

Host defense against *Salmonella* and rotaviral gastroenteritis: a serial study of transcriptional factors and cytokines

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Background and Purpose: Common etiologies of acute enterocolitis in childhood include the intracellular pathogens *Salmonella* and rotavirus, along with extracellular pathogens. In order to elucidate differentiating immunologic parameters in patients with acute gastroenteritis of different etiologies, we investigated interferon (IFN)-gamma, interleukin (IL)-12, and T-bet of T-helper type 1 subsets, and IL-4 and GATA-3 of T-helper type 2 subsets.

Methods: From June 1, 2003 to December 31, 2003, 32 patients with acute gastroenteritis were enrolled. Sequential heparinized blood samples were obtained on the day of presentation (day 1) and on day 3 of hospitalization. Using reverse transcriptase-polymerase chain reaction, the mean ratios of IFN-gamma, T-bet and IL-12 mRNA levels relative to beta-actin were determined.

Results: *Salmonella* infections induced stronger IFN-gamma and T-bet responses than either rotavirus infection or other enterocolitis ($p < 0.05$). However, poor IL-12 response in *Salmonella* infections implied failed T-helper type 1 immunity, and probably accounted for the prolonged clinical course. In contrast, by day 3 of hospitalization, most patients with rotavirus enterocolitis were symptom-free.

Conclusions: IL-12 is the key factor in determining host response against and, hence, disease activity of *Salmonella* infections.

Key words: Cytokines; Interferon type II; Rotavirus; *Salmonella*; T-lymphocytes, helper-inducer

Introduction

Infectious gastroenteritis is a common disease among children worldwide. Clinical manifestations depend on the organism and host response to infection and include asymptomatic infection, watery diarrhea, bloody diarrhea, chronic diarrhea, and extraintestinal manifestations of infection [1]. In Taiwan, as in other parts of the world, *Salmonella* and rotavirus are common causes of gastroenteritis in children. They are both intracellular pathogens, but often exhibit different clinical pictures; immune response is considered to be one major cause of the difference.

In humans, immune response to foreign pathogens commences with innate immunity. Innate immunity functions not only to protect the host from infection while slower adaptive immune responses are developing, but also to direct the qualitative and quantitative nature of subsequent adaptive immunity. Development of T-helper type 1 (Th1) or T-helper type 2 (Th2) cells from common precursor cells is augmented by signals derived from the innate immune system [2,3]. Cytokines play a key role in the interaction. Interleukin (IL)-12 produced by monocytes/macrophages and neutrophils as well as different accessory cells is required for Th1 development, whereas IL-4, produced by basophils and mast cells, is required for Th2 development [2,3].

Intracellular pathogens are well shielded from the effector cells of the cellular immune system. However, microbial proteins are processed and presented,

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promoting activation of T lymphocytes that determine resistance, susceptibility, and often immunopathogenesis of intracellular infections. Cellular immunodeficiency had been implicated in both chronic enteric virus infection and recurrent severe *Salmonella* infection [4,5]. T-cell immunity is therefore expected to play a key role in the interaction between host and infectious organisms in both rotavirus and *Salmonella* infections.

Naïve T-helper cells can differentiate into at least two functional classes of cell during an immune response, Th1 and Th2 cells, secreting effector cytokines interferon-gamma (IFN- γ) and IL-4, respectively [6,7]. Th1 cells are responsible for cell-mediated immunity, whereas Th2 cells are responsible for extracellular immunity. As well as their protective roles in host defense, both subsets of T-helper cell have been implicated in pathological responses. Th1 cells can mediate organ-specific autoimmunity and Th2 cells have been implicated in the pathogenesis of asthma and allergy. The final composition of the T-helper-cell response to antigen can, therefore, determine whether the outcome is favorable or not in infectious, inflammatory and autoimmune diseases.

Many factors influence the differentiation of a naïve T-helper cell to become a Th1 or Th2 cell. IL-12 and IL-4, acting through signal transducer and activator of transcription 4 (STAT4) and STAT6, respectively, at the initiation phase of T-helper cell differentiation, are key determinants of the outcome [6,7]. The commitment phase of T-cell differentiation is mediated by cell type-specific nuclear factors. The T-box transcription factor *T-bet* has been shown to have a central role in Th1 cell development, inducing both transcriptional competence of the locus encoding T-bet and selective responsiveness to the growth factor IL-12 [8,9]. By contrast, the zinc-finger transcription factor *GATA3* seems to be crucial for inducing some, but perhaps not all, key attributes of Th2 cells — in particular, transcriptional competence of the Th2 cytokine cluster, which includes the genes encoding IL-13, IL-4, and IL-5 [10-16].

Ectopic expression of T-bet in differentiating Th2 cells leads to strong expression of IFN- γ and several Th1-specific cell surface receptors [2,17-19]. Moreover, T-bet overexpression leads to down-regulation of the expression of IL-2 and several Th2-specific cytokines, suggesting that this transcription factor plays a central role in promoting not only the development and maintenance of Th1 phenotype but also suppression of Th2 phenotype. On the other hand, several in vitro studies have clearly demonstrated that GATA-

3 is sufficient, although not very potent, in directing developing and polarized T-helper cells to produce Th2 cytokines [10,14,15].

The objective of this study was to elucidate differentiating immunologic parameters in patients with acute gastroenteritis of different etiology. IFN- γ , IL-12, and T-bet of Th1 subsets, and IL-4 and GATA-3 of Th2 subsets were investigated.

Methods

Patient population and study design

From June 1, 2003 to December 31, 2003, patients admitted to the pediatric ward in Changhua Christian Hospital with symptoms suggesting acute gastroenteritis were enrolled. Informed consent was obtained from each patient's parents. Symptoms including onset and frequency of diarrhea, fever, and general activity were recorded at admission and during hospitalization. Stool was obtained for routine analysis, *Salmonella* culture, and rotavirus antigen detection. Sequential heparinized blood samples were obtained on the day of presentation (day 1) and on day 3 of hospitalization. Treatment of the gastroenteritis consisted mainly of supportive and symptomatic management. Antibacterial therapy for *Salmonella* infection was given at the discretion of primary care physicians. Complications were defined as bowel obstruction and/or perforation. Resolution of acute gastroenteritis was defined as afebrile for more than 24 h, stool passage frequency less than 4 times/day, and fair activity with fair oral feeding or intake.

Exclusion criteria

Patients with any one of the following conditions were excluded: (a) admitted two days or more after onset of diarrhea; (b) not admitted for acute gastroenteritis in the first place; (c) concurrent infections other than acute gastroenteritis; (d) gastroenteritis caused by concurrent infections of *Salmonella* spp. and rotavirus; (e) discharged before day 3 or sequential blood samples not collected completely; (f) history of congenital immunodeficiency or recurrent severe infections; (g) parents withdrew consent.

Determination of cytokine mRNA expression

Peripheral blood mononuclear cells were separated using the Ficoll-Hypaque method. Peripheral blood monocytes were then separated with the petri dish adherence method. Briefly, these mononuclear cell

suspensions were put into a 100% × 15 mm plastic petri dish and incubated in a humidified (37°C, 5% carbon dioxide) atmosphere for 1 h. The adherent cells were harvested with rubber policemen.

Cells were washed with phosphate-buffered saline three times, and a proper volume was transferred with a pipette into an Eppendorf tube containing a one-tenth volume of chloroform, vortexed vigorously for 20–30 s and placed on ice for 15 min. After centrifugation at 14,000 *g* for 15 min, the upper aqueous phase was transferred to a new tube, and an equal volume of isopropanol was added and gently shaken. The sample was placed at –20°C for 1 h, and then centrifuged at 12,000 *g* for 15 min. The RNA pellets were washed with 75% alcohol for 5 min, and then centrifuged at 7500 *g* for 10 min. The pellets were dissolved with diethylpyrocarbonate-water. After heating at 50–60°C for 10 min and cooled on ice, the quantity and purity of the RNA preparations were determined by measuring their absorbances at 260 (A_{260}) and 280 nm (A_{280}). An A_{260}/A_{280} ratio >1.9 was confirmed.

Reverse transcription

After the addition of 10 µg of RNA to diethylpyrocarbonate-water (to a total volume of 39 µL), 1 µL of oligo(dT) [0.5 µg/mL] was added. The mixture was then heated at 70°C for 10 min and immediately placed in an ice bath. Tris-hydrochloride, potassium chloride, magnesium chloride, manganese sulphate, dithiothreitol, deoxynucleotide triphosphate, ribonuclease inhibitor and reverse transcriptase (RT) were added. The reaction was held at 37°C for 1 h, and then at 65°C for 10 min, after which it was placed in the ice bath to interrupt the reaction. The cDNA product was stored at –70°C.

Polymerase chain reaction

The total amount of each sample was around 50 µL, including 5 µL of RT product, 0.2 mM deoxynucleotide triphosphate, 200 nM sense and antisense DNA probe (Table 1), 15 mM magnesium chloride, 50 mM potassium chloride, 10 mM Tris-hydrochloride (pH 9.0), 1% Triton X-100 and 2.5 U of *Taq* DNA polymerase. After being fully mixed, the samples were treated with the following protocol in each cycle: 94°C for 1 min; 65°C gradually decreasing to 55°C, with 1 min pause at each 0.5°C step-down; and 72°C for 1 min. A total of 21 cycles was performed. After that, another 19 cycles were done: 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The final step was 72°C for 5 min and then the product was stored at 4°C in a refrigerator.

Measurement of polymerase chain reaction products

The polymerase chain reaction (PCR) products from each sample, including IL-4, IL-12, IFN-γ, T-bet, GATA-3, and beta (β)-actin, were analyzed with a 1% agarose gel containing ethidium bromide. The final results of PCR were exposed with an ultraviolet light box and photographed with Kodak films. Density values were obtained with a laser-scanning densitometer SLR-2D/1D (Biomed Instrument Inc., Fullerton, CA, USA). The density of each band was compared to a 600-bp band which expressed the data of β-actin to obtain a ratio, and the ratios were used for further analysis.

Statistical analysis

Student's *t* test was used to determine the difference of immunologic factor level among different pathogen groups.

Table 1. Sense and antisense human cytokine primers used in polymerase chain reaction analyses

Cytokine	Primer sequence	Product size (base pairs)
IL-4	CTGCAAATCGACACCTATTA GATCGTCTTTAGCCTTTC	448
IL-12	AAGGAAGATGGAATTTGGTCCACTG GATGATGTCCCTGATGAAGAAGCTG	381
IFN-gamma	TTTTCAGCTCTGCATCGTTTTGGGT CCTTGAAACAGCATCTGACTCCTT	456
T-bet	ATGGGCATCGTGGAGCCGG CATTACTGTGGCCGGGCTG	242
GATA-3	AGGACGAGAAAGAGTGCCTCAA TGGGACGACTCCAGCTTCA	150
Beta-actin	ATTGCCGACAGGATGCAGAA GCTGATCCCACTGCTGGAA	150

Abbreviations: IL = interleukin; IFN = interferon

Table 2. Demographic characteristics and outcomes of the patients

Etiology	<i>Salmonella</i> (n = 12)	Rotavirus (n = 10)	Other (n = 10)
Age range (median)	16 days-9.5 years (11 months)	2 months-5 years (12 months)	6 days-15 years (13.5 months)
Gender			
Boys	6	5	4
Girls	6	5	6
Admission after diarrhea onset (days; mean)	0.96	0.75	0.86
Days before symptoms resolution (mean)	6.58	2.80	3.20
Complication	Nil	Nil	Nil

Results

Clinical characteristics

We classified eligible patients into three subgroups according to the identified infectious pathogens *Salmonella* spp., rotavirus, and other pathogens. Thirty two patients were enrolled. Demographic characteristics and outcomes of the patients of each subgroup are shown in Table 2.

Th1 cytokines and transcriptional factor mRNA profile

Using RT-PCR, the mean ratios of IFN- γ , T-bet, and IL-12 mRNA levels relative to β -actin are shown in Fig. 1, Fig. 2 and Fig. 3. On day 1, *Salmonella* infections induced stronger IFN- γ (109.02 ± 18.74 vs 65.86 ± 26.59 , $p < 0.05$) and T-bet (89.46 ± 27.27 vs 25.32 ± 14.18 , $p < 0.05$) immune responses than did nonspecific enterocolitis (Fig. 1 and Fig. 2). *Salmonella* infections also induced stronger T-bet responses than rotavirus infections (89.46 ± 27.27 vs 68.63 ± 12.83 , $p < 0.05$) [Fig. 2]. IFN- γ responses were higher in *Salmonella* infections than in rotavirus infections, although the difference was not statistically significant (109.02 ± 18.74 vs

75.92 ± 19.82) [Fig. 1]. IL-12 mRNA levels, however, were much higher in other enterocolitis (86.49 ± 51.67) than in *Salmonella* (19.89 ± 1.15 , $p < 0.01$) and rotavirus infections (62.04 ± 36.14) [Fig. 3].

On day 3, IFN- γ , T-bet, and IL-12 mRNA levels were lower relative to β -actin in all subgroups compared with day 1 (Fig. 1, Fig. 2 and Fig. 3). The inter-subgroup difference patterns of IFN- γ and T-bet remained the same as in day 1 measurements. However, IL-12 levels decreased at day 3 in all subgroups and were not statistically different between subgroups. Some of the RT-PCR results are shown in Fig. 4, Fig. 5 and Fig. 6.

Th2 cytokines and transcriptional factor mRNA profile

The mean ratios of IL-4 mRNA level relative to β -actin are shown in Fig. 7. On day 1, the mean ratios of IL-4 relative to β -actin were much higher in the rotavirus subgroup (64.59 ± 5.29) than in the *Salmonella* subgroup (36.85 ± 12.76) [$p < 0.05$]. IL-4 levels were higher on day 3 in the *Salmonella* subgroup, and contrasted with the downward trend in the rotavirus and nonspecific enterocolitis subgroups.

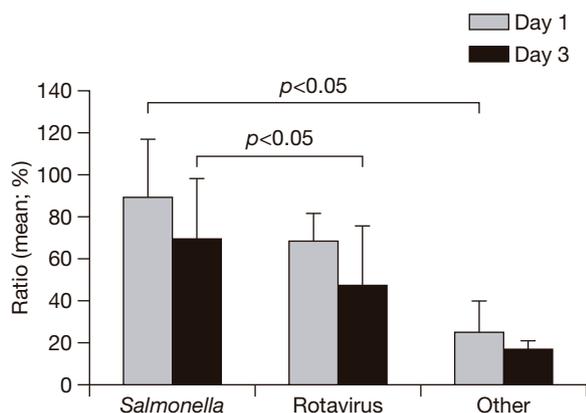


Fig. 1. Mean ratios of interferon-gamma mRNA to beta-actin in *Salmonella*, rotavirus, and other enterocolitis infections.

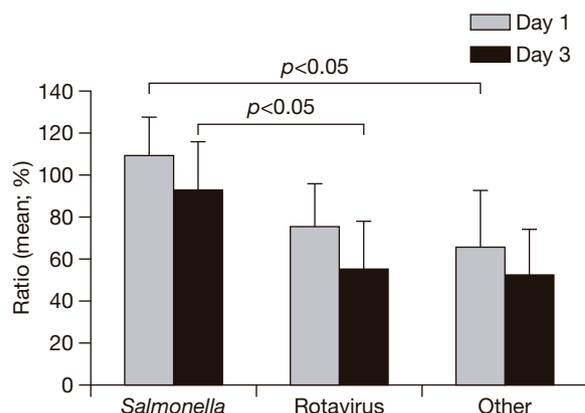


Fig. 2. Mean ratios of T-bet mRNA level to beta-actin in *Salmonella*, rotavirus, and other enterocolitis infections.

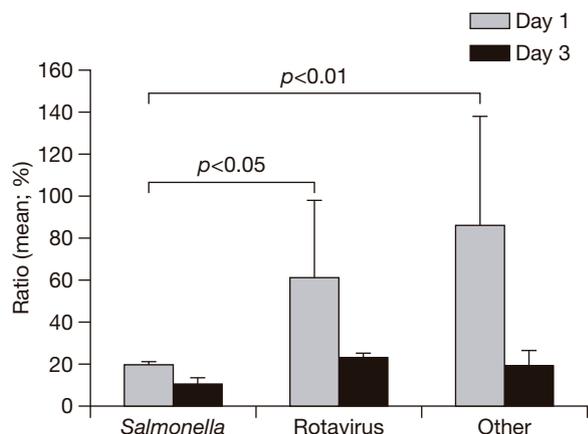


Fig. 3. Mean ratios of interleukin-12 mRNA to beta-actin in *Salmonella*, rotavirus, and other enterocolitis infections.

The mean ratios of GATA-3 relative to β -actin were comparable in all subgroups on both day 1 and day 3. The profile of GATA-3 was similar to that of IL-4, but without statistically significant differences between subgroups (data not shown).

Discussion

In this study, the main parameters of Th1 activity, i.e., T-bet, IFN- γ and IL-12, had the same downward trend between day 1 and day 3 measurements. This implies that adaptive immunity had been well activated before children presented with active symptoms.

We demonstrated that T-bet mRNA level was significantly higher in *Salmonella* infection than in rotavirus infection. T-bet represents the activity of activated T cells destined to be Th1 cells. IFN- γ gene remodeling and subsequent IFN- γ mRNA expression should be positively correlated with T-bet activity [20], as was the case in our study. IFN- γ levels mirrored T-bet

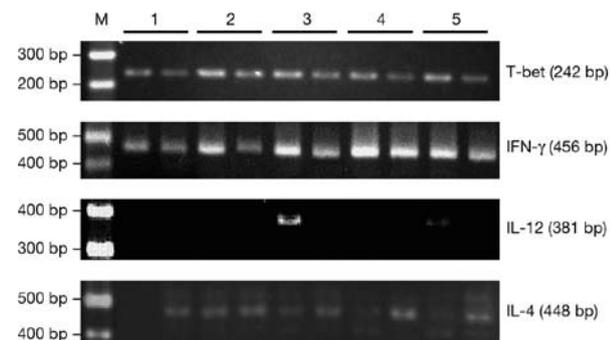


Fig. 4. Polymerase chain reaction results of cytokines and T-bet mRNA in five patients with *Salmonella* enterocolitis. M = marker; bp = base pairs; IFN- γ = interferon-gamma; IL-12 = interleukin-12; IL-4 = interleukin-4.

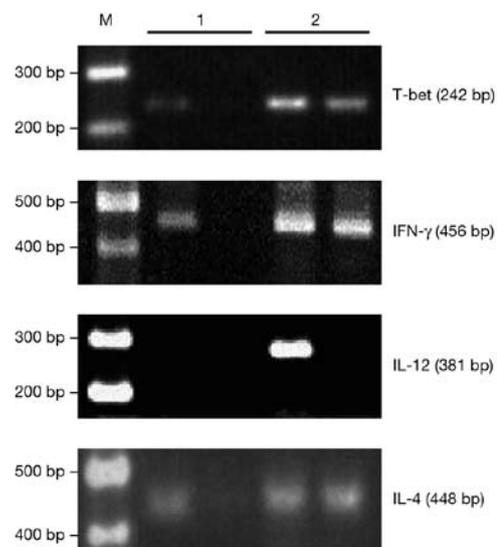


Fig. 5. Polymerase chain reaction results of cytokines and T-bet mRNA in two patients with rotavirus enterocolitis. M = marker; bp = base pairs; IFN- γ = interferon-gamma; IL-12 = interleukin-12; IL-4 = interleukin-4.

activity on day 1 and day 3, being higher in *Salmonella* infections than in rotavirus infections.

Our results showed that the other important cytokine in Th1 immunity, IL-12, was significantly lower in *Salmonella* infections than in rotavirus infections. IL-12 is centrally involved in promoting and linking innate and adaptive immunity. In response to most intracellular infections, IL-12 is the inducer cytokine of Th1 cells, and IFN- γ is the effector cytokine that mediates protection [21,22]. Although IL-12 has been described as the decisive cytokine in incipient Th1 immunity, impaired IL-12 response does not hinder T-bet and IFN- γ activities as our results show. This also implies that impaired IL-12 response alone probably accounts for the Th1 immunity failure in

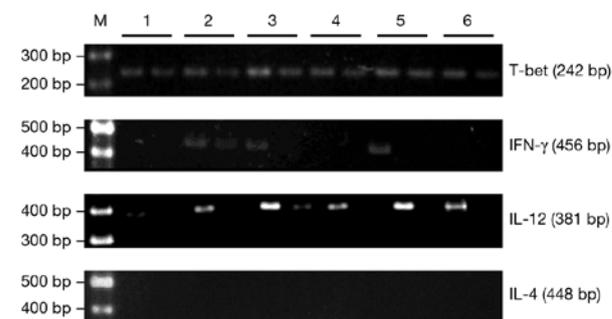


Fig. 6. Polymerase chain reaction results of cytokines and T-bet mRNA in six patients with other enterocolitis infections. M = marker; bp = base pairs; IFN- γ = interferon-gamma; IL-12 = interleukin-12; IL-4 = interleukin-4.

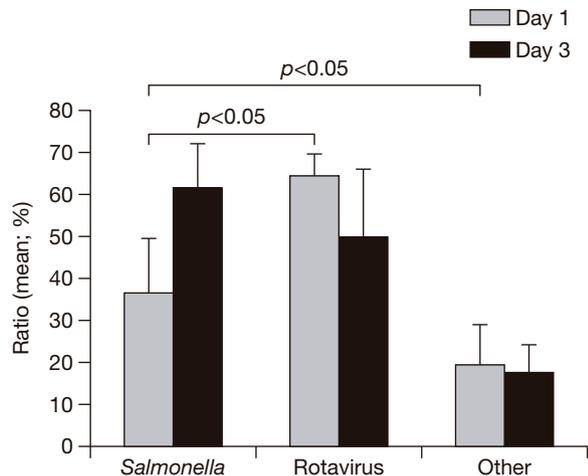


Fig. 7. Mean ratios of interleukin-4 mRNA to beta-actin in *Salmonella*, rotavirus, and other enterocolitis infections.

Salmonella infections and the resultant clinical differences between *Salmonella* and rotavirus infections.

In animal studies, neutralization of both IL-12 and IFN- γ increases *Salmonella* counts in the spleen and liver and decreases host survival [23-26]. Neutralization of IL-12 in *Salmonella*-infected mice is accompanied by a decrease in splenic IFN- γ mRNA expression and serum IFN- γ levels compared to infected mice not treated with anti-IL-12 [27]. IFN- γ treatment largely reverses the effects of IL-12 neutralization on splenic bacterial load [27]. This suggests that IL-12 plays a protective role in animal host defense against *Salmonella* via IFN- γ action. However, this may not be applicable in human hosts. In our study, IFN- γ expression did not correlate with IL-12 response in *Salmonella* infection. IL-12 seems to have a unique role in host immunity against *Salmonella* infection in humans.

Defects in IL-12 and its receptor have been demonstrated to cause recurrent severe *Salmonella* infections in human hosts [5]. A cause and effect relationship had been shown between impaired IL-12-mediated immunity and vulnerability to infections due to poorly virulent mycobacteria and *Salmonella* [28-30]. The role of human IL-12 in defense against mycobacteria and *Salmonella* cannot be compensated for by other immune interactions in vivo [28-30].

IL-4 response was much weaker in *Salmonella* infection than it was in rotavirus infection on day 1 in our study. This may indicate suppressed Th2 response in *Salmonella* infection. IL-4 has been shown to interfere with host immunity against *Salmonella* infections. In animal studies, neutralization of IL-4 functions

reduced the number of bacteria in the peritoneal cavity after intraperitoneal infection, and was accompanied by increased serum levels of IFN- γ and IL-12 [31]. In another study using IL-4 knock-out mice, lack of IL-4 was associated with delayed death after *Salmonella* infection and reduced liver abscess formation [32]. In our study, however, IL-4 level increased on day 3 in *Salmonella* infection, in contrast to the reduction trend in rotavirus infection. The presence of this phenomenon excluded IL-4 response defect in these patients. Whether this phenomenon occurred secondary to the amelioration of *Salmonella* infection and reduction of transient suppression of Th2 immunity or as a result of impairment of host immunity before the clinical course was completed cannot be determined in our study, due to the limited patient numbers.

In conclusion, our study results suggest that the immune responses triggered against *Salmonella* and rotaviral gastroenteritis in humans have different profiles. Impaired IL-12 response causes prolonged fever and diarrhea in *Salmonella* gastroenteritis. Further studies are needed to elucidate the innate immunity acting early in the course of infection, particularly the role of IL-12, and the inter-relationships between innate and adaptive immunities against intracellular pathogens.

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