BIOCHEMICAL AND GENETIC CHARACTERIZATION OF THREE HAMSTER CELL MUTANTS RESISTANT TO DIPHTHERIA TOXIN

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ABSTRACT

We describe here three different hamster cell mutants which are resistant to diphtheria toxin and which provide models for investigating some of the functions required by the toxin for expression of its cytotoxic activity. It is known that diphtheria toxin inactivates elongation factor 2 (EF-2). Cell-free extracts from mutants Dtx⁻¹ and Dtx⁻³ contained EF-2 that was resistant to inactivation by the toxin. In somatic cell hybrids, the phenotype of Dtx⁺⁻⁻¹ was recessive while that of Dtx⁻⁻³ was codominant. The evidence suggests that the codominant phenotype is the result of a mutation in a gene coding for EF-2. The recessive phenotype might arise by alteration of an enzyme which modifies the structure of EF-2 so that it becomes a substrate for reaction with the toxin. Another mutant, Dtx⁺⁻², contained EF-2 that was sensitive to the toxin and this phenotype was recessive.

Pseudomonas aeruginosa exotoxin is known to inactivate EF-2 as does diphtheria toxin and we tested the mutants for cross-resistance to Pseudomonas exotoxin. Dtx⁻¹ and Dtx⁻³ were cross-resistant while Dtx⁺⁻² was not. It is known that diphtheria toxin does not penetrate to the cytoplasm of mouse cells and that these cells have a naturally occurring phenotype of diphtheria toxin resistance. We fused each of the mutants with mouse 3T3 cells and measured the resistance of the hybrid cells to diphtheria toxin. Intraspecies hybrids containing the genome of mutants Dtx⁻¹ and Dtx⁻³ had some resistance while those formed with Dtx⁺⁻² were as sensitive as hybrids derived from fusions between wild-type hamster cells and mouse 3T3 cells.

KEY WORDS diphtheria toxin - pseudomonas aeruginosa exotoxin - elongation factor 2 - toxin resistant mutants - somatic cell hybridization - cell surface receptors

Diphtheria toxin is a protein of molecular weight 63,000 which kills sensitive cells by arresting protein synthesis (6, 25). Expression of the lethal activity of the toxin requires the cooperation of many gene products. There are three main steps in the intoxication process. First, the toxin interacts with a specific cell surface receptor, the nature of which is unknown (2, 3, 10, 18, 20, 28). Second, at least the A fragment of toxin crosses the plasma membrane and enters the cytoplasm. The details of this process are unknown; adsorptive endocy-
Tosis may be involved (24) or a pore in the membrane might be created through which the toxin passes (2). Third, once in the cytoplasm, fragment A catalyzes the transfer of the adenosine diphosphate ribose portion of NAD\(^+\) to elongation factor 2 (EF-2), rendering it inactive (16). ADP-ribose is covalently linked to an amino acid in EF-2 which is not commonly found in proteins and which remains unidentified (26). This reaction has been intensively studied in vitro and is relatively well understood (6). A similar reaction is catalyzed by Pseudomonas aeruginosa exotoxin (17). The function of any gene product involved in the intoxication mechanism might be defined in a cell which is resistant to the toxin. Mutants in both hamster and human lines selected for resistance to the toxin have been studied (11, 14, 15, 21–23). In this paper, we describe biochemical and genetic studies with three mutants from different complementation groups.

Cells derived from rats and mice are ~10,000-fold more resistant to diphtheria toxin than cells from sensitive species such as humans, rabbits, and hamsters. These cells are resistant because diphtheria toxin is unable to penetrate to their cytoplasm, but it is not clear whether they lack the toxin receptor or whether they are unable to internalize bound toxin (2, 3). We included mouse 3T3 cells in our genetic studies to determine the capacity of these cells to complement the defects in our mutants.

MATERIALS AND METHODS

Cells, Media, and Culture Conditions

All the cells used in this study were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 0.02 M N-2-hydroxyethylpiperazine-N’-2-ethanesulfonate buffer (HEPES), pH 7.4, penicillin, 50 U/ml, and streptomycin, 50 µg/ml. The wild-type cell line from which mutants were isolated was the pseudodiploid male Chinese hamster V79 lung fibroblast originally described by Ford and Yerganian (12). Mouse 3T3 cells deficient in thymidine kinase were obtained from Dr. Uta Francke (Yale University, New Haven, Conn.). Cell numbers were determined with a Coulter model ZF cell counter (Coulter Electronics, Hialeah, Fla.). Chromosomes were counted after Giemsa staining of fixed metaphase cells.

Assays for the Effect of Toxin on Cells

Diphtheria toxin (lot No. D298) was purchased from Connaught Laboratories (Willodale, Ontario, Canada) and further purified with DE-52 chromatography by the general procedures of Collier and Kandel (5). 95% of the toxin was estimated to be in the nicked form as determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and 2-mercaptoethanol.

Purified Pseudomonas aeruginosa exotoxin and diphtheria toxin A chain were provided by Stephen Lory, Gary Gilliland, and Dr. John Collier (University of California, Los Angeles).

The response of cells to toxin was measured by the ability of intact cells to synthesize protein and by plating efficiency tests. Protein synthesis was measured by the incorporation of \([^{35}S]\)methionine into trichloroacetic acid-insoluble material from duplicate samples. At 48 h before an experiment, the cells were plated in 24-well Falcon plates (Falcon Labware, Div. Becton Dickinson Co., Oxnard, Calif.) at 2 x 10⁴ cells/cm² in normal medium. Just before the assay, the medium was replaced with Dulbecco’s modified Eagle’s medium containing ½ the normal amount of methionine. The reduced concentration of methionine did not affect the growth rate of cells during the time of the assay. After either a 2-h or a 23-h incubation at 37°C, 1.0 µCi of \([^{35}S]\)methionine was added and the incubation was continued. 1 h later, the cells were washed twice with phosphate-buffered saline containing 1.0 mg/ml of unlabeled methionine and dissolved in 0.1 ml of a solution containing 0.05% sodium dodecyl sulfate, 1 mg/ml deoxyribonuclease I, and 1.0 mM CaCl₂ and MgCl₂. Aliquots were placed on numbered squares of Whatman 3M paper and soaked in 5% trichloroacetic acid for 30 min, followed by two washes with 95% ethanol. The papers were dried and assayed for radioactivity in a liquid scintillation counter. In the absence of toxin, there were at least 5,000 cpm of \([^{35}S]\)methionine incorporated into trichloroacetic acid-insoluble material per well and all assays were done in duplicate. Cells were plated in 6-well Linbro dishes for plating efficiency experiments. Diphtheria toxin was added the next day and colonies were allowed to form for 7–10 d. Cells were stained after removal of medium with 0.5% aqueous crystal violet for 20 min and air dried after washing gently with water.  

Mutant Selection and Cell Fusion

Toxin-resistant mutants Dtx'-1 and Dtx'-3 were obtained after a prolonged exposure to diphtheria toxin while Dtx'-2 was obtained after a short exposure as described previously (11). Thioguanine resistance, a recessive trait (4), and ouabain resistance, a codominant trait (1), were introduced in that order into cells so that cell fusion experiments could be performed. In each case, 5 x 10⁶ unmutagenized cells were exposed to either 5 µg/ml thioguanine or 2 mM ouabain. The frequency at which these markers appeared spontaneously in cell populations was ~10⁻⁴ mutants per cell plated. Thioguanine-resistant mutants were thereafter maintained in 5 µg/ml of the drug to prevent the accumulation of revertants. Neither of these markers affected the response of cells to toxin. Cells to be fused were plated...
together each at 5 x 10^6 cells/ml in one well of a 24-well Falcon dish. Fusion was mediated the next day with 50% polyethylene glycol 6000 in medium as described by Davidson et al. (8). 12-24 h later, the cells were trypsinized and replated in 60-mm culture dishes. 24 h later, the medium was replaced with selection medium. When a hamster cell containing both thioguanine and ouabain resistance was fused with another hamster cell lacking these markers, the selection medium consisted of normal medium containing hypoxanthine, 10^{-4} M, thymidine, 10^{-3} M, methotrexate, 5.5 x 10^{-5} M, and ouabain, 10^{-3} M. This medium without ouabain was used when thioguanine-resistant hamster cells were fused with the thymidine kinase-deficient 3T3 cells. The frequency of hybrid formation was at least 0.1% in all cases. Non-fused controls were carried through the procedure for each experiment. Parent cells escaping the selection procedure were rare and occurred at frequencies far lower than fusion. The resistance phenotype of hybrids obtained from intraspecies fusions was determined for at least two hybrid clones from each cross. Wild-type V79 cells and the mutants had 20-22 chromosomes while the hybrids had 35-42 chromosomes. To avoid artifacts arising from chromosome loss in individual hybrid clones of fusions with mouse and hamster cells, single clones were not isolated but the resistance phenotype was measured for the entire population surviving the selection. These cells were not karyotyped because of their heterogeneity.

**ADP-Ribosylation Assays**

EF-2 was extracted from cells by the general procedure of Gill and Dinius (13) as modified by Moehring and Moehring (21). Cells from three roller bottles (~5 x 10^8 cells) were trypsinized, washed with phosphate-buffered saline, and suspended in cold 0.25 M aqueous sucrose to a volume of 3.0 ml. The cells were broken with 100 strokes in a tight-fitting Dounce homogenizer. Ammonium chloride was added to give 0.5 M, followed by addition of 1.5 g of activated charcoal (Norit neutral, Fischer Scientific Co., Los Angeles, Calif.). The mixture was incubated at 0°C for 20 min with frequent vigorous agitation and centrifuged for 1 h at 100,000 g. The supernates were withdrawn, aliquoted, and stored at -80°C until use. The reaction mixture contained 1.0 mM NAD' (nicotinamide [U-14C]adenine dinucleotide; Amersham, Inc., Arlington Heights, Ill.), 0.18 M histamine, 0.25 M Tris buffer, pH 8.1, varying amounts of extract, and either 10 μg of diphtheria toxin A chain or 26 μg of activated Pseudomonas exotoxin in a final volume of 0.1 ml. The Pseudomonas exotoxin was activated by pre-incubation in 4 M urea and 5 mM dithiothreitol for 20 min at room temperature. The reaction mixture was incubated for 30 min at 25°C, and aliquots were placed on numbered squares of Whatman 3M paper that had been presoaked in a 50% trichloroacetic acid in ether solution (wt/vol) and dried. The paper was washed twice in aqueous 5% trichloroacetic acid followed by two washes in 95% ethanol. The dried papers were assayed in a scintillation counter with a counting efficiency of 50% of 89%. The time, toxin, and NAD' were not limiting in this reaction and the amount of ADP-ribosylated EF-2 was a linear function of the amount of extract added. Protein was assayed by the method of Lowry et al. (19).

**RESULTS**

**Phenotypes of the Mutants**

We previously isolated 20 clones selected in a single step for diphtheria toxin resistance and classified them into one of four groups based on their response to toxin in protein synthesis assays (11). We extended this study to include cells arising from an additional 22 clones. We chose one mutant from three of these groups for the studies presented here. The distribution of resistant clones in the groups represented by Dtx'-1, Dtx'-2, and Dtx'-3 was 48, 7, and 17%, respectively. The effects on protein synthesis in these three mutants after a 3-h exposure to dipheria toxin are shown at the top of Fig. 1. Dtx'-1 was totally resistant at all concentrations tested. Dtx'-2 had a dose response curve parallel to that of the wild-type, but shifted ten or 20-fold to higher concentrations. Dtx'-3 showed initial sensitivity similar to that of wild-type cells, but then maintained 50% of normal protein synthesis independent of increases in toxin concentration. Similar results were obtained when the cells were exposed to toxin for 24 h, except that the final level of protein synthesis for Dtx'-3 was 20–30% of control synthesis.

The plating efficiencies of these mutants as a function of toxin concentration are seen at the bottom of Fig. 1. The plating efficiency of Dtx'-1 was reduced as the toxin concentration was increased. This behavior is not the result of a genetically mixed cell population since all subclones of this mutant were similar. The plating efficiency curve of Dtx'-2 was similar to the curve seen in protein synthesis assays. Diphtheria toxin had little effect on the plating efficiency of Dtx'-3; however, the size of the colonies was markedly reduced above 5 x 10^{-11} M toxin. The small size of these clones suggested that the toxin might affect the growth rate of Dtx'-3. To test this, we measured the growth rate of this mutant in the presence and absence of toxin. In Fig. 2, upper curve, 10^{-11} M toxin was added to cells in mid-log phase and left in the medium. A decrease in growth rate was observed. In the lower curve of Fig. 2, Dtx'-3 was
challenged for 2 h with $10^{-7}$ M toxin followed by removal of toxin from the medium. The intoxicated cells did not recover to a normal growth rate during the next week. This suggested that the intracellular toxin remained active. Diphtheria toxin fragment A is relatively stable to proteolysis (6), and it was recently shown that a single molecule of fragment A within a cell could cause cell death (29). The growth rate of Dtx'-1 was not affected by toxin while Dtx'-2 ceased to grow above $3 \times 10^{-10}$ M toxin.

To determine whether the growth rate in the presence of toxin correlated with the rate of protein synthesis in the mutants, the incorporation of radioactive methionine into protein was followed as a function of time. As shown in Fig. 3 A and B, wild-type cells and Dtx'-2 ceased protein synthesis within 30 min after exposure to a high concentration of toxin. The rate of synthesis in Dtx'-3 initially decreased but then remained at a constant rate (Fig. 3 C). As expected, the toxin had no effect on protein synthesis in mutant Dtx'-1 (data not shown).

**Diphtheria Toxin Catalyzed ADP-Ribosylation of EF-2**

The amount of toxin-sensitive EF-2 in cell-free extracts from each of the mutants is shown in Table I. The EF-2 from Dtx'-1 accepted almost no ADP-ribose, that from mutant Dtx'-2 was normal, and that from mutant Dtx'-3 had about half the activity of that of wild-type cells. The ADP-ribose
accepting activity of wild-type extracts mixed with either Dtx'-1 or Dtx'-3 were additive, as seen in Table I. This suggested that diffusible inhibitors of the reaction were not present in the mutants.

**The Effect of Pseudomonas Aeruginosa Exotoxin on Wild-Type and Mutant Cells**

Pseudomonas aeruginosa exotoxin inactivates EF-2 by ADP-ribosylation as does diphtheria toxin (17). The dose response curves for wild-type and mutant cells exposed to Pseudomonas exotoxin for 24 h are shown in Fig. 4. These curves are similar to those seen with diphtheria toxin for Dtx'-1 and Dtx'-3, while Dtx'-2 shows no cross resistance to Pseudomonas exotoxin. The effect of Pseudomonas exotoxin on the ADP-ribosylation of EF-2 in cell free extracts is shown in Table I. The results are nearly identical to those obtained with diphtheria toxin.

| Extract source | pmol of EF-2 ADP-ribosylated per mg protein by diphtheria toxin | Percent of pmol of EF-2 ADP-ribosylated per mg protein by Pseudomonas exotoxin | pmol of EF-2 ADP-ribosylated per mg protein by diphtheria toxin | Percent of pmol of EF-2 ADP-ribosylated per mg protein by Pseudomonas exotoxin |
|----------------|---------------------------------------------------------------|---------------------------------------------------------------|---------------------------------------------------------------|---------------------------------------------------------------|
| V79            | 88                                                           | 100                                                          | 100                                                          | 100                                                          |
| Dtx'-1         | 3                                                             | 3                                                             | 4                                                             | 4                                                             |
| Dtx'-2         | 94                                                           | 107                                                          | 97                                                           | 94                                                           |
| Dtx'-3         | 37                                                           | 42                                                            | 43                                                           | 42                                                           |
| Dtx'-1 + V79   | 41                                                           | 46                                                            | 56                                                           | 54                                                           |
| Dtx'-3 + V79   | 66                                                           | 75                                                            | 65                                                           | 63                                                           |

Each assay contained 100 µg of total extract protein. Mixed extracts contained 50 µg of protein from each source. The pmol of EF-2 are the average of duplicate samples.

**Figure 2** The growth rate of Dtx'-3 with and without diphtheria toxin. No toxin, O; toxin added (10^{-7} M) and left in media at the time indicated by the arrow, X; toxin added (10^{-7} M) 2 h before first point and then removed.

**Figure 3** The relative rates of protein synthesis with (O) and without (●) 10^{-7} M diphtheria toxin. A, Wild-type; B, Dtx'-2; and C, Dtx'-3.
**Dominant-Recessive Tests in Hybrids**

Hybrids formed from wild-type cells and each of the mutants were constructed. Their phenotypes in protein synthesis assays are shown in Fig. 5. The mutations in Dtx'-1 and Dtx'-2 were recessive. The mutation in Dtx'-3 appeared to be codominant since hybrids derived from this mutant expressed a toxin-resistant component of protein synthesis. The level of protein synthesis in these hybrids was intermediate between that of Dtx'-3 and that of wild-type cells, which suggested that...
the gene dose of the mutated allele may be important. Since the plating efficiency of Dtx'-3 was not affected by toxin, we measured the plating efficiency of the hybrids formed with this mutant. The plating efficiency of these cells as a function of toxin concentration was identical to that of wild-type cells (data not shown). The phenotype of Dtx'-3 was codominant when hybrids were tested by protein synthesis assays but recessive when the same hybrids were tested in plating efficiency assays. Gupta and Siminovitch (15) described a similar phenomenon in diphtheria toxin-resistant CHO cells.

**Complementation between Mutants**

To determine whether these mutants were independent of one another, we constructed hybrids derived from each pair. The phenotypes of these cells in protein synthesis assays are shown in Fig. 6. All hybrids containing the genome of Dtx'-3 expressed toxin-resistant protein synthesis similar to crosses between Dtx'-3 and wild-type cells while the hybrids formed with Dtx'-1 and Dtx'-2 had a wild-type phenotype. This suggested that the mutations causing each phenotype were different.

**Complementation Tests with Mouse Cells**

We crossed our mutants with mouse cells to determine the patterns of complementation, and the results are shown in Fig. 7. The resistance of mouse cells is known to be recessive in hybrids prepared with sensitive cells (7, 9). We found that fusions involving 3T3 cells and either V79 wild-type cells or Dtx'-2 produced sensitive hybrids, while some fraction of protein synthesis was resistant in hybrids containing the genome of Dtx'-3. There was, however, almost no complementation in hybrids formed with Dtx'-1 and 3T3 cells.

**DISCUSSION**

Mutants Dtx'-1 and Dtx'-3 both had reduced levels of ADP-ribose accepting activity (Table I). However, the two mutants were fundamentally different. Diphtheria toxin decreased the rate of protein synthesis and the rate of proliferation of Dtx'-3 to a low but steady-state level (Figs. 2 and 3C). The biphasic nature of these curves and the fact that extracts from Dtx'-3 had about half of the normal ADP-ribose accepting activity could be explained by the presence of both toxin-sensitive and toxin-resistant EF-2. This interpretation was proposed by Gupta and Siminovitch (15) after their study of diphtheria toxin-resistant CHO cells and predicts the presence of a normal gene for EF-2 and a mutated gene which produces a structurally altered EF-2. Direct evidence for the altered gene product is lacking because an EF-2 which is resistant to ADP-ribosylation has not been directly

**Figure 6** The dose response curves in protein synthesis assays of hybrids formed with pairs of mutants. The hybrids were exposed to toxin for 3 h. The cell line listed first in each cross contained the thioguanine- and ouabain-resistant markers. Dtx'-2 × Dtx'-1, ○; Dtx'-3 × Dtx'-1, ■; Dtx'-3 × Dtx'-2, ●.
measured. However, our genetic evidence supports this proposal. If an allele for a toxin-resistant EF-2 were present, it should be expressed in hybrids. The codominance of the mutation was demonstrated by the expression of ~25% of normal protein synthesis after toxin treatment in all hybrids containing the genome of Dtx'-3 (Figs. 5 and 6).

In contrast, the phenotype of Dtx'-1 was recessive in interspecies hybrids (Figs. 5 and 6) and no evidence for a toxin sensitive EF-2 was found. This implies that a structural change in all copies of EF-2 can be produced by a mutation in a locus other than that for EF-2. This could be explained by considering the structure of the amino acid to which ADP-ribose is covalently linked in EF-2; this residue is not commonly found in proteins (26) and it probably is the result of a post-translational modification of EF-2. If this modification is required for the activity of the toxin, then the absence of the appropriate enzyme could produce a mutant with the phenotype of Dtx'-1. This interpretation is also tentative without a direct assay for the altered EF-2.

Hybrids formed with Dtx'-3 and V79 cells carried the allele for toxin-resistant protein synthesis but they did not form colonies when exposed to toxin. The rate of protein synthesis was apparently below some critical level required for cell proliferation. This suggested that, unlike the case of RNA polymerase II (27), the level of resistant EF-2 was strictly dependent on gene dose with no compensating regulatory mechanism to increase its synthesis. This was also demonstrated by the effect of toxin on the growth rate of Dtx'-3 (Fig. 2); the rate of growth did not tend to increase toward normal after toxin treatment. The possibility that EF-2 may become rate limiting in protein synthesis is not unreasonable; it is believed that there is only a slight excess of EF-2 over the number of ribosomes in a variety of rat cells (13).

Most of the mutants we found had dose response curves similar to either Dtx'-1 or Dtx'-3 (48 and 17%, respectively). The frequent appearance of mutants similar to Dtx'-3 was reasonable because this phenotype was codominant; however, the phenotype of Dtx'-1 was recessive. One possible explanation is that the Dtx'-1 locus may be hemizygous in this cell line.

The EF-2 derived from mutant Dtx'-2 was as sensitive to ADP-ribosylation as that from wild-type cells (Table I). Mutants of this general type have been classified as permeation mutants by Moehring and Moehring (21, 22); they cannot either properly bind toxin or efficiently transport the receptor bound protein to the cytoplasm. Dtx'-2 is interesting because we showed that the affinity of a toxin analogue for the receptor of Dtx'-2 was decreased about 13-fold (11). This could be the
result of a structural change in the receptor itself or the result of a change in the microenvironment around the receptor. Whatever the alteration is, the mutation producing it is recessive in hybrids with wild-type cells and with other mutants (Figs. 5 and 6). Unlike the mutations which probably affect the structure of EF-2, Dtx'-2 has no resistance to Pseudomonas exotoxin (Fig. 4). This clearly suggests that diphtheria toxin and Pseudomonas exotoxin do not share a common receptor. Diphtheria toxin-resistant CHOKI cells that are not resistant to Pseudomonas exotoxin have been noted before by Moehring and Moehring (21), but the basis for their resistance to diphtheria toxin is unknown.

Mouse cells have a naturally occurring phenotype of diphtheria toxin resistance. We prepared hybrids with mouse 3T3 cells and our mutants to examine the genetic character of this phenotype. The mouse phenotype was recessive in hybrids with wild-type cells, demonstrating that the normal hamster genome could confer sensitivity to the hybrids (Fig. 7). Dtx'-1 and mouse cells produced a population of hybrids whose capacity for protein synthesis was still 60% of normal after treatment with 10^{-8} M toxin. This was an unexpected result since the phenotype of Dtx'-1 was recessive in hybrids formed with other hamster cells. Several explanations are possible. If Dtx'-1 is missing an enzyme which modifies EF-2, the corresponding enzyme produced by a mouse gene in the hybrids may not recognize hamster EF-2. The unmodified EF-2 could account for the toxin-resistant portion of protein synthesis found in the hybrids. It is also possible that some genetic information from the mouse genome was silenced by the hamster genome or was lost as a result of rapid chromosome segregation. Protein synthesis in hybrids made with Dtx'-3 was depressed to 20 or 30% of normal by toxin, suggesting that EF-2 from both parental cells was present. Hybrids formed with Dtx'-2 were as sensitive to toxin as the hybrids derived from wild-type hamster cells and did not display the phenotype of Dtx'-2. There are conflicting reports on whether or not mouse cells have a diphtheria toxin receptor (2, 3). If they do, the sensitivity of these hybrids could be explained by expression of the mouse receptor. If they do not, the mouse genome at least has the information to restore the receptor or the environment around it to normal.

We thank John Collier, Gary Gilliland, and Stephen Lory for assistance with the EF-2 assays.

This work was supported by a California Research Coordinating Committee grant (CRCC-77SD11) and by the National Science Foundation (NSF PCM77-26862). R. Draper was supported by a National Institutes of Health Postdoctoral Fellowship (USPHS-GM-07199) and D. Chin by a National Institutes of Health Predoctoral Training Grant (USPHS-GM-7313).

Received for publication 15 January 1979, and in revised form 1 June 1979.

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