187 (not shown in Fig. 4), and seemed to reach a plateau above a concentration of the ionophore of 1 μM . Thus it seems that, although SW1116 cells are in a state of constitutive secretion which is not affected by cyclic AMP- or PKC-dependent stimuli, the secretion can be enhanced by Ca²⁺-mobilizing agents.

As noted above, the 19-9 antibody used to determine the mucin may detect other glycoproteins which carry the sialylated Le^a antigen. Although we have determined that under basal conditions SW1116 cells secrete predominantly mucin, we considered the possibility that on stimulation with A23187 the cells may also secrete other proteoglycans detected by this antibody. To examine this possibility, we determined the effect of chondroitin ABC lyase, which hydrolyses glycosaminoglycans (such as chondroitin 4- and 6-sulphate and dermatan sulphate proteoglycans) but not mucin, on the level of the 19-9-detectable material in the culture medium. Medium from either untreated or A23187-stimulated cells (1 μM for 1 h at 37 °C) was collected and subjected to the action of chondroitin ABC lyase (0.5 unit/ml for 3 h at 37 °C). The reaction mixture was then chromatographed on a column of Sephacryl-400 and the 19-9-detectable content in the fractions was determined. As shown in

Fig. 5. Effect of chondroitin ABC lyase treatment on the level of mucin secreted by control (■) and A23187-treated (●) cells

Culture medium from control or stimulated cells ($1 \mu\text{M}$ - Ca^{2+} ionophore for 30 min) was collected and treated with chondroitin ABC lyase. The untreated (a) and chondroitin lyase-treated (b) media were then chromatographed on a Sephacryl S-400 column and the mucin content in the fractions was determined by e.l.i.s.a. as described in the Experimental section. Results are means $\pm$ S.D. for three measurements.

Fig. 6. Effect of ionophore A23187 on secretion of β -glucuronidase from SW1116 cells

The same procedure as in the experiment of Fig. 1 was applied. Samples from the culture medium were taken at the desired times and 4MU glucuronide was added. Enzyme activity was determined by measurement of the 4MU fluorescence produced. ●, Medium of control cells, ■, medium of cells treated with $1 \mu\text{M}$ - Ca^{2+} ionophore A23187. Results are means $\pm$ S.D. for four measurements.

Fig. 7. Effect of arachidonic acid on secretion of β -glucuronidase from SW1116 cells

The same procedure as in Fig. 6 was applied. The Figure presents results obtained with medium of control cells (■) or medium of cells treated with $1 \mu\text{g}$ (▼), $10 \mu\text{g}$ (●) or $100 \mu\text{g}$ (◆) of arachidonic acid/ml. Results are means $\pm$ S.D. for four measurements.

Fig. 5, the level of mucin and its distribution were not significantly altered by the chondroitin lyase treatment of either control or stimulated cell medium. It is thus plausible to conclude that, under both basal conditions and ionophore stimulation, mucin is the major macromolecule secreted by SW1116 cells.

Comparison between secretion of mucin and lysosomal enzymes

The data described above i.e. the irregular response of mucin secretion to common secretagogues on the one hand, and its detection in lysosomal-like and vesicular structures on the other, led us to ask whether mucin in SW1116 cells is co-secreted with lysosomal contents. We therefore studied the effect of arachidonic acid and A23187, which were found to affect mucin secretion, on the secretion of β -glucuronidase, as a marker for lysosomal secretion. As shown in Fig. 6, β -glucuronidase secretion was enhanced by the Ca^{2+} ionophore, as found for mucin secretion. However, arachidonic acid, which inhibited mucin secretion, instead activated the secretion of the lysosomal enzyme, as shown in Fig. 7. It is therefore plausible to conclude that the

predominant route for mucin secretion in SW1116 cells is via a compartment separate from that containing the lysosomal contents.

DISCUSSION

The data presented in this study suggest that SW1116 colon carcinoma cells are uniquely specialized in mucin secretion, as they constitutively secrete considerable amounts of mucin as the major glycoprotein. Unlike in normal epithelial cells and T-84 colon carcinoma cells (McCool *et al.*, 1990), the secretion of mucin by SW1116 cells is not affected by stimuli that modulate the cyclic AMP level (forskolin, IBMX) or by a PKC-linked stimulus (PMA).

Regarding the regulation of basal mucin secretion, we found that arachidonic acid and some of its metabolites were clearly inhibitory. This is in contrast to previous reports (Marom *et al.*, 1981; Shelhamer *et al.*, 1982; Coles *et al.*, 1983) that the various kinds of eicosanoids (leukotrienes, prostaglandins and HETEs) all increased mucin and glycoprotein secretion in slices of normal airway gland (although the greatest increase was less than 2-

fold). However, these studies were performed with explants from normal subjects, using organ slices which contain more than one cell type. Thus it is difficult to differentiate direct and indirect effects on one cell type from those on another. This difficulty is circumvented by using a cell line culture, as in the present study.

The only stimulator of mucin secretion that we have found is the Ca^{2+} ionophore A23187, thus suggesting that, although the cells are already actively secreting mucin, the secretion may be further stimulated by Ca^{2+} mobilizing agents.

The lack of stimulation by typical secretagogues (arachidonic acid and its metabolites, as well as cyclic AMP and PKC-dependent stimuli) might suggest that regulation of mucin secretion from these cells occurs by processes different from those of other mucin-secreting cells (Phillips *et al.*, 1988; McCool *et al.*, 1990). Nonetheless, electron microscope studies suggest that at least some of the mucin is located in vesicular structures. Thus the exocytosis mechanism of mucin secretion cannot be excluded for SW1116 cells. The distinctiveness of the mucin secretory mechanism is further supported by the observation that arachidonic acid, while inhibiting mucin secretion, stimulates the secretion of the lysosomal contents. On the other hand, Ca^{2+} ionophore A23187 enhances the secretion of both mucin and the lysosomal contents. Taken together, these results might suggest that basal and ionophore-stimulated secretion may occur via separate mechanisms, possibly from more than one pool of mucin. Multiple mechanisms have been previously proposed for mucin secretion from epithelial cells (Kim *et al.*, 1987), and the detailed mechanisms of mucin secretion from SW1116 cells is yet to be studied.

The accumulation of mucin in airways is a major characteristic of the pathophysiology of CF. Although healthy people do not secrete appreciable amounts of airway mucin, unless stimulated exogenously or in illness, it is not clear whether the accumulation of mucin in CF is due to excessive secretion or to abnormal physical and chemical properties of the CF mucin (Boat & Cheng, 1989). The means of regulation of secretion in CF cells is not clear. However, it seems typical of cells derived from CF patients that they do not respond to cyclic AMP- or PKC-dependent stimuli. This has been shown for chloride secretion from lymphocytes (Chen *et al.*, 1989), intestinal (Berschneider *et al.*, 1988) and airway (Frizzell *et al.*, 1986; Hwang *et al.*, 1989; Li *et al.*, 1989) epithelial cells, and a pancreatic adenocarcinoma cell line (Shoumacker *et al.*, 1990). Similar results were reported for mucin secretion by McPherson *et al.* (1986), who found that in submandibular acinar cells obtained from a CF patient, the response of mucin secretion to a β -adrenergic stimulus was very poor compared with control cells.

Thus, unlike other colon carcinoma cell lines (T-84, HT-29), SW1116 cells exhibit a pronounced resemblance to epithelial cells from CF patients, mainly with regard to the marked, constitutive secretion of mucin and in the cells' response to secretory stimuli. The marked inhibition of mucin secretion from these cells by arachidonic acid and its metabolites might provide a means for regulation of mucin secretion. Taken together, these

observations make the SW1116 colon carcinoma cell line an especially interesting system for studying processes potentially related to disorders associated with excessive mucin secretion.

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