Cell polarization is required for ricin sensitivity in a Caco-2 cell line selected for ricin resistance

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It has been proposed that killing of mammalian cells by ricin requires efficient endocytic delivery to the trans-Golgi network (TGN) prior to retrograde transport to the endoplasmic reticulum and entry to the cytosol. In polarized epithelial cells, an efficient membrane-traffic pathway to the TGN is present from the basolateral but not the apical plasma-membrane domain. Thus one can hypothesize that a ricin-resistant phenotype might be demonstrated by polarized cells that fail to differentiate and thus fail to develop an efficient membrane-traffic pathway from the basolateral plasma membrane to the TGN. We have isolated and studied a ricin-resistant Caco-2 cell clone (Caco-2-RCA^r clone 2) which, when grown on plastic, was deficient in differentiation, measured by the development of polarized-cell-surface marker enzymes. The deficiency in differentiation was partially reversed, and ricin sensitivity was restored, when the cells were grown on filter supports. Our data provide the first evidence of a ricin-resistant cell line where resistance is due to the lack of development of polarized cell surfaces. The observed ricin resistance is consistent with the requirement that ricin is delivered to the TGN before its A chain enters the cytosol to mediate cell killing.

Key words: glycosylation mutant, polarized cell, trans-Golgi network.

INTRODUCTION

The toxic plant lectin ricin (*Ricinus communis* RCA_{60}), is a member of the AB-toxin family in which a catalytically active polypeptide (A) is associated with a cell-binding component (B). Through its B chain, ricin binds to cell-surface galactose-containing glycoproteins and glycolipids. After ricin endocytosis, the A chain, which is an N-glycosidase, enters the cytosol and inactivates ribosomes by depurination of a single adenosine in 28 S ribosomal RNA (reviewed in [1]). The site of translocation of the A chain into cytosol is a matter of dispute, with some research workers suggesting translocation across the endosome membrane [2,3]. However, a more widely presented view is that toxic ricin is a small proportion ($\leqslant 5\,\%$) of endocytosed ricin that is delivered to the trans-Golgi network (TGN), then transported in a retrograde pathway to the endoplasmic reticulum (ER) where translocation into the cytosol occurs, probably via a mammalian Sec61p-mediated route [1,4,5]. A variety of evidence has accumulated to suggest the importance of traffic to the TGN and ER for cell killing by ricin, including the ability of brefeldin A and ilimaquinone to block ricin intoxication of cells in which these compounds cause a disruption of Golgi morphology [6,7]. In addition, expression of mutant dynamin, which blocks endocytosis and traffic of ricin to the TGN [8], inhibits ricin intoxication. Moreover, addition of the ER retrieval motif KDEL to the C-terminus of the A chain results in an engineered polypeptide with an increased cytotoxicity of 10–100-fold, dependent on cell type [9]. However, the most compelling evidence was obtained from experiments using recombinant ricin in which the C-terminus of the A chain was modified by the addition of overlapping tyrosine sulphation and N-glycosylation sites [10]. It was found that only ricin A chain that had been both tyrosine-sulphated (an event occurring in the TGN) and core N-glycosylated (an event occurring in the ER) was translocated to the cytosol, implying that passage through both the TGN and ER was necessary for entry into the cytosol and inhibition of protein synthesis.

In polarized epithelial cells, an efficient membrane-traffic pathway to the TGN is present from the basolateral but not the apical plasma-membrane domain. This was demonstrated in polarized transfected Madin–Darby canine kidney (MDCK) and Caco-2 cells grown on filter supports, with experiments following the traffic of the TGN marker, TGN38, or constructs containing the TGN38 cytosolic tail [11,12]. Consistent with the importance of the cell surface to TGN pathway for ricin intoxication, $\approx$ 10-fold greater sensitivities of MDCK-I and Caco-2 cells to ricin were demonstrated when the toxin was added to the basolateral compared with the apical side of filter-grown cells [13]. Further evidence for the importance of delivery to the TGN for ricin intoxication would be provided if an epithelial cell line deficient in polarization was shown to have reduced ricin sensitivity. In the present study, we generated a ricin-resistant Caco-2 cell line (Caco-2-RCA^r clone 2), which, when grown on plastic, was defective in differentiation and development of polarized cell surfaces. When the Caco-2-RCA^r clone 2 cells were grown on filter supports, increased differentiation, measured as expression of polarized-cell-surface enzyme markers, was observed and the ricin-resistance phenotype was reversed.

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; FCS, fetal calf serum; Caco-2-RCA^r, ricin-resistant human colon adenocarcinoma cell line; MDCK, Madin–Darby canine kidney; TGN, trans-Golgi network.

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EXPERIMENTAL

Cell culture, ricin binding and endocytosis

Culture of Caco-2 cells on plastic or filter supports, ^{125}I-labelling of ricin, measurement of ricin binding to cells and of ricin endocytosis were carried out as described previously [14,15]. Analytical methods were also as described previously [14–16], unless stated below. The affinity of ricin binding and amount of binding per cell were calculated using the 'LIGAND' software of Munson and Rodbard [17]. Analysis of ricin binding to separated glycoproteins was carried out after SDS/PAGE (10% gel), by electrophoretic transfer on to nitrocellulose, blocking with PBS/0.1% BSA and incubation with ^{125}I-ricin (4×10^6 d.p.m./μg of protein).

Preparation of ricin-resistant Caco-2 clones

For the first selection of ricin-resistant Caco-2 cells, parental Caco-2 cells were seeded at a density of 1.5×10^7 cells/150-cm^2 plastic flask. After seeding cells (24 h), ricin was added at 0.5 ng/ml of Dulbecco's modified Eagle's medium (DMEM). After 6 h at 37 °C, the ricin-containing DMEM was removed and replaced with fresh DMEM supplemented with 20% fetal calf serum (FCS; this medium was replaced every 3 days). All surviving cells were passaged in DMEM supplemented with 10% FCS 14 days after ricin intoxication, to enable seeding of enough 150-cm^2 plastic flasks for the next selection in higher ricin concentrations (4 ng/ml). This selection procedure was repeated using, successively, ricin at 8 ng/ml and then 16 ng/ml. Caco-2-RCA^r cells surviving after the final selection were cloned twice by limiting dilution in DMEM supplemented with 20% FCS and 50% conditioned medium (conditioned medium was taken from flasks containing confluent Caco-2 cells and was filtered to remove any cells and debris). Twelve ricin-resistant clones were selected by this procedure. It should be noted that no parent Caco-2 cells survived when treated with ricin at 2 ng/ml under the conditions used to select ricin-resistant clones.

Measurement of protein synthesis

The rate of protein synthesis was measured for 4 h at 37 °C using [^{14}C]leucine (0.1 μCi/ml per well with cells growing on plastic, or 0.1 μCi/ml, 2 ml added apically and basolaterally, with cells growing on filters) and leucine-free DMEM. The medium was then removed and plastic wells/filters were washed once with PBS containing 0.5 mM Mg^{2+} and 0.9 mM Ca^{2+}. Two successive aliquots (2 ml) of 10% (w/v) trichloroacetic acid at 4 °C were added to wells/filters and precipitated protein was dissolved in 0.1 M KOH before measuring radioactivity associated with the cells. Inhibition of protein synthesis was determined by incubating cells with ricin for 6 h at 37 °C before adding [^{14}C]leucine.

Formaldehyde fixation and isobutanol extraction of cells

Cells grown in 24-well disposable trays were fixed using formaldehyde, and lipids were extracted using isobutanol, as described by Sandvig et al. [18]. Both treated and untreated cells were incubated with ^{125}I-ricin and washed as described for lectin-binding studies.

Analysis of glycolipids

Glycolipids were extracted from Caco-2 cells using the method of Folch et al. [19], as described by Smith [20]. In a single experiment, glycolipids were extracted from cells grown in a 75-cm^2 plastic flask for 7 or 14 days. After phase separation, gangliosides were prepared from the upper phase as described by Stein and Smith [21] and, after mild alkaline hydrolysis, dialysis and freeze drying, were resuspended in 10 μl of a chloroform/methanol/water mixture (3:48:47). Aliquots (2 μl) were subjected to TLC on silica gel 60 F_{254} HPTLC plates using methyl acetate/*n*-propanol/chloroform/methanol/ 0.25% aq. KCl (25:20:20:20:17) as neutral solvent [21]. The HPTLC plates had been pre-run with the same solvent before samples were loaded. Asialoganglioside G_{M1}, monosialoganglioside G_{M1} and disialoganglioside G_{D1a} (all from Sigma) were run as standards. Gangliosides were visualized after TLC by spraying with a reagent prepared immediately before use by adding 4 ml of 18.3 M H_2SO_4 to 96 ml of the following solution: 0.2 g of naphthoresorcinol (naphthalene-1,3-diol)/0.4 g diphenylamine in 100 ml of 95% ethanol. After spraying, plates were baked for 10 min at 150 °C. Glycosylceramides were prepared from the lower phase according to Stein and Smith [21] following Folch extraction. After mild alkaline hydrolysis and evaporation to dry, glycosylceramides were resuspended in 60 μl of a chloroform/methanol mixture (4:1) and aliquots (4 μl) subjected to TLC on silica gel 60 F_{254} HPTLC plates. Once samples were loaded, the plates were first run with chloroform and then ethyl acetate before separation of the glycosylceramides using chloroform/methanol/water (65:25:4). Lactosylceramide, glucosylceramide and galactosylceramide (from Sigma) were used as standards. Glycosylceramides were also visualized after TLC by spraying with the same α-naphthol staining reagent.

Enzyme assays

Cells were washed twice with PBS containing 0.5 mM Mg^{2+} and 0.9 mM Ca^{2+} and lysed by freeze–thawing in 0.1% Triton X-100/40 mM Hepes in PBS, pH 7.4 (2 ml per 25-cm^2 plastic flask). Alkaline phosphatase [22] and sucrase-isomaltase [23] activities in the lysates were assayed as described previously.

Statistical methods

Unless otherwise stated, data are presented as means $\pm$ S.D. with the number of observations (n) in parentheses.

RESULTS

Caco-2-RCA^r cells were prepared by successive positive selection, in increasing concentrations of ricin, of cells growing on plastic. Twelve clones were isolated, all being more resistant to ricin than parent Caco-2 cells (results not shown). These clones all had a reduced number of ricin-binding sites ($0.54–1.98 \times 10^7$/cell) when compared with parent cells (3.7×10^7/cell).

One clone, Caco-2-RCA^r clone 2, was studied further to determine the basis of the ricin resistance. This clone showed a $\approx$ 7 fold difference in ricin sensitivity when compared with parent cells grown on plastic (Figure 1a). The Caco-2-RCA^r clone 2 cells had 1.2×10^7 binding sites per cell. The K_d value for ricin binding was as expected, similar for parent (4.22×10^7 M) and Caco-2-RCA^r clone 2 cells (4.01×10^7 M). No difference in glycoprotein labelling of parent cells and Caco-2-RCA^r clone 2 cells was observed after incorporating [^{3}H]galactose by exogalactosylation using the method of Brandli et al. [24] (results not shown), suggesting that glycoproteins on the Caco-2-RCA^r clone 2 cells were not significantly less sialylated than those on parent cells. No differences in ricin binding to glycoproteins could be detected between Caco-2-RCA^r clone 2 and parent Caco-2 cells by SDS/PAGE followed by transfer on to nitrocellulose, incubation

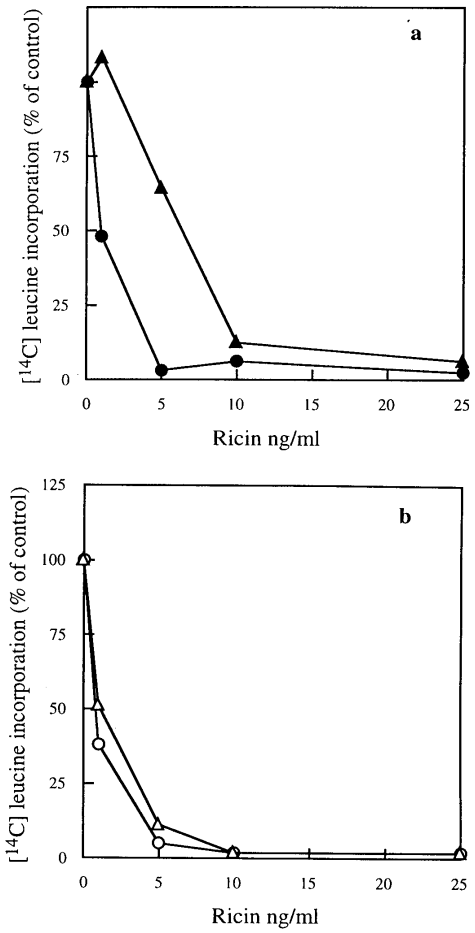

Figure 1 Sensitivity of parent Caco-2 cells and Caco-2-RCA^r clone 2 cells grown on (a) plastic and (b) filter supports

Caco-2 parent cells (●, ○) and Caco-2-RCA^r clone 2 cells (▲, △), grown on (**a**) 24-well plastic disposable trays and (**b**) filters, were exposed to 0–25 ng/ml ricin for 6 h at 37 °C. Protein synthesis was measured during a 4-h interval after ricin incubation as described in the Experimental section. Each point is the mean of triplicate measurements. S.D.s were $< 10\%$.

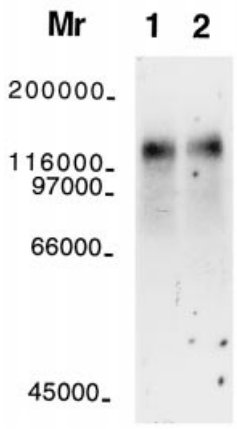

Figure 2 Binding of ^{125}I-ricin to glycoproteins of parent Caco-2 cells and Caco-2-RCA^r clone cells

Glycoproteins in whole-cell lysates (1×10^5 cells/lane) from parent Caco-2 cells (lane 1), and Caco-2-RCA^r clone 2 cells (lane 2), were separated by SDS/PAGE (10% gel), transferred on to nitrocellulose, incubated with ^{125}I-ricin and subjected to autoradiography using KODAK X-Omat AR film at −70 °C. Incubating with ^{125}I-ricin in the presence of 0.2 M lactose gave no binding of ^{125}I-ricin to glycoproteins, indicating that binding of ^{125}I-ricin was specific for galactose-containing glycoproteins. The positions of molecular-mass markers (M_r) are shown on the left.

Table 1 Endocytic uptake of surface-bound ^{125}I-ricin in Caco-2 cells

Uptake of ^{125}I-ricin was measured as the percentage of surface-bound ricin after warming cells to 37 °C for 30 min. Values are the mean ± S.D. from four separate cell cultures.

	Parental Caco-2 cells	Caco-2-RCA^r clone 2
Plastic grown	7.7 ± 0.2	5.9 ± 0.3
Filter grown (apical uptake)	5.3 ± 0.1	3.7 ± 0.4
Filter grown (basolateral uptake)	7.3 ± 0.3	11.6 ± 0.6

with ^{125}I-ricin and autoradiography (Figure 2). Moreover, endocytosis of surface-bound ^{125}I-ricin in 30 min at 37 °C was similar in 7-day plastic-grown cultures of both parent cells and Caco-2-RCA^r clone 2 cells (Table 1). Since ricin also binds to glycolipids, these were extracted with isobutanol prior to ^{125}I-ricin binding to formaldehyde-fixed 7-day plastic-grown cell layers. It was found that ricin binding to glycolipids accounted for 71 ± 5 ($n = 3$) and $48 \pm 10\%$ ($n = 3$) of the total amount of ricin bound to parent and Caco-2-RCA^r clone 2 cells, respectively. Despite the difference in binding of ^{125}I-ricin to parent and Caco-2-RCA^r clone 2 cells, no differences were seen in the pattern of either gangliosides or glycosylceramides extracted from these cells after culture for 7 or 14 days and separation by TLC (Figure 3). There was also no observable difference when the TLC plates were overlaid with ^{125}I-ricin and subjected to autoradiography (results not shown). On the basis of these data it was difficult to explain the ricin resistance of the Caco-2-RCA^r clone 2 cells on the basis of alterations in ricin binding or endocytic uptake.

When grown on plastic in the absence of ricin, Caco-2-RCA^r cell clones maintained their ricin resistance for at least 6 months through many passages (> 20). However, all twelve of the Caco-2-RCA^r cell clones, including Caco-2-RCA^r clone 2, lost their ricin resistance when grown as confluent monolayers on filter supports. When comparing the concentrations of ricin that would cause 50% inhibition of protein synthesis, Caco-2-RCA^r clone 2 cells showed an increase in sensitivity to ricin of $\approx$ 7-fold when grown on filters compared with growth on plastic (Figure 1b). Parent Caco-2 cells also showed a small ($\approx 30\%$) increase in sensitivity to ricin. The $\approx$ 7-fold increase in ricin sensitivity of the Caco-2-RCA^r clone 2 cells grown on filter supports was greater than any change in endocytic uptake of ricin observed in the filter-grown compared with plastic-grown cells (Table 1). The reversal of phenotype was not due to an incomplete or 'loose' monolayer being formed, since measurements of transepithelial resistance were $504 \pm 45\ \Omega \cdot cm^2$ for the Caco-2-RCA^r clone 2 cells compared with $536 \pm 55\ \Omega \cdot cm^2$ ($n = 3$) for the parent cells. Growth on filters resulted in small changes in gangliosides and glycosylceramides compared with growth on plastic (Figure 3), and no differences were seen between filter-grown parent and Caco-2-RCA^r clone 2 cells. There were no differences in morphology of filter-grown parent and Caco-2-RCA^r clone 2 cells observed by transmission electron microscopy with the development of a brush border on the apical surfaces of the cells (results not shown). The development of a brush border was consistent with increased differentiation, known to occur when epithelial cells are grown on permeable filter supports [25,26]. We therefore measured the activities of alkaline phosphatase and sucrase-

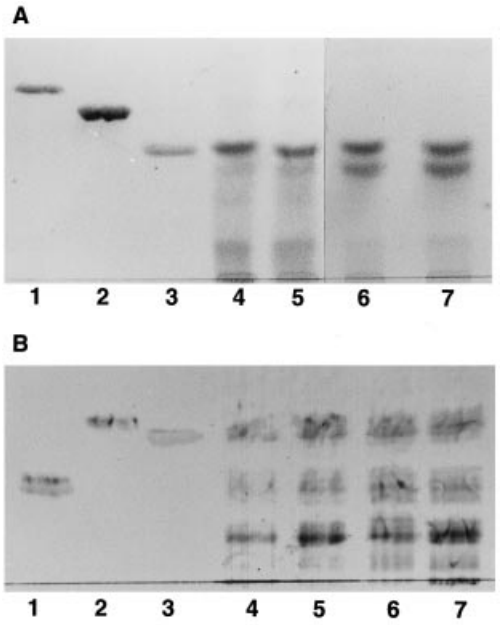

Figure 3 TLC analysis of glycolipids from parent Caco-2 cells and Caco-2-RCA^r clone 2 cells

(**A**) Gangliosides. Lanes 1–3 show standards (1, asialoganglioside G_{M1}; 2, monosialoganglioside G_{M1}; and 3, disialoganglioside G_{D1a}). Gangliosides from parent Caco-2 cells (lane 4) and Caco-2-RCA^r clone 2 cells (lane 5) grown on filters for 14 days, and from parent Caco-2 cells (lane 6) and Caco-2-RCA^r clone 2 cells (lane 7) grown on plastic for 14 days were extracted, separated by TLC and stained as described in the Experimental section. (**B**) Glycosylceramides. Lanes 1–3 show standards (1, lactosylceramide; 2, glucosylceramide; and 3, galactosylceramide). Glycosylceramides from parent Caco-2 cells (lane 4) and Caco-2-RCA^r clone 2 cells (lane 5) grown on filters, and from parent Caco-2 cells (lane 6) and Caco-2-RCA^r clone 2 cells (lane 7) grown on plastic were extracted, separated by TLC and stained as described in the Experimental section.

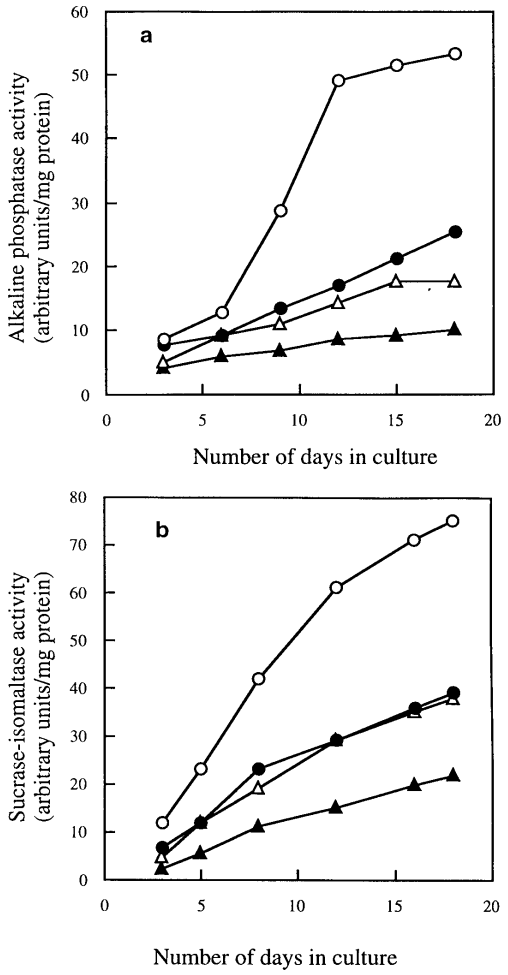

Figure 4 Development of alkaline phosphatase (a), and sucrase-isomaltase (b), activity in parent Caco-2 cells and Caco-2-RCA^r clone 2 cells, grown on plastic and filter supports

Enzyme activity was measured in whole-cell lysates taken from parent Caco-2 cells grown on filters (○) and plastic (●), and from Caco-2-RCA^r clone 2 cells grown on filters (△) and plastic (▲), for 3–18 days in culture, as described in the Experimental section. Each point is the mean of triplicate measurements. S.D.s were $< 10\%$.

isomaltase, two enzymes used previously as markers of cell differentiation in this cell type [27]. The increase in specific activity of both enzymes relative to age of cell culture was greatest when parent cells were grown on filters. Caco-2-RCA^r clone 2 cells grown on filters developed slightly more enzyme activity than parent cells grown on plastic but, whether grown on plastic or filter supports, the clone 2 cells showed much less enzyme activity than parent cells grown on the same support (Figure 4).

DISCUSSION

In the present experiments, since we wished to reduce the possibility of producing cloned cells exhibiting multiple mutations, we avoided mutagens which have been used by others to raise lectin-resistant mutant cell lines [28–30]. Nevertheless, the selected cells were at least 7 times more resistant to ricin than parent Caco-2 cells, i.e. similar to the resistance shown by MDCK-II-RCA^r cells [24,31].

In previous studies with other cell types, resistance has been observed at one of three stages of ricin toxicity: cell-surface binding [28,32]; endocytosis [33]; or inhibition of protein synthesis [29]. In contrast, the data described above suggest that the underlying defect accounting for ricin resistance in the Caco-2-RCA^r clone 2 cells grown on plastic is linked to their state of differentiation, with them being unable to reach the threshold of differentiation necessary for efficient ricin intoxication achieved by the parent cells. This phenotype provides additional evidence for the importance of delivery to the TGN for ricin intoxication, since only in more polarized cells will an efficient basolateral-plasma-membrane-to-TGN-membrane traffic pathway exist. The observation that parent Caco-2 cells are able to partially differentiate when grown on plastic is not surprising, since previous studies have shown the development of partially polarized domed structures in post-confluent monolayers of these cells grown on impermeable supports [34]. The novel feature of the Caco-2-RCA^r clone 2 cell line is that it is unable to achieve such polarization when grown on plastic but this defect is partially reversed when grown on filter supports. The accompaniment of the partial reversal of the polarization defect with an increase in ricin sensitivity demonstrates the coupling of these two phenomena.

Although experiments on HT29 cells have suggested previously that ricin intoxication may be related to the state of differentiation in some cell types [35], our data provide the first evidence that it can be related to the development of polarized cell surfaces. The data also suggest that in addition to binding, endocytosis and

protein-synthesis inhibition mutants, a further class of ricin-resistant cell mutants exists in which resistance may be due to inefficient delivery to the TGN. Such mutants may be useful in studying delivery of proteins from endosomes to the TGN. This is a pathway of much current interest since there appear to be routes to the TGN from both early [36,37] and late [38] endosomes.

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