Correlation analysis of Treg/Th17 cells and related cytokines in patients with psoriasis vulgaris

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OBJECTIVE: To investigate potential differences in circulating levels of T regulatory (Treg)/T helper 17 (Th17) cells, related inflammatory cytokines and specific transcription factors in healthy individuals and patients with psoriasis conforming to one of three Traditional Chinese Medicine (TCM) syndromes: blood-heat syndrome (BHS), blood-stasis syndrome (BSS) and blood-dryness syndrome (BDS).

METHODS: Sixty-seven patients with psoriasis were recruited and assigned to one of three corresponding TCM syndrome groups: BHS (n = 40), BSS (n = 14) and BDS (n = 13 patients). The control group comprised 21 healthy individuals. The circulating levels of Treg/Th17 cells in peripheral blood were assessed using flow cytometry; the levels of inflammatory cytokines interleukin (IL)-10 and tumor necrosis factor (TNF)-α by enzyme-linked immunosorbent assay; and the mRNA expression of T cell-specific transcription factors retinoic acid-related orphan receptor γt (RORγt) and forkhead box P3 (Foxp3) by quantitative real-time PCR.

RESULTS: The ratio of Th17 cells and the levels of TNF-α and RORγt were all significantly higher in the BHS and BSS groups than the control group (P < 0.05), while the ratio of Treg cells and the levels of IL-10 and Foxp3 mRNA in the BHS group were significantly lower compared with the control group (P < 0.05). No significant differences were seen between the BSS group and the control group. The ratio of Th17 cells and the levels of TNF-α and RORγt in the BDS group were not significantly different from those of the control group; however, the ratio of Treg cells and the levels of IL-10 and Foxp3 were all lower than those in the healthy controls (P < 0.05).

CONCLUSION: Compared with healthy individuals, the ratio of Th17 cells and the levels of related cytokines were higher, while the ratio of Treg cells and the levels of related cytokines were lower, in the peripheral blood of psoriasis/BHS patients; corresponding results for the BSS and BDS groups also showed differences. We propose that patterns of differentiation of immunological cells in psoriasis patients are reflected in corresponding TCM blood syndromes.

Keywords: Psoriasis; vulgaris; Blood-heat; Blood stasis; T-Lymphocytes, regulatory; Th17 cells; Medical informatics
INTRODUCTION

Psoriasis is a type of scaling erythematosus dermatitis with complex etiology and pathogenesis. Recent studies have widely acknowledged that psoriasis is an immune disease under the background of polygenic inheritance disorders with abnormal T cells. While the mechanisms by which innate and adaptive immune cytokines regulate inflammation in psoriasis are not completely understood, many studies have indicated that T helper 17 (Th17) cells play an important role in the development of psoriasis lesions. Tumor necrosis factor (TNF)-α also stimulates and induces keratinocytes to secrete proinflammatory cytokines, which may represent another key pathway in the pathogenesis of psoriasis.

Regulatory T (Treg) cells are major regulators of immune homeostasis through their immunosuppressive function. Activation by stimulation of T cell antigen receptor (TCR) shows the characteristics of inhibition of the proliferation of CD4+ and CD8+ T cells. Transforming growth factor beta (TGF-β) can induce T cells to differentiate into Treg cells and subsets of Treg produce interleukin-10 (IL-10) and TGF-β. IL-10 is an important immune mediator, with multiple effects on anti-inflammatory and immune regulatory functions. Several lines of evidence suggest that IL-10 has anti-psoriatic abilities. Retinoic acid-related orphan receptor γt (RORγt) and forkhead box P3 (Foxp3) are transcription factors specific to Th17 and Treg cells, respectively and each are necessary for activation. In patients with psoriasis, hyperactivation of Th17 cells is responsible for abnormalities in the Th17/Treg balance and the impaired function of Treg cells also causes the hyperactivation of Th1 and Th17, each of which can cause psoriatic inflammation. Zhang et al. revealed that an increasing amount of Th17 and FOXP3+/+Treg in the blood and lesions of psoriatic patients were positively correlated with the severity of the disease. The correlation between Psoriasis Area and Severity Index (PASI) score and Th17/Treg ratio was inverse in skin lesions, but positive in blood. Imbalances in Treg cells and Th17 cells and their major cytokines IL-10/TNF-α, together with the specific transcription factors Foxp3/RORγt, play an important role in the pathogenesis of psoriasis with immune injury. Oral systemic medicines have been used successfully for the treatment of psoriasis for over 50 years and include methotrexate, cyclosporin, acitretin and fumaric acid esters; however, the most effective treatment is injection of biologics. Most of the oral drugs and biologics for the treatment of psoriasis are limited by a diverse array of toxicities. Some data have shown associations between biologics and serious adverse events and increased risk of infection in the first 6 months. Furthermore, there is a lack of clinical data on biologics with regard to long-term effects, safety and even potential side effects on various organs. Compared with traditional non-biologic systemic and biologic treatments for psoriasis, Traditional Chinese Medicine (TCM) treatment for psoriasis is safe, effective and well tolerated, with fewer side effects over the long term.

TCM discriminates between psoriasis conditions based on blood syndrome, mainly involving the area, color, distribution, intensity of itching and isomorphic response of psoriatic lesions. The classification and treatment of psoriasis vulgaris in TCM focus on three TCM syndromes: blood-heat syndrome (BHS), blood-stasis syndrome (BSS) and blood-dryness syndrome (BDS). The BHS type is treated with the clearing heat and cooling blood method; the BSS type with the activating blood and resolving stasis method; and the BDS type with the nourishing blood and moistening dryness method. In the early stage, most patients have BHS, which later may transform into BSS or BDS. BHS is the key link in the transformation of pathological changes. There has been little information concerning the relationships between TCM syndromes and T cells and circulating cytokines, although they have been regarded as playing putatively important roles in modulating inflammatory responses. In this study, we examined Treg/Th17 cell ratios using the levels of related cytokines IL-10 and TNF-α, as well as the T cell-specific transcription factors Foxp3 and RORγt, to explore the relevant distributions of Treg/Th17 cells among psoriasis patients with three TCM syndromes.

MATERIALS AND METHODS

The study was approved by the Beijing University of Chinese Medicine Ethics Committee (Approval No. BJZYYDX-LL-2014017) and written informed consent was obtained from all of the study participants.

Diagnostic criteria and PASI standard

The diagnostic criteria for psoriasis vulgaris were based on the Guideline for the Treatment of Psoriasis (2008), issued by the Chinese Medical Association. Skin lesions were assessed according to the PASI standards formulated by Fredriksson and Petersson.

TCM standards

The diagnostic criteria for the three TCM syndromes of psoriasis were based on The Differential Diagnostics of TCM Syndrome Complex. (a) The BHS involves drop-shaped lesions that occur and develop quickly, accompanied by erythema in the damaged skin area. New skin lesions appear continuously, with more silvery-white scales that cannot cover the red spots and can easily strip in the surface; they are positive for the Auspitz sign and isomorphic response. BHS often involves severe itching accompanied by general symptoms of dry mouth and tongue, constipation, emotional irritability, yellow urine, red or dark-red tongue with
a thin white or slightly yellow coating and a wiry-slippery or rapid pulse. (b) BDS features quite a long duration of illness with rare appearances of new skin lesions of pale-red color. The lesions can expand into the shape of a coin or ring, typically manifesting as infiltrated erythema and thin white scales. Patients have a pale tongue with a thin white or minimal coating and a deep-thready pulse. (c) BSS lesions are dark red in color and have a long course, manifesting as infiltrated erythema and thickened white, like-scale lesions. The patients have dark purple tongue with ecchymosis and a hesitant or uneven pulse.

Inclusion criteria
The criteria for patient recruitment included: age between 18 and 65 years; willingness to sign an informed consent form; diagnosis of psoriasis vulgaris with BHS, BSS or BDS; no co-morbid conditions that might interfere with the results; no hematological abnormalities; no active systemic therapy within the previous month; and no topical application of corticosteroid or other topical treating medications within the previous 2 weeks.

Exclusion criteria
Patients with the following conditions were excluded from the study: PASI score standard < 1; women who were pregnant, lactating or trying to become pregnant; exhibition of psoriasis arthritis, pustular psoriasis or erythrodermic psoriasis; or serious systemic disease, cancer, immune deficiency, or mental disorders.

Subjects
Beginning in 2014, a total of 67 patients from the Beijing Dongzhimen Hospital Dermatology Department were recruited and assigned into one of three groups according to the TCM syndrome. The patient demographics, psoriasis and syndrome profiles are presented in Table 1. Twenty-one healthy volunteers (9 male and 12 female) from the Beijing university of Chinese medicine were recruited into the control group.

Peripheral blood samples
Peripheral blood samples (3-5 mL) were taken from the patients and control group participants and transferred to heparin anticoagulant tubes, followed by centrifugation at 3000 r/min for 10 min. Serum (400 μL) was collected and stored at −80°C for later use.

Isolation of human peripheral blood mononuclear cells (PBMCs)
PBMCs were isolated from the peripheral blood samples of the psoriasis patients and controls by density gradient centrifugation. Cells were collected from the interface, washed with phosphate-buffered saline and maintained in RPMI 1640 medium containing 10% fetal bovine serum and 1% penicillin streptomycin solution.

Flow cytometry analysis
The serum levels of Treg and Th17 cells were measured by a multiplex flow cytometric assay with a Beckman Coulter EPICS XL/FC500/Altra (Beckman Coulter, Miami, FL, USA). The cells were stained with CD3-FITC, CD8-EC and CD4-PC5 (BD Pharmingen, New Jersey, USA) and CD4/CD25-FITC/PE (eBioscience, San Diego, CA, USA). Prior to IL-17A staining, the cells were incubated for 6 h, then suspended in fixation/permeabilization solution (Beckman Coulter, USA) and stained with anti-human IL-17A-PE (eBioscience, California, USA), anti-human IFN-γ-FITC (mouse IgG1k, 4S.B3, Beckman Coulter, USA) and Foxp3-PE-cy5 (eBioscience, California, USA). The concentrations of cytokines in the peripheral blood were assessed using a special kit (Intra-Prep Permeabilization Reagent, Cat. No. 2388/2389, Beckman Coulter, USA) according to the manufacturer’s instructions.

Measurement of cytokines
We measured the serum levels of TNF-α by a human TNF-α immunoassay kit (HS400C, R & D Systems, Minneapolis, MN, USA) and IL-10 using the enzyme-linked immunosorbent assay (ELISA) method (Multi Sciences Biotech, Hang Zhou, China) according to the manufacturer’s instructions.

RNA isolation and quantitative real-time PCR
We used Primer-BLAST from the National Center for Biotechnology Information to design the primers of target genes. Total RNA was isolated from PBMCs using an ultra-pure RNA kit (CW0581, CWBio Co. Ltd., Kang Century Biotech Companies, China) and cDNA was synthesized with a first-strand cDNA synthesis kit (HiFi-MMLV cDNA, CWbio CW0744, CWBio Co. Ltd.). Quantitative real-time PCR was performed using SYBR Green PCR Mixture (CW0957, CWBio Co. Ltd., Kang Century Biotech Companies, China) under the following PCR conditions: 65°C for 5 min followed by 37°C for 40 min. The results were normalized using an 7500 RT-PCR apparatus, using the 2-ΔΔct method.

Statistical analysis
All statistical analysis was performed using the SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). The data are shown as mean ± standard deviation (x ± s). They were analyzed with the one-way analysis of variance (ANOVA). Numerical data were compared using the χ2 test. A P value of less than 0.05 was considered statistically significant. Independent samples were subjected to nonparametric testing using the Kruskal-Wallis test methods.

RESULTS

Clinical characteristics
There were no significant differences among the three
TCM syndrome groups and the control group regarding age, number of years since diagnosis, sex ratio or PASI ($P > 0.05$; Table 1).

**Comparison of the ratios of Treg/Th17 cells in the TCM syndrome patients and healthy controls**

In the BHS and BDS groups, the ratios of Treg cells were significantly lower than those in healthy controls ($P = 0.0003$; $P = 0.14$, respectively). There was no statistical difference between the BSS group and the healthy control group ($P = 0.22$). There was no difference between the three TCM syndrome groups ($P = 0.17$; Table 2).

Conversely, the peripheral blood ratio of Th17 cells was higher in the patients with psoriasis compared with the healthy control group ($P < 0.05$, $P < 0.01$). There were no differences between the BDS groups ($P = 0.005$; $P = 0.001$), and were also not significant between the BSS and BDS groups ($P = 0.22$). The mRNA expression of Th17 cells was higher in the BHS and BDS groups compared with the BDS group, but not significantly ($P = 0.02$; $P = 0.01$; Table 2).

**Comparison of IL-10 and TNF-α levels in the TCM syndrome patients and healthy controls**

Compared with the healthy control group, the serum levels of IL-10 were significantly lower in the BHS and BDS groups ($P = 0.0005$; $P = 0.005$) and were also lower in the BSS group but the difference was not significant ($P = 0.10$). There were no differences between the three TCM syndrome groups ($P = 0.20$; Table 2).

Conversely, the serum levels of TNF-α were higher in all three psoriasis patient groups compared with the healthy controls. In the BSS and BDS groups, the differences were significant ($P = 0.001$ for $P = 0.005$), while the difference between the BDS group and the healthy control group was not significant ($P = 0.07$). Again, there was no difference between the three TCM syndrome groups ($P = 0.2$; Table 2).

**Comparison of Foxp3 and RORγt mRNA expression in the TCM syndrome patients and healthy controls**

The mRNA expression of T cell-specific transcription factor Foxp3 was lower in TCM syndrome patients compared with healthy controls. The differences in Foxp3 mRNA expression were significant in the BHS ($P = 0.001$) and BDS ($P = 0.09$) groups compared with the healthy control group; the difference was not significant for the BSS group ($P = 0.66$). Among the three TCM syndrome groups, the levels of Foxp3 mRNA were much higher in the BSS group compared with the BHS and BDS groups ($P = 0.002$; $P = 0.01$, respectively; Table 3).

The mRNA expression of T cell-specific transcription factor RORγt were higher in the psoriasis patients compared with the healthy controls, particularly in the BHS group ($P = 0.0001$). In the BSS group, there was no difference between the BHS group and the healthy control group ($P = 0.02$), while no statistical difference was found between the BDS and the healthy control group ($P = 0.24$). Among the three TCM syndrome groups, the level of RORγt mRNA was significantly higher in the BHS group compared with the BDS group ($P = 0.01$), but no difference with the BSS group ($P = 0.43$; Table 3).

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**Table 1** Patient demographics and Traditional Chinese Medicine profiles of the four study groups ($\bar{x} \pm s$)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Age (years)</th>
<th>Gender [n (%)]</th>
<th>Progress (years)</th>
<th>PASI</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Male</td>
<td>Female</td>
<td></td>
</tr>
<tr>
<td>BHS</td>
<td>40</td>
<td>38.2±1.9</td>
<td>22 (55.0)</td>
<td>18 (45.0)</td>
<td>13.2±1.4</td>
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<tr>
<td>BSS</td>
<td>14</td>
<td>43.1±3.6</td>
<td>8 (57.0)</td>
<td>6 (42.9)</td>
<td>13.4±2.9</td>
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<tr>
<td>BDS</td>
<td>13</td>
<td>39.4±2.1</td>
<td>8 (61.5)</td>
<td>5 (38.5)</td>
<td>11.2±1.6</td>
</tr>
<tr>
<td>Healthy control</td>
<td>21</td>
<td>38.3±3.1</td>
<td>9 (42.9)</td>
<td>12 (57.1)</td>
<td>-</td>
</tr>
</tbody>
</table>

Notes: BHS: blood-heamt syndrome; BSS: blood-stasis syndrome; BDS: blood-dryness syndrome; PASI: Psoriasis Area and Severity Index.

**Table 2** Comparison of the ratios of Treg/Th17 cells and serum levels of IL-10 and TNF-α among the three TCM syndrome groups and the healthy control group (pg/mL, $\bar{x} \pm s$)

<table>
<thead>
<tr>
<th>Item</th>
<th>BHS (n=40)</th>
<th>BSS (n=14)</th>
<th>BDS (n=13)</th>
<th>Healthy control (n=21)</th>
<th>F value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treg</td>
<td>2.5±1.4</td>
<td>3.6±1.7</td>
<td>2.7±1.2</td>
<td>4.3±2.4</td>
<td>5.32</td>
<td>0.002</td>
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<tr>
<td>Th17</td>
<td>3.1±1.6</td>
<td>3.6±2.6</td>
<td>1.9±1.3</td>
<td>1.5±1.0</td>
<td>6.71</td>
<td>0.001</td>
</tr>
<tr>
<td>IL-10</td>
<td>2.4±1.2</td>
<td>3.2±1.9</td>
<td>2.6±1.2</td>
<td>4.1±1.7</td>
<td>6.48</td>
<td>0.001</td>
</tr>
<tr>
<td>TNF-α</td>
<td>1.6±0.7</td>
<td>1.8±1.1</td>
<td>1.4±0.5</td>
<td>0.9±0.3</td>
<td>5.81</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Notes: TCM: Traditional Chinese Medicine; BHS: blood-heamt syndrome; BSS: blood-stasis syndrome; BDS: blood-dryness syndrome; TNF: tumor necrosis factor; IL: interleukin. $^aP < 0.01$, $^bP < 0.05$, compared with the healthy control group; $^cP < 0.05$ compared with the BHS group; $^dP < 0.05$ compared with the BSS group.
maintaining immune balance. Clinical and cell inflammatory responses. The function and progression of inflammatory reactions. Increased serum levels of IL-17, TNF-α, IL-1α, IL-10, and related cytokines and mobilize, raise and activate lymphocytes that secrete IL-22, TNF-α, IL-1α, and Foxp3 (3). Treg cells and expression of IL-17, TNF-α, and Foxp3 were all lower, compared with healthy control participants (P < 0.05). One explanation for why the levels of Treg cells and related cytokines remained normal in the BSS group but not the BHS group is that Treg cells play a role in regulating Th17 cells, influencing the immune function of the related cytokines. TCM maintains that the pathogenesis of this syndrome is insufficiency of blood with nutritional effect. Specifically, the blockage of Qi and its stagnation in the skin, with the coexistence of blood stasis and pathogenic toxins, appear to be a struggle between the vital energy and pathogenic factors. Previous studies showed that the Th17 expression level in both BHS and BSS patients was higher than normal, with the most obvious differences in the BHS group. However, Treg cell expression did not increase significantly at the same time, indicating that the levels of Th17 cells and related cytokines could become a reference index for the immunological diagnosis and treatment of psoriasis and that the detection of changes in Th17/Treg levels could be used as objective criteria for assessing the condition of patients. In this study, the expression patterns of Th17 and Treg in the three TCM syndromes of the Th17 and Th17 cells agreed with previous findings. BDS could be transformed from BHS and BSS and was always in a resolution or latent phase clinically. Our results also con-

<table>
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<tr>
<th>mRNA</th>
<th>BHS (n = 11)</th>
<th>BSS (n = 8)</th>
<th>BDS (n = 8)</th>
<th>Healthy control (n = 9)</th>
<th>F value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Foxp3</td>
<td>0.71±0.21</td>
<td>1.16±0.55a</td>
<td>0.78±0.23c</td>
<td>1.29±0.30</td>
<td>6.33</td>
<td>0.002</td>
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<tr>
<td>RORγt</td>
<td>2.67±0.85</td>
<td>2.14±0.68a</td>
<td>1.66±0.90b</td>
<td>1.23±0.41</td>
<td>6.97</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Notes: TCM: Traditional Chinese Medicine; BHS: blood-heat syndrome; BSS: blood-stasis syndrome; BDS: blood-dryness syndrome. *P < 0.01, †P < 0.05, compared with the healthy control group; ‡P < 0.01, compared with the BHS group; ‡P < 0.05, compared with the BDS group.

**DISCUSSION**

In this study, we examined the circulating levels of Treg/Th17 cells, related inflammatory cytokines (IL-10, TNF-α) and T cell-specific transcription factors, (Foxp3, RORγt) and found differences between the three TCM syndrome groups and the healthy control group that can provide clinical guidance for TCM treatment principle and formula design. Operating under guiding rules of signs and symptoms to differentiate syndromes, TCM is recognized as an important approach to manage psoriasis vulgaris. For example, TCM proposes that the basic pathogenesis of psoriasis is “interior heat accumulation and stagnation of blood,” while Jin et al. considered the core pathogenesis of this disease to be “heat in blood and excessive toxin.” In recent years, research that integrates TCM syndromes and biological molecular networks has established an organic connection between TCM syndrome phenotypes and microscopic biological indicators. The pathogenesis of psoriasis is closely related to the dysfunction of immune cells, which is caused by imbalances in the immune network. T cell activation has been proven to be a central pathogenic mechanism of the immune cell-inflammatory network in psoriasis, with the most supportive evidence being the detection of activated CD4+ and CD8+ T lymphocytes in the epidermis and dermis of psoriasis patients. Th17 cells are a newly-discovered subset of CD4+ T lymphocytes that secrete IL-17, IL-6, IL-22, TNF-α and other cytokines and mobilize, raise and activate neutrophils to participate in the occurrence and progression of inflammatory reactions. Increased serum levels of IL-17 and IL-23 in psoriasis indicates that Th17 cells and associated cytokines are involved in the pathogenesis of psoriasis. In recent years, Treg cells, isolated for study as an independent T lymphocyte subset, were found to be distinct from Th1 and Th2 cells. TGF-β could stimulate native T cells to differentiate into Treg cells, which mainly secrete IL-10 and TGF-β. Treg cells are differentiated from CD4+ T cells and play the role of immune tolerance mediator, while Th17 cells participate in a series of immune and inflammatory responses. The function and progress of differentiation are mutually antagonistic. Under normal circumstances, homeostasis is usually maintained. Keeping the Th17/Treg ratio balanced is essential to maintaining immune balance. Clinical and cell experiments indicate that imbalance of the Th17/Treg ratio might be one pathogenetic mechanism of autoimmune diseases.
firmly that, in the BDS group, the levels of pro-inflammatory Th17 cells, cytokine TNF-α and the transcription factor RORγt were relatively normal; at the same time, the levels of Treg cells, cytokine IL-10 and the transcription factor RORα remained in a declining state. We propose that dysfunction of Treg cells could be the defining immunological property of the BDS.

In conclusion, the three TCM syndromes associated with psoriasis vulgaris presented entirely different types of lesions and clinical manifestations. The expression of Treg and Th17 immune cells, together with specific cytokines and transcription factors, was complex, with each syndrome showing unique key immune indicators. The differentiation of Th17 cells accounted for major advantages in BHS patients, but without significant changes in Treg cells. The differentiation of Th17 cells also accounted for major advantages in BSS patients, with concomitant decreases in Treg cells. The differentiation of Th17 cells accounted for major advantages in BDS patients, whereas there was no practical significance for Th17 cells. We conclude that there is a certain correlation between the blood regulating therapy and the immune balance of Treg/Th17 cells, wherein each syndrome may possess a different immune status. The detection of Treg/Th17 cells, related cytokines and transcription factors corresponded, to some extent, to the biological characteristics of the TCM syndromes. Disordered immune cells are an important factor in the pathogenesis of psoriasis. Syndrome differentiation and treatment involves a large number of objective and microscopic substances and immune cells and immune network theory may be seen as representing just a part of the whole organism. If we want to accurately explore the objective laws in the classification of TCM syndromes, our research must include the detection of the levels of various types of cells whenever possible. By clarifying this point, we can treat psoriasis more pertinently through different therapeutic targets of different Chinese medicines.

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