Lipopolysaccharide enhances HSV-1 replication and inflammatory factor release in the ARPE-19 cells

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ABSTRACT

Purpose: During acute retinal necrosis (ARN), retinal pigment epithelial (RPE) cells could be stimulated by both herpes simplex virus (HSV) and lipopolysaccharide (LPS). We aim to investigate the impact of LPS on HSV-1 infection and inflammatory factors in human retinal pigment epithelial cell lines (ARPE-19 cells).

Methods: ARPE-19 cells were infected by HSV-1F strain and HSVg4 strain, a modified HSV strain with GFP genes cloned in, for 1 h. Different concentrations of LPS were added. Green fluorescence protein (GFP) of HSVg4 and the infected cell protein 4 (ICP4) expression were observed. Cell culture supernatants were collected to detect 34 kinds of related cytokines and chemokines by multiplex immunoassay assay.

Results: Under LPS treatment, the cytopathic effect displayed as enlarged multinucleated cells, and the GFP fluorescence intensity and ICP4 expression increased in the HSV-1-infected ARPE-19 cells. HSV-1 infection stimulated cytokines IL-1α, IL-1β, IL-1RA, IL-2, IL-4, IL-6, IL-9, IL-12P70, IL-15, IL-18, IL-21, IL-27, TNF-α, IFN-γ and chemokines CXCL1, CXCL8, CXCL10, CXCL12, CCL2, CCL3, CCL4, CCL5, CCL11 while LPS further enhanced their expression.

Conclusion: LPS promoted HSV-1 infection and inflammatory factor release in ARPE-19 cells, indicating that ARN could deteriorate when complicated with endotoxemia.

1. Introduction

Herpes simplex virus type 1 (HSV-1) is a linear double-stranded DNA virus with 152 kb genome. HSV-1 infects the majority of the human population and distributes worldwide (Smith and Robinson, 2002). Common ocular manifestations of HSV-1 infection include blepharitis, conjunctivitis, keratitis, uveitis and retinitis (H and C, 2013). HSV-1 is the second common cause of acute retinal necrosis (ARN) (Lau et al., 2007; Roy et al., 2014), and the leading cause of bilateral ARN (Miserocchi et al., 2018). ARN is a significant cause of visual impairment in human immunodeficiency virus (HIV)-positive patients and always leads to severe complications such as occlusive vasculopathy, retinal detachment and optic neuropathy (Ganatra et al., 2000; Guex-Crosier et al., 1997; Jabs et al., 1987; Lau et al., 2007; Roy et al., 2014; Stewart, 2013). Moreover, herpes virus lesions can be complicated by inflammation and secondary bacterial infections.

Lipopolysaccharide (LPS) is a central component of the outer membrane in Gram-negative bacteria and closely related to bacterial pathogenicity (Needham and Trent, 2013; Scott et al., 2017). During infection, it binds to the toll-like receptor 4 (TLR4) on the cellular surface and induces vigorous immune responses (Sassi et al., 2010; Seeley and Ghosh, 2017). In the outer blood-retina barrier, LPS induces inflammation and cell death in retinal pigment epithelial (RPE) cells (Ozal et al., 2018). It is also the common cause of sepsis (Pérez-Hernández et al., 2021). LPS may get into the bloodstream by multiple methods and cause endotoxemia (Munford, 2016). Endotoxemia is found in various situations, including obesity, nonalcoholic fatty liver disease, acquired immune deficiency syndrome (AIDS) and bacterial infection (Boutagy et al., 2016; Munford, 2016; Sandier and Douek, 2012; Xue et al., 2017).

RPE cells are monolayer cells that perform various functions, such as light absorption, phagocytosis, and maintenance of immune privilege.
They could be infected by HSV-1 during ARN and play a role in the pathogenesis of HSV-1 retinitis (Kashiwase et al., 2000; Moses et al., 2014; Tiwari et al., 2008). Zheng et al. found that RPE cells migrated into the ARN lesion and produced cytokines in vivo (Zheng and Atherton, 2005). Besides, ARN might be complicated by endotoxemia in AIDS patients, thus RPE cells, part of the outer blood-retina barrier between the systemic circulation and the retina, may be influenced by LPS during ARN (Dillon et al., 2014; Sandler and Douek, 2012). It is still unknown whether LPS stimulation affects HSV-1 replication and subsequent immune responses such as cytokine secretion in RPE cells. Our previous research elucidated that LPS promoted HSV-1 infection in corneal epithelial cells and African green monkey kidney (VERO) cells when added after 1-hr

**Figure 1.** Lipopolysaccharide (LPS) enhanced herpes simplex virus 1 H129-G4 strain (HSVg4) infection in human retinal pigment epithelial cell lines ARPE-19 cells. The cells were infected with HSVg4 at MOIs of 0.01, 0.1 and 1 for 1 h and then treated with LPS at 0, 10, 50 and 100 μg/ml for 24 h. The control cells were not infected with HSVg4. A. The cytopathic effects (CPE) of ARPE-19 cells under different conditions. The CPE is presented as enlarged multinucleated cells with diaphanous margins. Typical CPE was magnified in Figure 1A. Scale bar = 100 μm. B. The correlated green fluorescent protein (GFP) image of HSVg4 in ARPE-19 cells. GFP fluorescence represents HSV-1 replication in ARPE-19 cells. C. The relative fluorescence values in ARPE-19 cells under different conditions. Compared to the group of LPS 0 μg/ml in different HSV-1 concentrations, **p < 0.01. n = 5.
infection (Zeng et al., 2020). In this study, we aimed to investigate the impact of LPS on HSV-1 replication and cytokine/chemokine release in ARPE-19 cells.

2. Material and methods

2.1. Cells, virus infection and LPS stimulation

ARPE-19 cells (human retinal pigment epithelial cells, ATCC® CRL-2302™) were cultured in DMEM/F12 supplemented with 10% fetal bovine serum (Gibco, NY, USA) at 37 °C in a 5% CO₂-95% air incubator. HSV-1 F strain and HSV-1-H129-G4 strain (HSVg4) were used to infect ARPE-19 cells as previously described (Duan et al., 2012a, 2012b; Zeng et al., 2020). HSVg4 is a replication-competent recombinant virus with green fluorescent protein (GFP) genes inserted in the genome modified by Professor Minhua Luo from Wuhan Institute (Zeng et al., 2017). Briefly, ARPE-19 cells were cultured to 80% confluence, then the cells were infected with HSV-1 for 1 h at multiplicities of infection (MOIs) of 1, 0.1, 0.01 and gently shaken every 15 mins to allow viral absorption. After 1 h, the inoculum was removed and the medium was replaced with serum-free DMEM/F12 medium containing different dosages of LPS (Sigma-Aldrich, St. Louis, MO, USA, 0 μg/ml, 10 μg/ml, 50 μg/ml and 100 μg/ml). Photography and assays were conducted at the indicated time points.

2.2. Morphological observation

As HSV-1 infected ARPE-19 cells, cells displayed typical morphologic changes called cytopathic effect (CPE). Photography of cells was captured at 24 h post infection (p.i.) under light microscopy to observe CPE at different MOIs (Duan et al., 2012b; Enlander et al., 1974; Zeng et al., 2020). In typical CPE, infected cells round up, coalesce and become flat enlarged cells containing several nuclear prominences. The margins of infected cells turn diaphanous. Besides, virus release is consistent with the appearance of rounded and vacuolated cells (Pereira, 1961). As the virus titer grows, the ratio of viruses and cells per area increases, and more cells are infected and coalesce into a bigger syncytial cell. Therefore, the diameter of multinucleated cells increases under higher virus titers. Photos of infected cells were taken under a phrase contrast microscope (Nikon Eclipse TS100F, Tokyo, Japan) and the comparison of virus titers was made based on the diameters of multinucleated cells.

2.3. Fluorescence microscopy

With GFP genes cloned in the genome, green fluorescence could be observed during HSVg4 replication in host cells. ARPE-19 cells were infected by HSVg4 and then stimulated by LPS as described above. At 24 h p.i., cells were observed using a fluorescence microscope with a digital camera Nikon Eclipse TS100F (Nikon, Tokyo, Japan) and the parameters were as followed: excitation wavelength 465 nm–495 nm, emission wavelength 515 nm–555 nm, binning 2x2, power of the mercury lamp 50 W, exposure time 10 ms. Each experiment was repeated in triplicate. The fluorescence values were analyzed by ImageJ software (NIH, Bethesda, Maryland). All images were transformed to 8-bit black and white images, the auto threshold was set, and then mean gray values were automatically measured when limited to the threshold.

2.4. Western blot analysis

Western blotting was performed as we previously described (Duan et al., 2012b). Briefly, ARPE-19 cells were infected by HSV-1 F strain at MOIs of 1 and 0.1, and then stimulated by LPS as described above. At 24 h p.i., cells were harvested and lysed with lysate buffer (20 mM tris-HCL). The samples were freeze-thawed 3 times and then centrifuged at 15777 × g for 30 min at 4°C to remove cellular debris. The protein content in the supernatant was determined by the bicinchoninic acid method using bovine serum albumin (BSA) as the standard. The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted on polyvinylidene fluoride (PVDF) membranes (Merck Millipore, Billerica, USA). The membranes were incubated with 1 μg/mL of mouse anti-human monoclonal antibodies (Abcam, Cambridge, UK) that recognize HSV-1 infected cell protein (ICP4) or 0.2 μg/mL of mouse anti-human β-actin antibodies (Kang-Chen, Shanghai, China) respectively at 4 °C overnight. Then, they were exposed to secondary goat anti-mouse IgG antibodies (Zhongshan Goldenbridge, Beijing, China) for 30 min at 4°C to remove cellular debris. The protein content in the supernatant was determined by the bicinchoninic acid method using bovine serum albumin (BSA) as the standard. The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted on polyvinylidene fluoride (PVDF) membranes (Merck Millipore, Billerica, USA). The membranes were incubated with 1 μg/mL of mouse anti-human monoclonal antibodies (Abcam, Cambridge, UK) that recognize HSV-1 infected cell protein (ICP4) or 0.2 μg/mL of mouse anti-human β-actin antibodies (Kang-Chen, Shanghai, China) respectively at 4 °C overnight. Then, they were exposed to secondary goat anti-mouse IgG antibodies (Zhongshan Goldenbridge, Beijing, China) for 30 min at 4°C to remove cellular debris.

Figure 2. Infected cell protein 4 (ICP4) expression was promoted by LPS in HSV-1-infected ARPE-19 cells. A. ICP4 expression in ARPE-19 cells at the multiplicity of infection (MOI) of 0.01. The uncropped images of blots were displayed in S1. Fig. B. The ratio of ICP4/β-actin band intensities in different groups in A. C. ICP4 expression at the MOI of 0.1. The uncropped images of blots were displayed in S1. Fig. D. The ratio of ICP4/β-actin band intensities in different groups in C. n = 3. The unedited images of blots in the second and third repeated experiments were shown in S2. Fig.
Danvers, MA, USA) and exposed by Kodak Imaging Station 4000MM (Kodak, Rochester, NY, USA).

2.5. Multiplex immunoassay assay

ARPE-19 cells were infected by HSV-1 F strain at MOIs of 1 and 0.1 and then stimulated by different dosages of LPS (0, 10, 50 and 100 μg/ml). At 6 and 12 h p.i., the cell culture supernatants were collected. Measurement of 34 types of immune-related proteins was performed using ProcartaPlex Human Cytokine & Chemokine Panel 1A 34plex (Thermo scientific Ltd., LA, USA) following the manufacturer’s instructions. Cytokines listed below were detected in our study: IL-1β, IL-1α, IL-1RA, IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 p70, IL-13, IL-15, IL-17A, IL-18, IL-21, IL-22, IL-23, IL-27, IL-31, IFN-α, IFN-γ, TNF-α, TNF-β/LTA. The detected chemokines are listed below: Eotaxin/CCL11, GM-CSF, GRO-α/CXCL1, IL-8/CXCL8, IFN-γ/CXCL10, MCP-1/CCL2, MIP-1α/CCL3, MIP-1β/CCL4, RANTES/CCL5, SDF1-α/CXCL12. The assay’s principle of quantitative analysis was based on the standard provided in the kit. The concentrations of immune-related proteins in the samples were measured using the standard curve generated by the standard. The prepared incubated plates (containing samples, standard and quality control) were read on a Luminex analyzer (Thermo scientific Ltd., LA, USA). The data obtained from the analyzer were processed by the MILLIPLEX analyst v5.1 software using five parameters logistic regression (Merck Millipore, Darmstadt, Germany).

2.6. Statistical analysis

All experiments were biologically repeated for a minimum of 3 times, and the quantitative data were expressed as means ± standard deviation (SD). Comparisons among the relative fluorescence intensities in different groups were conducted by One-way Analysis of Variance (ANOVA). Differences among the ratios of band intensities were analyzed by Mann-Whitney test. The discrepancies among the concentrations of cytokines and chemokines in different groups were analyzed by Kruskal-Wallis test. Statistical analysis of data was performed using SPSS 26.0 software (SPSS Inc., Chicago, USA). A *p* value less than 0.05 was considered statistically significant.

3. Results

3.1. LPS increased HSV-1 replication in ARPE-19 cells

ARPE-19 cells were infected with HSVg4 at MOIs of 0.01, 0.1 and 1 and stimulated by LPS at 0, 10, 50, 100 μg/ml, while the morphology and GFP fluorescence intensity of HSVg4 in cells were observed at 24 h p.i. As shown in Figure 1A, cells without virus infection showed normal cellular morphology, while the CPE was observed in HSVg4-infected cells. Under the condition of MOI 0.01 and LPS absence, only a few cells were infected and displayed in clusters, while many cells were uninfected and displayed normal cellular morphology. Under the increase of LPS concentration, CPE increased. In addition, CPE also increased along with LPS concentration at MOIs of 0.1 and 1. The HSVg4 replication was shown comparatively in Figure 1B. GFP expression increased with LPS concentration. The fluorescence intensity of GFP was analyzed and shown in Figure 1C. The GFP fluorescence of HSVg4 in cells treated with LPS significantly increased in a dose-dependent manner compared to cells without LPS treatment (*P* < 0.01, respectively).

3.2. LPS increased ICP4 expression in HSV-1 infected ARPE-19 cells

The ICP4 expression was detected by Western blot analysis (Figure 2). ARPE-19 cells were infected with HSV-1 F strain at MOIs of 0.01, 0.1 and stimulated by LPS at 100 μg/ml. Cells in the control and LPS only groups were uninfected. ICP4 was detected at 24 h p.i. No ICP4 expression was observed in control and LPS alone groups, while ICP4 expression was found in HSV-1 and HSV-1 plus LPS stimulation groups (Figure 2A and Figure 2B). Moreover, the expression of ICP4 was higher in LPS-treated RPE cells than that of LPS-untreated RPE cells at MOIs of 0.01 and 0.1, but the differences were not significant (*p* > 0.05, respectively) (Figure 2C and Figure 2D).

![Figure 3. Cytokine secretion in ARPE-19 cells under LPS treatment and HSV-1 infection. ARPE-19 cells were infected with HSV-1 F for 1 h and incubated with different concentrations of LPS for 12 h. The supernatant of cell culture medium was collected and analyzed at 6 and 12 h post-infection. Statistical analysis was conducted using Kruskal-Wallis test. Compared to the control group under the same LPS levels and at the same time point, ## *p* < 0.01; Compared to the LPS 0 μg/ml group at the same MOI and time point, *p* < 0.05, **p** < 0.01, ***p** < 0.001. Each data point is the average value of 3 independent assays.](image-url)
3.3. LPS increased cytokine secretion in HSV-1 infected ARPE-19 cells

To investigate the immune response of ARPE-19 cells under LPS and HSV-1 stimulation, 24 cytokines were analyzed in our study, including IL-1β, IL-1α, IL-1RA, IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 p70, IL-13, IL-15, IL-17A, IL-18, IL-21, IL-22, IL-23, IL-27, IL-31, IFN-α, IFN-γ, TNF-α and TNF-β/LTA. ARPE-19 cells were infected with HSV-1 F strain at MOIs of 0.01, 0.1 and LPS at 0, 10, 50, 100 μg/ml were added subsequently. At 6 and 12 h p.i., cytokines in the medium were detected. As shown in Figure 3, IL-27 and TNF-α levels were affected by HSV-1

Figure 4. Chemokine secretion in ARPE-19 cells under LPS treatment and HSV-1 infection. Kruskal-Wallis test was used in statistical analysis. Compared to the LPS 0 μg/ml group at the same MOI and time point, *p < 0.05, **p < 0.01, ***p < 0.001. Each data point is the average value of 3 independent assays.

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concentration (Figure 3). In detail, compared to control groups under the same LPS concentration, cytokines mentioned above dramatically increased in HSV-1 infected cells at the MOI of 1 (P < 0.05, respectively). The secretion of these cytokines was further promoted by LPS (P < 0.05, respectively). In addition, the release of IL-1α, IL-1β, IL-1RA, IL-2, IL-4, IL-6, IL-9, IL-12P70, IL-15, IL-18, IL-21 and IFN-γ also enhanced under LPS stimulation in a dose-dependent manner (P < 0.05, respectively). The cytokine levels were affected by incubation time. Cytokines IL-5, IL-7, IL-10, IL-13, IL-17A, IL-23, TNF-α, IFN-γ and IL-31 did not increase or decrease under LPS treatment or HSV-1 infection.

3.4. LPS increased chemokine release in HSV-1 infected ARPE-19 cells

To analyze the chemokines released by HSV-1-infected ARPE-19 cells under LPS stimulation, 10 kinds of chemokines were tested in our study: Eotaxin/CCL11, GM-CSF, GRO-α/CXCL1, IL-8/CXCL8, IP-10/CXCL10, MCP-1/CCL2, MIP-1α/CCL3, MIP-1β/CCL4, RANTES/CCL5, SDF1-α/CXCL12. As shown in Figure 4, no chemokine significantly increased in HSV-1 infected cells compared to the control group under the same LPS concentration. In addition, HSV-1-infected cells under LPS treatment (10, 50 and 100 μg/ml) produced significantly higher levels of chemokines compared to those without LPS treatment, including CXCL1, CXCL8, CXCL10, CXCL12, CXCL2, CCL3, CCL4, CCL5 and CCL11. The expression of GM-CSF was unaffected under HSV-1 or LPS treatment.

4. Discussion

HSV-1 is a common pathogen in all age groups and may cause ARN during ocular infection. As the outer layer of blood-retina barrier, RPE cells can be affected by both HSV-1 infection and LPS stimulation under such conditions. However, limited information was available on the effect of LPS on HSV-1 replication and subsequent immune response in RPE cells. In the current study, we found that LPS increased HSV-1 replication and the release of cytokines IL-1α, IL-1β, IL-1RA, IL-2, IL-4, IL-6, IL-9, IL-12P70, IL-15, IL-18, IL-21, IL-27, TNF-α, IFN-γ and chemokines CXCL1, CXCL8, CXCL10, CXCL12, CCL2, CCL3, CCL4, CCL5 and CCL11 in a dose-dependent manner in ARPE-19 cells.

It was reported that LPS impacted the infectivity of several viruses, such as poliovirus, mouse mammary tumor virus, human immunodeficiency virus 1 and dengue virus, and immune responses of host cells (Kamaladasa et al., 2016; Liu et al., 2016; Robinson et al., 2014; Wilks et al., 2015). The effect of LPS on HSV-1 infection has also been investigated in immune-related cells in previous studies (Domke-Opitz and Kirchner, 1999; Hung et al., 2012; Kirchner et al., 1976). For example, Kirchner et al. found that under LPS stimulation, HSV-1 infection occurred in spleen cells while no infection was observed in untreated cells (Kirchner et al., 1976). Domke-Opitz et al. reported that LPS promoted HSV-1 reactivation in macrophages (Domke-Opitz and Kirchner, 1990). Hung et al. elucidated that LPS increased the production of IL-8 among HSV-1-infected neutrophils (Hung et al., 2012). In the current study, ARPE-19 cells, cell lines of human RPE cells were studied as RPE cells form the outer blood-retina barrier that could be stimulated by LPS and HSV-1 during ARN in AIDS patients. The results showed that LPS significantly enhanced HSV-1 replication in ARPE-19 cells, which was consistent with cornea epithelial cells and VERO cells (Zeng et al., 2020). Furthermore, we investigated changes in the expression of inflammatory factors comprehensively in ARPE-19 cells to reveal their immunological roles during the challenge of virus and bacterial components.

Cytokines IL-6, IL-8, IL-18, TNF-α, IFN-γ and chemokines CCL2 were widely studied in both HSV-1 infection and LPS stimulation in RPE cells. The release of inflammatory factors IL-6, IL-8, IL-18 and CCL2 increased in serum and aqueous humor samples of ARN patients, while RPE cells released TNF-α and IFN-γ during HSV-1 infection (de Visser et al., 2017; Zheng and Atherton, 2005). In LPS-treated ARPE-19 cells, the levels of IL-6, IL-8, IL-18, TNF-α, IFN-γ and CCL2 also elevated (Fernandez-Robredo et al., 2020; Qiu et al., 2021). These inflammatory factors were pro-inflammatory factors and involved in progressive retinal pathology, such as RPE degeneration and neovascularization (Ijima et al., 2014; Leung et al., 2009; Li et al., 2009; Naik et al., 2021; Taghavi et al., 2019). TNF-α could induce RPE cells to migrate, which could be seen in the retina of HSV-1 infected mice (Liu et al., 2012; Zheng and Atherton, 2005). Previous researches provided only partial profiles of the altered cytokines and chemokines in ARPE-19 cells under HSV-1 infection and LPS stimulation. Our study covers the changes of 24 types of cytokines and 10 types of chemokines in HSV-1-infected and LPS-treated ARPE-19 cells, offering basic profiles of inflammatory factor changes and indicating potential interaction among cytokines and chemokines.

Two HSV-1 strains, HSV-1 F strain and HSV-1-H129-G4 strain, were used in this study. We used HSV-1 F strain to investigate the effect of LPS on ICP4 protein expression and cytokines/chemokines release. HSV-1-H129-G4 strain was used to directly observe levels of HSV-1 in cells because GFP was expressed in the HSVg4-infected ARPE-19 cells. We found that LPS enhanced the replication of both HSV-1 F and HSVg4 in ARPE-19 cells. However, there are some limitations in our study as well. Only the in-vitro RPE cell line model was included in our study. ARPE-19 is a human cell line evolved by selective tryptophanization of a primary RPE culture (Dunn et al., 1996). ARPE-19 cells share the same morphology characteristics of RPE and similar biochemical functions, such as the synthesis of mRNAs encoding cellular retinaldehyde binding protein (CRALBP) and the RPE-specific protein RPE65 and tight junction formation. However, ARPE-19 cells are not immortal, heterogeneous and have an unrepresentative transcriptome (Pfeffer and Fliesler, 2022). In present studies, ARPE-19 cells were used to observe epithelial-mesenchymal transition, oxidative stress, high glucose-induced injury and inflammation response (Qian et al., 2019; Ravi et al., 2021; Yang et al., 2021; Yuan et al., 2020). Therefore, ARPE-19 cells were selected to evaluate the viral replication and subsequent inflammatory response in this research. The experimental study in ARPE-19 cells could be different from ARN patients infected by HSV-1, cytomegalovirus or Herpes Zoster Virus. In addition, ocular HSV-1 infection causes various lesions, including blepharitis, conjunctivitis, keratitis, uveitis and ARN, while only ARN was studied in our research.

5. Conclusion

In this study, we showed that LPS promoted HSV-1 infection and enhanced the release of inflammatory factors in human ARPE-19 cells in a dose-dependent manner, indicating that ARN might deteriorate when complicated by endotoxemia. As a preliminary research, further analysis is needed to reveal the subsequent impacts of RPE-secreted cytokines in the retina in vivo.

Declarations

Author contribution statement

Fang Duan: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.
Weiting Zeng: Analyzed and interpreted the data; Wrote the paper.
Yafang Zhang: Performed the experiments.
Dai Li: Contributed reagents, materials, analysis tools or data.
Kaili Wu: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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