A mutant herpes simplex virus 1, mtHSV, was constructed by inserting the E. coli beta-galactosidase gene into the loci of icp34.5, the apoptosis-inhibiting gene of HSV. The mtHSV replicated in and lysed U251 (human glioma cells), EJ (human bladder cells), and S-180 (mice sarcoma cells), but not Wish (human amnion cells) cells. With its intact tk (thymidine kinase) gene, mtHSV exhibited susceptibility to acyclovir (ACV), which provided an approach to control viral replication. An in vivo test with mtHSV was conducted in immune-competent mice bearing sarcoma S-180 tumors, which were treated with a single intratumoral injection of mtHSV or PBS. Tumor dimensions were measured at serial time points, and the tumor volumes were calculated. Sarcoma growth was significantly inhibited with prolonged time and reduced tumor volume. There was microscopic evidence of necrosis of tumors in treated mice, whereas no damage was found in other organs. Immunohistochemical staining revealed that virus replication was exclusively confined to the treated tumor cells. HSV-1 DNA was detected in tumors, but not in the other organs by a polymerase chain reaction analysis. From these experiments, we concluded that mtHSV should be a safe and promising oncolytic agent for cancer treatment.

Keywords: Cancer gene therapy, Herpes simplex virus-1, icp34.5, Oncolysis, Sarcoma

Introduction

In 1996, McCormick’s group proposed that dl1520 (ONYX-015), an E1B-55kDa-deleted adenovirus mutant, would selectively replicate in p53-deficient cells (Bischoff et al., 1996). The molecular basis is that the E1B-55KD protein can bind and inactivate P53 in normal cells in order to initiate virus replication, so that infection of the E1B-55kDa-deleted mutants will lead to apoptosis in normal cells before viral replication. Only cancer cells with nonfunctional P53 are permissible for replication of these mutants. Although this hypothesis was subsequently challenged (Edwards et al., 2002), the use of replication-conditional (oncolytic) viruses to deliver anticancer genes and to lyse tumors cells has become one of the most intensive research areas in virus-based cancer therapy (Alemany et al., 2000).

Adenovirus, reovirus, and herpes simplex virus type 1 (HSV-1) were used to treat various experimental tumors in animal models. Compared to other viruses, HSV-1 has its own advantage since it has a large volume of foreign genes, a wide host range, and high infection efficiency (Roizman, 1996). In fact, since 1996, engineered replication-conditional HSV-1 has been used to treat a wide variety of experimental malignancies, including the brain, breast, ovarian, and other cancers (Andreansky et al., 1996; Mckie et al., 1996). Most of these studies demonstrated the efficacy of HSV mutants as anti-cancer agents, both in vitro and in vivo.

Two types of oncolytic HSV-1 mutants have been studied. One consists of viral mutants with defects in the function for nucleic acid metabolism, including thymidine kinase (tk), ribonucleotide reductase (RR), or uracil-N-glycosylase. The other consists of mutants with deletions of the neurovirulence gene, icp34.5 (infected-cell protein 34.5 gene). The icp34.5 gene of HSV is absolutely required for a neurovirulent phenotype of HSV-1 in animal models, but is not required for viral replication in some cell lines in vitro (Roizman, 1997). It is thought to block apoptosis in some cells by regulating the interferon-inducible PKR pathway (He et al., 1997). An in vivo experiment indicated that these attenuated HSVs, with

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Abbreviations: PFU, plaque-forming unit; HSV, herpes simplex virus; S-180, sarcoma-180; ACV, acyclovir; IHC, immunohistochemistry; tk, thymidine kinase.
deletions of the icp34.5 gene, still retain their ability to replicate in malignant tumor cells, but not in normal tissues (Mackie et al., 1996; Varghese et al., 2001).

Soft tissue sarcomas comprise approximately 1% of all adult malignancies and can have life-threatening outcomes (Beach, 1999). The overall prognosis is poor in spite of multimodality therapies such as surgery, chemotherapy, and radiotherapy. New methods need to be explored to treat this disease. In the present study, we show that a mutant herpes simplex virus (mtHSV) was constructed by insertion of a bacterial-galactosidase gene (lacZ) into both copies of icp34.5 of the HSV-1 genome. It replicates preferentially in some tumor cells with an activated ras in vitro and replicates selectively in mice sarcoma and bone tumors in vivo. Tumor regression was achieved by a single intratumoral injection of mtHSV. No toxicity or adverse side effects were observed. Here, mtHSV serves as a safe and highly efficacious anti-cancer agent to target malignant mice soft tissue and bone tumors in immune-competent mice, and it is a promising anti-cancer strategy for the future.

Materials and Methods

Cell lines, plasmids, viruses, and animals Vero (African green monkey kidney), U251 (human glioma), EJ (human bladder tumor), and Wish (human amnion) cells were obtained from CCTCC (Chinese Center of Typical Culture Conserve, Wuhan, China). All of the cells were grown in Dulbecco’s minimal essential medium (DMEM, Gibco/BRL, Grand Island, USA) that was supplemented with 2 mM L-glutamine, 8% fetal bovine serum (FBS), 100 units of penicillin, and 0.1 mg of streptomycin per ml of medium in an atmosphere containing 5% carbon dioxide at 37°C. Mice sarcoma S-180 cells were also obtained from CCTCC and were maintained by intraperitoneal passage in BALB/c mice (Experimental Animal Center, Wuhan, China).

The HSV-1 strain F (wild type) and plasmid pRB4789 were kindly provided by B. Roizman. The plasmid pRB4789 contained a 2.9 kb BamHI S fragment of HSV-1 genome sequences, including the icp34.5 gene. Plasmid pSV-beta-galactosidase, in which the beta-galactosidase gene was under the control of a SV40 early promoter, was purchased from the Promega (Madison, USA).

To produce virus stocks, subconfluent monolayers of Vero cells were infected with virus at an MOI of 0.1. Then 48 h after infection, the cells and supernatant were harvested and subjected to freeze-thaw cycles. Viral output was determined by a plaque assay on the Vero cells and stored at −70°C.

Specific pathogen-free BALB/c mice were obtained from the Experiment Animal Center (Wuhan, China) and used at approximately 4-5 weeks of ages.

Engineering of recombinant virus The plasmids that were used for the engineering of mtHSV by the homologous recombination were designed to insert the β-galactosidase gene (lacZ) into the icp34.5 genes of the HSV-1 genome. The recombinating plasmid was engineered as follows: The full-length lacZ gene and SV40 early promoter were excised as an EcoRI-BamHI fragment from plasmid pSV-β-galactosidase and subcloned into pFastBac1 (Promega) to get the first transfer plasmid pFL, in which a NotI restriction endonuclease site is upstream of the lacZ cassette. Then the Xhol-BamHI fragment of pFL that contained the lacZ castee was subcloned into pBluescript (Promega) to construct the second transfer plasmid, named pBL, so that another NotI site was downstream of the lacZ cassette. The plasmid pRB4789 that contained the icp34.5 gene was digested with NotI to remove the 750bp coding sequence of icp34.5, except that 650 nucleotides at the 5’ end and 1500 nucleotides at the 3’ end remained as homologous flanking sequences. The gel purified-NotI fragments from pBL (lacZ) and pRB4789 were ligated, resulting in the formation of recombinating plasmid pIL.

HSV-1 F DNA was extracted after the lysis of infected Vero cells with SDS-proteinase K, repeated phenol-chloroform extraction, and ethanol precipitation. The plasmid pIL DNA was extracted by the standard large-scale alkaline lysis procedure. Monolayer Vero cells were co-transfected by HSV-1 F DNA and plasmid pIL DNA with lipofectAMINE™, according to instructions that were provided by the manufacturer (GIBCO/BRL). Three days later, the cytopathic effect (CPE) was obvious and virus progeny was harvested by freeze-thawing cycles. The progeny was replated on Vero cells at various MOI. Then 36-48 h later, the selective medium (0.5% agar and 300 μg/mL X-gal) was changed into the dish. The blue plaques were selected and purified through two additional rounds of plaque purification, followed by a PCR procedure with the primer pairs within the deleted region, “gac gag gac tgg cgg gac ag” (367nt of the icp34.5 coding sequence) and “acg gcc ctc ggg ccc cag gc” (878nt of the coding sequence). One plaque without the targeted 510 bp product was chosen and identified as mtHSV. Infected titers (PFU/ml) of the parent and mutant virus were determined by standard plaque assays.

A Southern blot analysis was performed on viral genomes after digestion with NcoI. Biotin-labeled probes included the BbsI fragment of lacZ from the plasmid pSV-beta-galactosidase and NotI fragment of the icp34.5 gene from pRB4789. After the viral genomes were transferred to hybrid membranes, hybridization was performed using a commercial kit, following the suppliers instructions.

In vitro cytotoxic and viral replication assay For the cytotoxic assays, U251, EJ, Wish, and S-180 cells in 96-well plates were infected with mtHSV at MOI of 0.1 and 1. The cells were harvested at 0, 24, 48, 72, and 96 h after infection. Viable cells, which were trypan blue excluded, were counted on a hemocytometer on the postinfection days and expressed as a percentage of mock-infected cells.

For the virus replication assay, a monolayer culture of U251, EJ, Wish cells in 24-well plates, and freshly-harvested S-180 cells, which were also seeded into 24-wells plate and adjusted to 2.5 x 10^7/ml with DMEM containing 1% FBS, were infected with mtHSV at the MOI of 0.1 PFU/cell. The cells and medium were harvested at 24, 48, and 72 h and titered on Vero cells by a standard plaque assay.

For the acyclovir (ACV) sensitivity assay, acyclovir (Qianjiang Pharmacy, China) was dissolved with DMEM into a concentration range. Subconfluent monolayers of the Vero cells that were grown in 96-well plates were exposed to the icp34.5-defective HSV
(mtHSV) or wild type HSV-1 F. After absorption for 1.5 h at 37°C, the cells were rinsed and incubated with a range of concentrations (0.001-10 μg/ml) of acyclovir in DMEM for 24 h. DMEM of a similar volume served as the untreated control. The supernatant and cells were harvested 24 h after infection, and titred on Vero cells by the standard procedure.

Treatment of mice bearing sarcoma tumors For the survival studies, each BALB/c mouse (age, 4-5 weeks; weight, 22-25 g) was inoculated with freshly-harvested 2 x 10⁶ S-180 cells in the armpit of the left upper limb. On day 4, when the average diameter of the tumors reached about 4 mm, the tumor-bearing mice were randomly divided into groups (30 mice/group) and were given a single intratumoral injection of 5 x 10⁶ PFU mtHSV or PBS in the volume of 0.1 ml. Every 2 days the tumor growth was determined by measuring the tumor with external calipers. Tumor volumes were calculated using the formula 0.53 x length x width (Yamamura et al., 2001). The mice were assessed daily, moribund mice were killed, and the date of death was recorded as described (Andreansky et al., 1998).

Pathology and immunohistochemistry (IHC) analysis The tumor-bearing mice treatment (30 mice/group) was previously described. After mtHSV was injected into the tumors, the mice were sacrificed at 7 day intervals. The tumor, brain, heart, lung, both kidneys, and liver of each mouse were removed. The specimens were fixed in 10% formalin and embedded in paraffin blocks, followed by section and hematoxylin-eosin staining. A pathology analysis was carried out under a light microscope. An immunohistochemistry (IHC) assay was also applied to the slides of these tissues. Rabbit polyclonal antiserum to HSV-1 (DAKO, B0114) was used to detect replicating HSV at a dilution of 1:2000 (Kesari et al., 1998). A biotinylated secondary antibody was then added and visualized with streptavidin-labeled horseradish peroxidase and chromogen solutions. Counterstaining with harris hematoxylin was performed. The tumor cells that stained brown or red are positive for the HSV-1 infection.

PCR Genomic DNA of the removed tissues was isolated as previously described. The extracted DNA (100 ng) was subjected to a PCR analysis using two sets of primers that were specific for HSV-1. The first set was described by Lakemany (Lakemany et al., 1995), 5'-ata cac ttc gac tgg ccc tt-3' and 5'-cgg tac atg tgg ttc ac-3', which coded for the DNA polymerase gene (UL30) giving a 189 bp product. The other one, 5'-acg acg aca tct gag gac ca-3' and 5'-gtt ctt ctt ctt gac ac-3', described by Puchhammer Stock in 1993 (Puchhammer et al., 1993), was specific for the DNA polymerase accessory protein (UL42) region of HSV-1, which generates a 279 bp product after amplification.

The reactions were performed in 100 μl volumes that contained 200 mM each deoxynucleoside triphosphate (Promega), 2 units of Taq polymerase with a PCR buffer (Promega). After one cycle of a 5 min denaturation at 94°C, then 35 cycles of amplification was carried out as follows: 94°C for 1 min, 58°C for 1 min, and 72°C for 2 min. The final cycle was terminated with a 5 min extension at 72°C. The DNA negative controls were prepared from uninfected animals and positive controls that consisted of DNA from virus stock. The experiments were repeated twice.

Statistical analysis A statistical analysis was performed using the Student's t-test. The difference was considered statistically significant with p<0.05.

Results

Construction and identification of mtHSV Mutant HSV was engineered by inserting the lacZ gene into both copies of the icp34.5 gene of the parent virus, HSV-1 F. As illustrated in Fig. 1, most of the icp34.5 gene coding sequence was deleted (from 132nt to 952nt) in mtHSV. The β-galactosidase gene under the control of the SV40 early promoter was expressed in Vero cells after viral infection. The virus construct was verified by a Southern blot analysis (Fig. 2).

Cytotoxicity of mtHSV and viral replication in vitro To measure the cell viability, four cell lines were infected with mtHSV, and typhblue-excluded assay was used to determine the viral cytotoxicity. As illustrated in Fig. 3, at MOI of 1, the mortality of U251 and EJ cells was 70% and 54% in the 48-h post-infection, 100% and 95% in the 72-h post-infection, respectively. S-180 was also sensitive to the mtHSV infection. The mtHSV had only a small influence on the Wish cells. At MOI of 0.1, U251 was again the most sensitive cell line to mtHSV, followed by EJ and S-180 cells, but the Wish cells were resistant to the mtHSV infection. All of the cell lines, except Wish, were completely destroyed by mtHSV within 5-6 days at MOI of 0.1. The mtHSV was able to proliferate in all of the cancer cell lines that were tested (Fig. 4). U251, the most sensitive cell line to mtHSV cytotoxicity, displayed the earliest time of peak viral production on day 2, with an approximately 10⁷ PFU viral titer. EJ and S-180 showed peak

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viral proliferation with 10^6 PFU viral titers from days 3-4. The mtHSV did not replicate as efficiently in Wish cells. There was almost no increase in viral titer noted by day 6.

To determine whether mtHSV remained sensitive to antiviral drugs, the effect of ACV on the virus-infected Vero cells was examined. At 24 h after infection, the virus was harvested, and the ACV sensitivity of mtHSV was compared with that of the parental strain by a plaque assay (Fig. 5). The result demonstrated that mtHSV retained similar sensitivity as wtHSV to acyclovir (p>0.05). One µg/ml of acyclovir inhibited 40% mtHSV and 45% wtHSV replication. When the concentration of acyclovir reached 10 µg/ml, the replication of both mtHSV and wtHSV were substantially inhibited. Such sensitivity provided a method to control the unanticipated infection during the clinical application.

Anticancer efficiency of mtHSV in vivo In order to determine the potential of mtHSV for treatment of mice sarcoma, the mice sarcoma model was established by a subcutaneous implantation of S-180 cells into immune competent mice. The tumors were then treated by an intratumoral injection of mtHSV or PBS. The antiviral potency of mtHSV was evaluated by tumor volume, scores of tumor necrosis, and survival time of the mice. Compared with the mock-infected controls, mtHSV caused a significant reduction in S-180 tumor growth (p<0.05) (Fig. 6, Table 1). Survival was significantly prolonged in the mtHSV-treated group with mean survival times of 55.8 days, as compared to animals receiving PBS that showed a mean survival time of 34 days (p<0.01). The average volume of mtHSV-treated S-180 tumors was 306.5 mm^3 when compared to 562.6 mm^3 for the PBS-treated control tumors on day 60 (p<0.05) (Fig. 6). Two out of nineteen tumor-bearing animals demonstrated complete tumor regression.
Pathologic and histological findings The pathology analysis result showed that organs (except tumors) were damaged by mtHSV. There was evident necrosis in the injected tumors (Fig. 7); whereas the brains, hearts, kidneys, lungs, and spleens showed no pathological changes even after 4 weeks of treatment.

The IHC assay showed that positive cytoplasmic and nuclear staining existed in treated tumor cells with patch areas of necrosis, but not in brains or other removed tissues. In all of the animals, the antigen was seen in sarcoma cells from the virus treated nodules with no antigen staining in the adjacent normal connective tissue. Because the antibody can detect the major glycoprotein that is presented in the virus envelope and at least one core protein, it was used to detect the replicating virus (Kerasi et al., 1998). In the S-180 bearing mice that were treated by an injection of mtHSV, an immunohistochemical examination of the excised mtHSV-injected nodules showed evidence of viral replication within the limits of the tumor mass. Necrosis was evident, even at day 7 of the injection, and this region was devoid of expression. The extent of antigen presentation in the sarcoma nodules supports the assumption that tumor necrosis was induced by virus replication.

PCR The spreading of viral DNA in the treated tumor tissues, but not in brains, lungs, livers, kidneys, and hearts, was demonstrated by PCR amplification with the primers that are specific to the genes that are associated with DNA replication, which amplify the sequences of 189 and 279 bp, (Fig. 8). The persistence of viral DNA in tumor tissue was detected by PCR from 7-28 days after an injection; whereas no targeted fragment was detected in other tissues.

Table 1. Therapeutic effect of mtHSV for mice bearing S-180 tumors

<table>
<thead>
<tr>
<th></th>
<th>Mean days</th>
<th>Survival</th>
<th>Cure ratio</th>
<th>Mean tumor volume when died (mm³)</th>
<th>Mean tumor weight when died (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mock group</td>
<td>34</td>
<td>0/20</td>
<td></td>
<td>562.6</td>
<td>0.667</td>
</tr>
<tr>
<td>mtHSV treated</td>
<td>55.8</td>
<td>2/19</td>
<td></td>
<td>306.5</td>
<td>0.334</td>
</tr>
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Discussion

A major challenge of cancer gene therapy for solid tumors is to increase efficacy of the intratumoral distribution of viral vectors. A replication-defective viral vector cannot diffuse well in a large tumor mass, which would lead to low therapeutic efficiency. The oncolytic viruses, also known as conditional-replicative viruses, can spread from initially-infected cells to surrounding tumors cells, thus enhancing the antitumor effects (Alemany et al., 2000). As a well-studied virus, HSV-1 is an ideal candidate to be modified as an oncolytic virus for its large volume for foreign genes and ability to establish latency (Roizman, 1996). Genetic engineering techniques have allowed disease-associated genes to be deleted while retaining the virus’s ability to infect, replicate in, and lyse a variety of malignant tumor cells. Moreover, these mutants with the intact tk gene are sensitive to antiviral drugs, such as acyclovir and ganciclovir. Therefore, these drugs can be used as a safety measure to treat HSV infection in the event of undesired infection or toxicity, and may improve therapeutic efficacy through the bystander effect.

Since 1996, several HSV icp34.5 mutants have been studied as oncolytic virus (Andreansky et al., 1996). The molecular mechanism of targeting oncolysis of the icp34.5-/- mutant is not fully understood, although ICP34.5 has been shown to prevent the shut-off of protein synthesis in human cells (He et al., 1997). Normally, protein synthesis inhibition in virally-infected cells is a result of the protein kinase R (PKR)-mediated phosphorylation and inactivation of eIF-2 alpha, which prevents the premature termination of protein synthesis. ICP34.5 binds protein phosphatase 1 (PP1) through its carboxyl terminus, forming a high-molecular-weight complex that dephosphorylates the alpha subunit of the translation initiation factor eIF-2 (eIF-2alpha). Therefore, this enables sustained protein synthesis and results in the inhibition of the translation of viral transcripts (He et al., 1996; 1997). Moreover, Leib et al. found that the growth of the icp34.5 mutant was enhanced up to 1,000-fold in the eyes and trigeminal ganglia of the IFNR (interferon-receptor)-knocked mice. It exhibited wild-type replication and virulence in a host from which the PKR gene was deleted (Leib et al., 1999). Thus, the in vivo target of ICP34.5 may be the host IFN response, and the malignant cells with a defect in the IFN-PKR pathway would be susceptible targets to the HSV icp34.5 mutant. As we know, there are many cancer cells with defects in the IFN-mediated pathway (Lee, 2002). By regulating the IFN-inducible PKR pathway, infection of
Another oncolysis virus, reovirus, was shown to exploit the ability of an element of the cellular ras signaling pathway to down-regulate PKR (Strong et al., 1998). Transformed cells with an activated ras signaling pathway are therefore particularly susceptible to reovirus infection. Recently, another independent group found that as is the case with reovirus, the ras signaling pathway is also the major determinant of host cell permissiveness to the HSV-I-derived oncolytic mutant (Frassati et al., 2001). The icp34.5 mutant was also found to preferentially infect both the ras-transformed cells and PKR−/− (but not PKR+/+) mouse embryo fibroblasts. Inhibitors of the ras signaling pathway, such as the farnesyl transferase inhibitor 1 and PD98059, effectively suppressed the HSV-1 infection of ras-transformed cells. The malignant cell lines that were used in our experiment demonstrated this preference. Sequence analyses had previously revealed that U251 cells contained a mutation in the N-ras gene (Tsuda et al., 1995). EJ-ras is a well-used oncogenic mutant of the cellular Ha-ras (valine substituted for glycine at amino acid 12) (Parada et al., 1985) and S-180, it is also in a constitutive “p21ras-on” state of the transformed cells (Efferth and Volm, 1992). Considering the fact that approximately more than 30% of all human cancers have a ras mutation that results in constitutive activation, and that other cancers may have defects in their ras signaling pathways, even without the ras gene mutation, then a ras-selective oncolytic virus is of great significance.

HSV-1 is a prevalent pathogen that infects 60% to 90% of the adult population. During the acute infection or reactivation of the latent virus, the immune response is significant. The ultimate result could be corneal blindness or fatal sporadic encephalitis (Kerasi et al., 2001). It is unclear whether the host immune system complements or compromises tumor cell

Fig. 7. IHC staining of section from the post-injection nodule (A, B, and C) and brain (C, C ×400) excised from the treated mouse. Tumor tissue immunostaining revealed the presence of viral antigen in the remaining tumor cells. Treated tumors displayed areas of necrosis (B), whereas the brain remained normal (C). No brown reaction product is seen in the brain (C) and untreated tumor cells (D, D ×400).

Fig. 8. Distribution of viral DNA in the tissues of treated mice by PCR amplification. Isolated DNA from the tumor, brain, kidney, lung, liver, and heart of the treated mice were subjected to PCR analysis. DNA were obtained at day 21 after the injection of mHSV. The 189-bp and 279-bp reaction product is indicated.

mHSV leads to apoptosis in some non-permissive “normal” cells and oncolysis in IFN-response-defected cells, respectively.
killing by oncolytic viruses. Experiments in an immune-
competent animal will provide this important information
since the overwhelming majority of the in vivo tests were
carried in immune comprised animals. It is encouraging that
no induction of encephalitis, no adverse symptoms, and no
damages to important organs were observed in the treated-
BALB/C mice in our experiment. In a study that was reported
by Kesari et al., the icp34.5 deficient HSV-1 replication was
observed in normal cells in the basal layer (Keraki et al.,
1998), but we found that HSV antigen was exclusively
confined to the tumor cells in the injected nodules.

HSV icp34.5 mutants have been used to treat experimental
tumors. Recent phase I clinical trials indicate that the icp34.5
deletion mutant-based vectors are safe (Varghese et al., 2001).
Our study also demonstrated that mtHSV may take advantage
of an activated ras pathway in some tumor cells and lead to
selective oncolysis. In mice that are infected with mtHSV, the
brain and other organs were not infected or damaged, even
after 3 weeks of treatment. This observation supports the
notion that it is a safe viral vector. We concluded that mtHSV
may serve as a good anti-cancer agent. We anticipate that
some modifications will enhance the therapeutic safety and
efficacy of the HSV-based oncolytic virus. These
modifications would include inserting the anti-cancer gene into
icp34.5 instead of the lacZ gene, inserting a cancer-
associated antigen promoter before a replication-required gene such as icp4 or icp27, deleting some other neurovirulent
genes, etc.

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