

Th1/Th2 profile in peripheral blood in atopic cough and atopic asthma

T. Shirai, K. Suzuki, N. Inui*, T. Suda*, K. Chida* and H. Nakamura*

Department of Internal Medicine, Fujinomiya City General Hospital, Fujinomiya and *Second Department of Internal Medicine, Hamamatsu University School of Medicine, Hamamatsu, Japan

Summary

Background Eosinophilic tracheobronchitis with cough hypersensitivity, abbreviated as atopic cough, is an important cause of chronic cough. The reason for the absence of airway hyper-responsiveness is unknown, differing from asthma, a Th2 cytokine-mediated disorder.

Objective To compare the type 1 helper T cell (Th1)/Th2 balance in the peripheral blood from subjects with atopic cough and atopic asthma, we assessed the intracellular cytokine production at the single-cell level.

Methods Thirty-six subjects (10 patients with atopic cough, 18 with atopic asthma, and eight control subjects) were included. Intracellular IL-4 and IFN- γ were detected in CD4⁺ T cells by flow cytometry.

Results A significantly lower ratio of IFN- γ /IL-4-producing CD4⁺ T cells after phorbol 12-myristate acetate/ionomycin stimulation was found in patients with atopic cough and atopic asthma compared with normal subjects. In comparison between atopic patients, the ratio of IFN- γ /IL-4-producing cells was significantly higher in atopic cough than in atopic asthma. However, the proportion of IL-4-producing CD4⁺ T cells was significantly higher in patients with atopic asthma than in normal control subjects and no significant difference was detected between patients with atopic cough and normal subjects. No significant difference in the proportion of IFN- γ -producing cells was found between the subjects. Overall, the total IgE levels were positively correlated to the IL-4-producing cells and inversely correlated to the ratio of IFN- γ /IL-4-producing cells.

Conclusion These results show the lower degree of Th2 cytokine predominance in atopic cough compared with atopic asthma and suggest the relation between the Th1/Th2 balance and atopic status.

Keywords airway hyper-responsiveness, capsaicin, CD4⁺ T cells, flow cytometry, IFN- γ /IL-4, intracellular cytokine

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Introduction

Non-asthmatic eosinophilic airway inflammation is a common cause of chronic cough [1–3]. Eosinophilic tracheobronchitis with cough hypersensitivity, also known as atopic cough, is a condition proposed by Fujimura et al. in which atopy is common, cough response to inhaled capsaicin is increased [4–6], eosinophils are documented in the induced sputum [6], and the submucosa of biopsied trachea and bronchi but not the bronchoalveolar lavage [4], cough is non-productive, corticosteroids but not bronchodilators are effective, and airway responsiveness to methacholine is not heightened [4–6]. The presence of eosinophilic inflammation and corticosteroid-responsiveness are common to asthma, but the absence of airway hyper-responsiveness (AHR) is distinct. The number of eosinophils in the induced sputum and the biopsied bronchial submucosa is found to be smaller in atopic cough than in

asthma [4–6]. In addition, submucosal eosinophilic inflammation, which is limited to the central bronchi, is more intensive in patients with more severe atopic cough compared to patients with a milder form [7]. However, the pathogenesis of atopic cough, in particular what makes differences between atopic cough and bronchial asthma, is unknown.

Asthma is characterized by airway inflammation with eosinophils, mast cells, and T lymphocytes. It is known that an increased production of type 2 helper T (Th2) cell-derived cytokines (IL-4, IL-5, and IL-13) and/or a decreased production of Th1 cell-derived cytokines (IFN- γ and IL-2) are essential for developing airway inflammation [8–10]. A study of the balance of Th1/Th2 in asthma and other eosinophilic inflammatory disorders is one way to investigate the pathogenesis.

Therefore this study was performed to compare the Th1/Th2 balance in the peripheral blood from subjects with atopic cough and atopic asthma. We also sought to examine the relationship between the Th1/Th2 balance and atopic status in these conditions. We have used the flow cytometric method to examine intracellular cytokine production in CD4⁺ T cells at the single-cell level [11]. This method is sensitive and does not

Correspondence: Toshihiro Shirai, Fujinomiya City General Hospital, 3–1 Nishiki-cho, Fujinomiya, 418–0076 Japan. E-mail: fmyhsp@lilac.ocn.ne.jp

need purification of peripheral blood mononuclear cells which might modify the cell activation.

Materials and methods

Subjects

Thirty-six subjects aged 19–75 years participated in the study. Subjects consisted of patients with atopic cough ($n = 10$), atopic asthma ($n = 18$), and normal control subjects ($n = 8$). Atopic cough was diagnosed by the following criteria [4]: (i) chronic non-productive cough without wheeze lasting for more than 8 weeks; (ii) presence of one or more of the following findings indicative of atopy, including past history and/or complication of allergic diseases except for bronchial asthma, peripheral blood eosinophilia, elevated total IgE level in serum, positive specific IgE antibody to at least one inhalant allergen including *Dermatophagoides pteronyssinus*, cat fur, Japanese cedar pollen, grass pollen, and *Candida albicans*, positive allergen skin test, and induced sputum eosinophilia; (iii) no bronchial reversibility defined as $< 10\%$ improvement in forced expiratory volume of 1 s (FEV₁) 10 min after inhaling 300 µg salbutamol; (iv) normal airway responsiveness; (v) increased sensitivity of cough response to inhaled capsaicin; (vi) bronchodilator-resistant non-productive cough; (vii) a normal chest radiograph; and (viii) normal spirometric values. Other causes of cough including gastroesophageal reflux, post-nasal drip, and chronic bronchitis were excluded. The patients with atopic asthma satisfied the American Thoracic Society criteria [12], had presence of AHR, and presence of findings indicative of atopy. Severity of asthma was classified according to the classification of the Global Initiative for Asthma [13]. The normal control subjects had none of the findings indicative of atopy and normal sensitivity of cough response to inhaled capsaicin. No subject was a current smoker, had been taking angiotensin-converting enzyme inhibitors, or had received oral or inhaled corticosteroids for at least 1 month before entry into the study. All subjects gave informed consent. The protocol was approved by the local ethics committee.

Study design

Patients suspected of having atopic cough attended the hospital on at least two occasions. At the first visit they had a capsaicin inhalation test, spirometry, bronchial reversibility test, a chest radiograph, and blood tests. At the second visit the subjects had a methacholine inhalation test. This study was done before the cough resolved with successful treatment. Patients with atopic asthma and normal control subjects also had the above tests. However, the capsaicin inhalation test was not performed in patients with atopic asthma because its relevance is not certain.

Capsaicin inhalation test

Cough threshold to inhaled capsaicin was measured according to the method reported previously (14, 15) and defined as the lowest concentration of capsaicin that elicited five or more coughs. A concentration ≤ 3.9 mM was defined as increased airway cough sensitivity.

Methacholine inhalation test

Bronchial challenge tests to methacholine were performed according to the standard method proposed by the Japanese

Society of Allergology (16, 17). A provocation concentration of methacholine causing a 20% fall in FEV₁ < 10 mg/mL was defined as AHR (18).

Reagents and antibodies

Ionomycin, phorbol 12-myristate acetate (PMA), brefeldin-A, and bovine serum albumin (BSA) were purchased from Sigma Chemical Co (St Louis, MO, USA). The following antibodies were used: peridin chlorophyll protein (PerCP)-conjugated anti-CD4, fluorescein isothiocyanate (FITC)-conjugated anti-IFN- γ , and phycoerythrin (PE)-conjugated anti-IL-4 antibodies (Becton Dickinson, San Jose, CA, USA). Isotype-matched, conjugated (FITC, PE, and PerCP) control antibodies, fluorescence-activated cell sorting (FACS) Lysing Solution, and FACS Permeabilizing Solution were obtained from Beckton Dickinson.

Preparation of peripheral blood cells

Ten milliliters of heparinized blood were collected from each patient or control subject and diluted 1 : 20 with a culture medium consisting of RPMI-1640 supplemented with 10% fetal calf serum (FCS, Gibco, Rockville, MS, USA), 100 U/mL penicillin (Gibco), and 100 µg/mL streptomycin (Gibco). In several experiments, PBMCs were isolated by means of density centrifugation of heparinized blood on Lymphoprep (Nycomed, Oslo, Norway). To further deplete B cells and natural killer (NK) cells, non-adherent cells were incubated with saturating concentrations of anti-CD 11b, anti-CD 16, anti-CD19, anti-CD36, and anti-56 mAbs conjugated with superparamagnetic microbeads (Miltenyi Biotec, Gladbach, Germany) for 15 min on ice and washed in phosphate-buffered saline (PBS) containing 5 mM EDTA and 0.5% human serum. Unlabelled cells were then isolated by eluting from magnetic columns (Miltenyi Biotec), routinely resulting in greater than 98% purity of CD3⁺ and less than 1% CD56⁺ cells.

Stimulation of cells and intracellular cytokine staining

One milliliter of peripheral blood diluted 1 : 20 in the culture medium were placed in each well of a 24-well culture plate. They were then stimulated with PMA (25 ng/mL) and ionomycin (1 mg/mL) in the presence of brefeldin-A (10 mg/mL) for 4 h at 37 °C in a humidified 5% CO₂ incubator. Brefeldin-A was used to interrupt intracellular transport mechanisms, thereby leading to accumulation of cytokines in the cells.

After stimulation with PMA/ionomycin, the cells were incubated with either PerCP-conjugated anti-CD4 antibody for 15 min at room temperature. In the preparation of peripheral blood cells, erythrocytes were lysed by adding FACS Lysing Solution. After centrifugation, the cells were washed with 0.1% BSA-PBS and subsequently incubated in FACS Permeabilizing Solution for 10 min at room temperature. After washing with 0.1% BSA-PBS, the permeabilized cells were then incubated with FITC-conjugated anti-IFN- γ , and PE-conjugated anti-IL-4 antibodies for 30 min at room temperature. After staining, they were washed with 0.1% BSA-PBS, fixed in 1% paraformaldehyde, and stored at 4 °C. The cells were analysed within 12 h after storage. Negative controls consisted of unstimulated cells. We confirmed that the viability of the cells freshly obtained from peripheral blood was greater than 97% by using trypan

blue exclusion. After PMA/ionomycin activation, the viability of each cell population was greater than 95%.

Flow cytometric analysis

The samples were analysed on a FACScan flow cytometer (Beckton Dickinson) equipped with a 15-mW argon ion laser and appropriate filters for FITC (530 nm), PE (585 nm), and PerCP (> 650 nm) by using the Cell Quest Software (Beckton Dickinson). Lymphocytes were first gated on the basis of forward- and side-light scatter. Subsequently, within this gated area, in which CD3⁺ cells fell, a second gate was further selected. The following analyses were done on cells in the second gate. For three-color analysis, we measured the number of positive cells for anti-IFN- γ (FITC), anti-IL-4 (PE), or both antibodies simultaneously in 50 000 gated cells that were stained with anti-CD4 antibody (PerCP). The number of positive cells for each cytokine was expressed as a percentage of CD4⁺ T cells. In all experiments, parallel incubations were performed with FITC- or PE-conjugated irrelevant antibodies matched for the isotypes of the anticytokine antibodies. The cut-off level for definition of positive cells was thus set so that less than 1% of irrelevant-antibody stained cells were positive.

Statistics

Results are expressed as mean $\pm$ SEM. Comparisons between groups were made with the Kruskal–Wallis test, followed by the Mann–Whitney *U*-test. Correlations between variables were done using the Spearman rank correlation coefficient. A *P*-value of < 0.05 was considered significant. Power analysis was performed with a sample size estimation program (SSEP) [19] and the power of this study was 37.9%.

Results

All the subjects completed the study. Clinical characteristics of the subjects are shown in Table 1. Blood eosinophil counts were significantly higher in atopic asthma than in atopic cough and normal control subjects. Total IgE levels were significantly increased in patients with atopic cough and atopic asthma compared with control subjects. FEV₁ and FEV₁/FVC were significantly higher in atopic cough and normal controls compared with atopic asthma. AHR was detected in atopic asthma

but not in atopic cough and normal control subjects. Capsaicin cough threshold was significantly lower in atopic cough than in control subjects. Eighteen atopic asthmatic patients were classified as: 10, intermittent (step 1); seven, mild persistent (step 2); and 1, moderate persistent (step 3).

Under unstimulated conditions, we could find no cytokine-expressing cells in the CD4⁺ T cell population of peripheral blood in patients or normal control subjects.

In response to PMA/ionomycin, no significant difference was found in the proportion of IFN- γ -producing CD4⁺ T cells between patients with atopic cough and atopic asthma and normal control subjects (atopic cough, 19.8% $\pm$ 1.7%; atopic asthma, 17.3% $\pm$ 1.0%; normal control, 21.5% $\pm$ 2.1%; Fig. 1, upper panel). However, the percentage of IL-4-producing CD4⁺ T cells was significantly higher in patients with atopic asthma than in normal control subjects (4.0% $\pm$ 0.4% vs. 2.0% $\pm$ 0.3%; *P* = 0.0205; Fig. 1, middle panel). In addition, the ratio of IFN- γ /IL-4-producing CD4⁺ T cells was significantly lower in patients with atopic cough and atopic asthma than in normal control subjects (7.4 $\pm$ 1.0, 5.0 $\pm$ 0.5 vs. 12.1 $\pm$ 1.9, respectively; *P* = 0.0011; Fig. 1, lower panel). The difference between patients with atopic cough and atopic asthma was also significant.

No correlation was found between the eosinophil counts and the intracellular cytokine production in CD4⁺ T cells. However, the total IgE levels were positively correlated to the proportion of IL-4-producing CD4⁺ T cells (*r* = 0.49, *P* = 0.0046) and inversely correlated to the ratio of IFN- γ /IL-4-producing CD4⁺ T cells (*r* = -0.60, *P* = 0.0005; Fig. 2). No correlation was found between the total IgE levels and the proportion of IFN- γ -producing CD4⁺ T cells. If the normal subjects were excluded from analysis, there was a significant correlation between the total IgE levels and the ratio of IFN- γ /IL-4-producing CD4⁺ T cells (*r* = -0.41, *P* = 0.0321). No other significant correlation was found.

Discussion

We found a significantly lower ratio of IFN- γ /IL-4-producing CD4⁺ T cells in peripheral blood after PMA/ionomycin stimulation in patients with atopic cough as well as in patients with atopic asthma compared with normal subjects. In contrast,

Table 1. Characteristics of subjects

	Atopic cough	Atopic asthma	Normal controls
Number	10	18	8
Sex (male/female)	2/8	7/11	1/7
Age*	54 (29–75)	46 (19–71)	43 (26–48)
Ex-smoker/non-smoker	3/7	7/11	2/6
Blood eosinophils (cells/ μ L)*	187 (0–832)	548 (0–2988)‡	112 (0–330)
Total IgE (U/mL)†	231 (5–1200)§	542 (10–2300)§	15 (5–29)
FEV ₁ (% of predicted)*	120 (98–151)	97 (67–128)‡	118 (95–141)
FEV ₁ /FVC (%)*	88 (85–93)	82 (72–98)‡	87 (82–95)
PC ₂₀ (mg/mL)†	> 20.00¶	1.35 (0.31–6.59)	> 20.00¶
Capsaicin cough threshold (μ M)	2.58 (0.49–3.90)§	ND	20.20 (7.80–125.00)

PC₂₀, provocative concentration of methacholine causing a 20% fall in FEV₁; ND, not done. *Mean with range shown in parentheses. †Geometric mean with range in parentheses. ‡*P* < 0.05 versus atopic cough and normal controls; §*P* < 0.05 versus normal controls. ¶All subjects showed PC₂₀ > 20 mg/mL.

there was a significantly higher percentage of IL-4-producing CD4⁺ T cells in patients with atopic asthma but not atopic cough compared with normal subjects. These findings suggest that the prominent shift toward a type 2 phenotype, relative to normal subjects, may occur in CD4⁺ T cells in atopic cough, although to a lower degree than in atopic asthma.

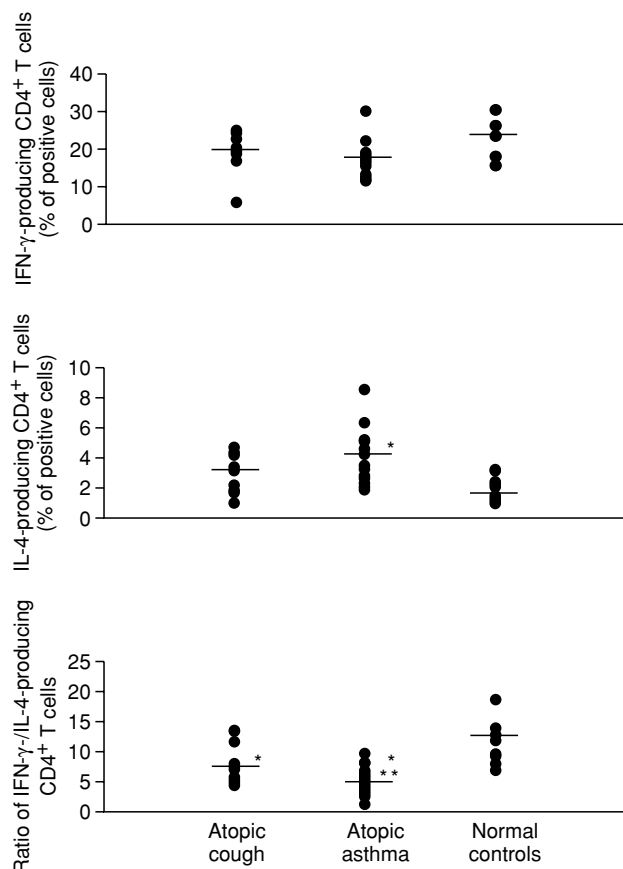


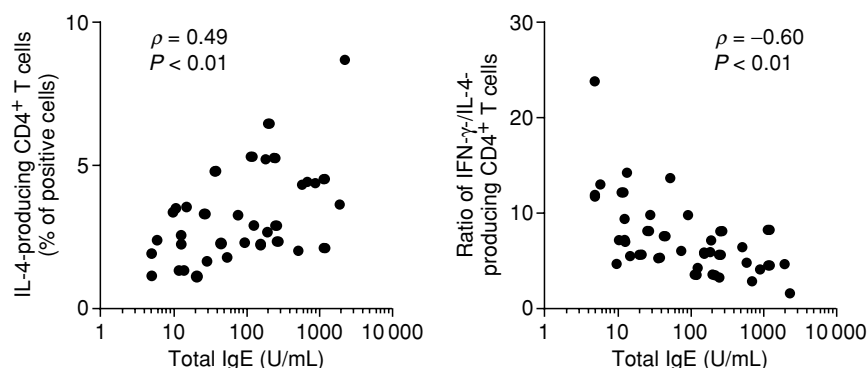
Fig. 1. The percentage of IFN- γ - and IL-4-producing CD4⁺ T cells and the ratio of IFN- γ /IL-4-producing CD4⁺ T cells obtained from peripheral blood after stimulation with PMA/ionomycin. The percentage of IL-4-producing CD4⁺ T cells is significantly higher in patients with atopic asthma than in normal controls. The ratio of IFN- γ /IL-4-producing CD4⁺ T cells is significantly lower in patients with atopic cough and atopic asthma than in normal controls. The difference between patients with atopic cough and atopic asthma was also significant. However, no significant difference is found in the proportion of IFN- γ -producing CD4⁺ T cells between the patients and control subjects. * $P < 0.05$ versus normal controls; ** $P < 0.05$ versus atopic cough.

Intracellular cytokine production was assessed by using three-colour flow cytometry. This technique permits the simultaneous detection of multiple cytokines at the single-cell level in different cell populations, contributing to the clinical evaluation of immune status in terms of the cytokine profiles. The patterns of cytokine expression in T cells activated with a combination of PMA and ionomycin used in this study were shown to generally agree with their physiologically regulated expression [20]. A previous study reported that there was no difference in the profiles of cytokine expression among activating signals, such as PMA/ionomycin, superantigen, and anti-CD3 plus anti-CD28 mAbs, because the profiles could reflect intrinsic characteristics of the T cells *in vivo* after fully activating them [21]. Thus this method enables us to study cells with little *in vitro* incubation, decreasing the potential for artifacts arising from prolonged culture. In preliminary experiments, we isolated highly purified population of CD3⁺ T cells from whole blood using magnetic cell sorting, and compared the cytokine profiles of purified CD3⁺ T cells with those of unseparated whole blood. We found no difference in cytokine profiles of CD4⁺ cell population between purified CD3⁺ T cells and unseparated whole blood. Although the unseparated whole blood contained NK cells which were likely to express IFN- γ in response to PMA/ionomycin, we put the gates on CD4⁺ cells, and the contamination of NK cells was negligible. These findings are consistent with those of a study on intracellular cytokine expression with unseparated whole blood [22]. Taken together, it is reasonable to measure the intracellular cytokine expression by unseparated whole blood, instead of purified cells.

Because PMA/ionomycin stimulation has been shown to down-regulate CD4 expression, there may be difficulty in recognizing a clear CD4⁺ population with anti-CD4 mAb [21, 23], resulting in the misreading of the true population of CD4⁺ T cells. Indeed, we found that the mean fluorescent intensity (MFI) of CD4 expression by blood cells was decreased by 62% after 4 h of stimulation with PMA/ionomycin (MFI of unstimulated cells, 601; MFI of stimulated cells, 226). In our assays, however, we could still detect clearly bimodal profiles for CD4 after stimulation, and there was no difference in the proportion of CD4⁺ cells between unstimulated and stimulated cells (36.0% vs. 35.4%). Thus we used the above protocol for measurement of cytokine expression in CD4⁺ T cells.

Total IgE levels, served as a marker of atopic status, are correlated positively to the percentage of IL-4-producing CD4⁺ T cells and negatively to the ratio of IFN- γ /IL-4-producing CD4⁺ T cells. Previous studies also demonstrated

Fig. 2. Correlations between total IgE levels and proportion of IL-4-producing CD4⁺ T cells and ratio of IFN- γ /IL-4-producing CD4⁺ T cells. Total IgE levels are positively correlated to the proportion of IL-4-producing CD4⁺ T cells ($\rho = 0.49$, $P < 0.01$) and inversely correlated to the ratio of IFN- γ /IL-4-producing CD4⁺ T cells ($\rho = -0.60$, $P < 0.01$). Total IgE levels are log-transformed.



the correlation between atopy or total IgE levels and IL-4, although their methods were different from ours [24,25]. We could not detect the reduced proportion of IFN- γ -producing CD4⁺ T cells in atopic patients. The impairment of CD4⁺ T cells to produce IFN- γ is suggested to be related to atopy as opposed to the overproduction of IL-4 [25]. However, a predominant shift to IL-4-producing cells in the balance of IFN- γ /IL-4-producing cells demonstrated in this study is compatible with previous studies, which suggest that atopy is the result of an impaired IFN- γ production leading to IL-4 synthesis and IgE-dependent sensitization to allergens [24–26].

In atopic asthma, an increased proportion of IL-4-producing CD4⁺ T cells is found together with the lower ratio of IFN- γ /IL-4-producing cells. Other investigators also reported the lower ratio of IFN- γ /IL-4-producing CD4⁺ T cells in whole blood culture at the single-cell level in atopic asthma compared with normal controls. However, Wong et al. found a reduced proportion of IFN- γ -producing cells but no increase of IL-4-producing cells [27], while Kuo et al. did not find any significant differences in proportions of IFN- γ - or IL-4-producing cells between atopic asthmatic children and normal controls [28]. Basically, there is no difference in methodology between theirs and ours. The diversity of studied population or atopic status may be the cause of the discrepancy.

Fujimura et al. reported that atopic cough and bronchial asthma share a common pattern of eosinophilic inflammation in the airway [4, 6]. However, the number of eosinophils in the induced sputum and the biopsied bronchial submucosa was smaller in atopic cough than in asthma, and eosinophils in bronchoalveolar lavage fluid were not increased in patients with atopic cough, suggesting the possibility that the pathogenesis of atopic cough is different from that of atopic asthma in the view of site and intensity of eosinophilic inflammation. Thus, it would be interesting to assess the Th1/Th2 balance in the airway samples in addition to peripheral blood. The limitation of this study is in using only peripheral blood cells.

The small numbers of the subjects also limit the power of this study, suggesting the possibility of false negative results. However, the significant differences found among atopic cough, atopic asthma, and normal controls are, at least partly, relevant despite the low power of the study. Further studies with more subjects will clarify the difference between atopic cough and atopic asthma.

In conclusion, the CD4⁺ T cell population in the peripheral blood of patients with atopic cough had a prominent shift to a Th2 phenotype compared with those of normal subjects, but to a lower degree than those of atopic asthma. Our data also suggest the relationship between the Th1/Th2 balance and atopic status.

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