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Original Article

Serum Cytokine Profile and Mechanism of Eight Patients with Chronic Idiopathic Urticaria

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Background: Chronic urticaria (CU) is short-lived itching maculopapular skin lesions with or without angioedema for more than 6 weeks. Sixty percent of CU patients were classified as chronic idiopathic urticaria (CIU) with an underlying autoimmune mechanism.
Objective: This study aims to evaluate the serum cytokine profiles of CIU and investigate the possible mechanism of autologous serum therapy (AST) for autologous serum skin test (ASST) positive CIU patients.
Methods: In total 8 CIU patients, 6 were ASST positive and 4 of the 6 patients received AST for 9 weeks. All patients in study group are subjected to detect anti-nuclear antibodies and autoimmune thyroid disease. Anti-histamines were allowed to be consumed when needed during weekly serum therapy. Clinical symptoms and the serum cytokine levels including IL-4, IL-5, IL-9, IL-13, IL-17, IL-6, TNF-α, IL-12(p70), IL-17A and INF-γ were monitored and analyzed.
Results: The CIU patients showed significantly increased IL-9 (6.29±4.29 versus 2.28±1.17 pg/ml) and IL-6 (6.16±5.58 versus 2.23±0.96 pg/ml) serum level compared to control group. CIU patients with positive ASST had higher IFN-γ compared to negative ASST. Furthermore, higher TNF-α and IFN-γ in ASST positive patients had better response to AST. In addition, IL-9 was the only cytokine that decreased gradually after AST.
Conclusion: Based on our results, the cytokines IL-9, IL-6 and TNF-α are involved in mechanism of CIU inflammation. The IFN-α increase potentially leads to CIU patients with ASST positive; whereas higher level of TNF-α might be crucial for successful AST.

Keywords: Chronic urticaria; Chronic idiopathic urticaria; Autologous serum skin test; Autologous serum therapy; Inflammation.

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1. Introduction:

Urticaria is a kind of skin lesion caused by histamine release from degranulated mast cells which affect 15-25% of population worldwide.[1] Urticaria renders

patients stress due to disrupted sleep and recurrent itching or even persistent skin lesion.[2] When the urticaria lasts more than 6 weeks, the condition is called chronic urticaria which covers 30% of the urticaria cases.[3] Although urticaria is a common disease with a long history;[4] however, the mechanisms of urticaria, especially CU, are not well-studied.

There are three main types of CU, including chronic idiopathic urticaria (CIU, 60%), physical urticaria (35%) and urticaria vasculitis (5%).[5] CIU has been suggested to be an autoimmune response since 50% of the CIU patients produced IgG, IgE auto-antibodies, binding to IgE receptors (FcεRI) on mast cells and basophils which activated the cells to release histamine.[6,7] Due to the autoimmune characteristics of CIU, autologous serum therapy (AST) has been reported to ameliorate the histamine inflammatory effect in 21-60% of CIU patients.[8] Although potential treatment for CIU has been documented; however, there are also controversial reports against the effect of AST.[9,10] In this regard, the controversial result of AST is due to lack of understanding of the mechanism involved.

The basophil histamine release assay is a formal in vitro test to detect functioning autoantibodies circulating in CIU patients, but it is not practical to be performed in various parts of the world. [11,12] On the contrary, autologous serum test (ASST) is more practical to detect relevant autoantibodies ASST is a method depicted in 1986 which test patients` response to inflammatory mediators in the autologous serum.[13,14] ASST positive patients produce the visible hives on skin. Not all CIU patients are ASST positive and it has been suspected that ASST positive CIU patients had higher chance of successful AST. AST is still an effective method of treatment for CIU; however, studies on the AST-associated mechanism are lacking. Therefore, the goal of this study is to examine the effect of AST in ASST positive patients based on the change of cytokine profile before and after AST. We investigated the cytokines related to positive ASST and the possible predictor for successful AST in ASST positive CIU patients. Understanding the mechanisms of AST is crucial for improved CIU treatment.

2. Materials and Methods

2.1. Study design

This paper follows ethical approval of the Show Chwan Memorial Hospital and informed consent was obtained from all the subjects included in this study. All recruited CIU patients received ASST and the patients with positive ASST underwent 9 consecutive weeks of AST by intramuscular injections. The patients with negative ASST continued to participate by follow-up visit at the hospital without AST. All patients in study group are subjected to detect anti-nuclear antibodies (ANA), thyroglobulin, microsomal, and thyroid stimulating hormone (TSH) receptor antibodies. To secure patients` health during the trial, anti-histamines were allowed to be consumed when needed to counter inflammatory response after every weekly serum therapy. After the 9 consecutive weeks of AST, patients were further followed up for 3 months on response to the serum therapy by any need of anti-histamine drugs; in case of any needed medical attention, the patients were attended to immediately to ensure safety. The healthy individuals not including pregnant women were included in the control group. Blood samples were collected from study and control group for analysis of serum interleukins. The interleukins including IL-4, IL-5, IL-9, IL-13, IL-17, IL-6, TNF-α, IL-12(p70), IL-17A and INF-γ were measured by enzyme-linked

2.2. Immunosorbent assay.

During the weekly serum therapy, patients were evaluated the activity of urticaria by symptom score (range from 0-15, evaluating the symptoms of pruritus, number of hives, size of largest hive, interference with sleep and interference with daily activities) which was modified from Urticaria Activity Score Over 7 Days. Patients were also asked the need for emergency anti-histamine drugs during the period of AST.

2.3. Serum collection

To prepare serum from patients for ASST and AST, 5 mL venous blood was collected from the participants. The blood was centrifuged 3000 rpm, 5 min, and 2 mL serum collected by a syringe

for AST. Immediately after collecting serum, serum was injected back into the CIU patients by intramuscular injection.

2.4. Autologous serum skin test

The ASST is performed to determine if the flaring developed in patients was due to release of auto-antibodies which indicates auto-immune urticaria. To perform the ASST, 0.05 ml of the freshly-isolated serum was injected by intramascular method. The 10 mg/mL histamine drug was injected on the same arm as a positive control and injection with normal saline was a negative control on the identical arm. Observation of reddish colored flaring response, hive having diameter more than 1.5 mm indicated the participant was positive. All serum, histamine and normal saline were injected in equal volumes. The responses of injected area were measured 30 min after the injections (Figure 1B).

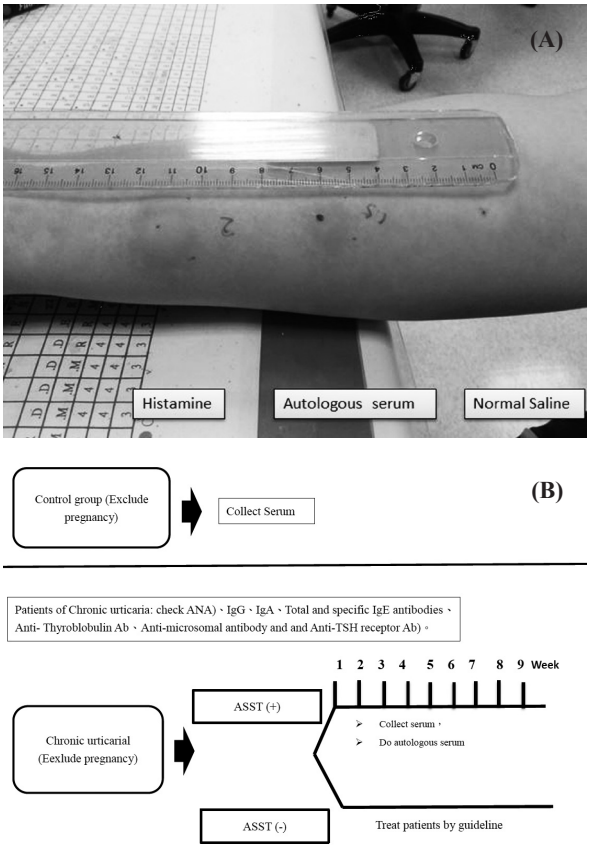


Figure 1. Autologous serum skin test (ASST) for autologous serum therapy in chronic idiopathic urticarial patients. **A)** Picture showing: (1) negative control injected with normal saline; (2) ASST positive injected with serum; (3) positive control injected with histamine. **B)** Flowchart depicting the control and study groups which excluded the pregnant individuals.

2.5. Enzyme-linked immunosorbent assay

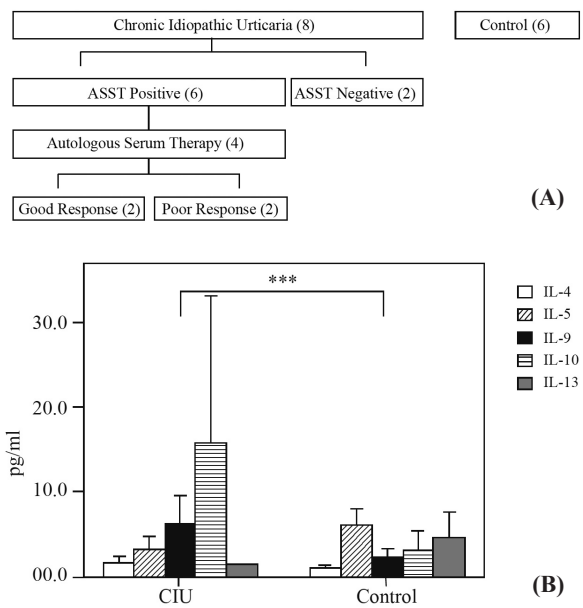
Enzyme-linked immunosorbent assay (ELISA) was used to analyze the cytokine in blood samples of CU and control participants. Blood samples were prepared no dilution, and 1:10 dilutions in microplate, absorbance measured at 450 nm. The samples were prepared in duplicates, cytokine concentration calculated based on standard curve.

2.6. Statistical analysis

The mean value of ELISA measurements were derived for control and CIU groups. Results are represented as mean ± standard error of the mean (SEM). Student`s t test, two-sided and Mann-Whitney U-test were used to compare the mean values obtained from two independent conditions; **P*<0.05 indicates a significant result, ***P*<0.01 indicates a very significant result, ****P*<0.001 indicates a highly significant result.

3. Results

3.1. Autologous serum test



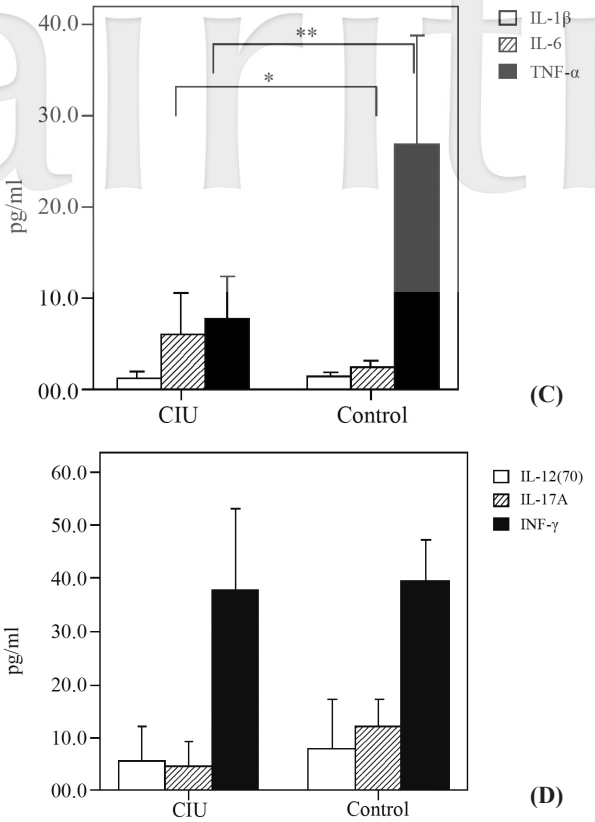


Figure 2. CIU patients had higher IL-6 and IL-9 compared to healthy controls. **A)** Illustration showing the number for study and control groups. **B)** IL-4, IL-5, IL-9, IL-10 and IL-13; **C**) IL-1β, IL-6, TNF-α; **D**) IL-12(p70), IL-17A and IFN-γ.

There were 8 CIU patients included in study group. Out of 8, 6 patients were ASST positive, 4 patients participated the AST. In the control group, 6 healthy adults were recruited (Figure 2B). The average age for control and CIU groups had no difference. The white blood count, hemoglobin level, platelet count and eosinophil percentage between the two groups showed no significant difference. In CIU group, three patients showed ANA over 1:80 and one had thyroglobulin antibody (Table 1, Table 2).

3.2. Increased IL-6, IL-9 in CIU patients

Before AST, the cytokines related to TH2 response in the CIU patients showed significantly increased IL-9 level compared to control (6.29±4.29 versus 2.28±1.17 pg/ml) (Figure 2B). The pro-inflammatory cytokine IL-6 was higher in CIU (6.16±5.58 versus 2.23±0.96 pg/ml) whereas TNF-α was significantly lower in CIU patients (7.79±6.70 versus 26.79±14.83 pg/ml) (Figure 2C). The TH1 response-related cytokines such as IL-12(p70), IL-17A, IFN-γ showed no significant difference between CIU patients and control groups (Figure 2D).

3.3. High expression of IFN-γ in CIU patients with positive ASST

ASST was performed in all CIU patients. Out of the 8 CU patients, 6 were positive for ASST. The level of IFN-γ was significantly higher in ASST positive patients (41.00±24.07 versus 27.69±12.60 pg/ml) (Figure 3A). Meanwhile, the serum level of IL-9, IL-6 or TNF-α showed no significant difference between CIU patients with ASST positive and CIU patients with ASST negative (Figure 3A).

3.4. Response of ASST positive patients to autologous serum therapy

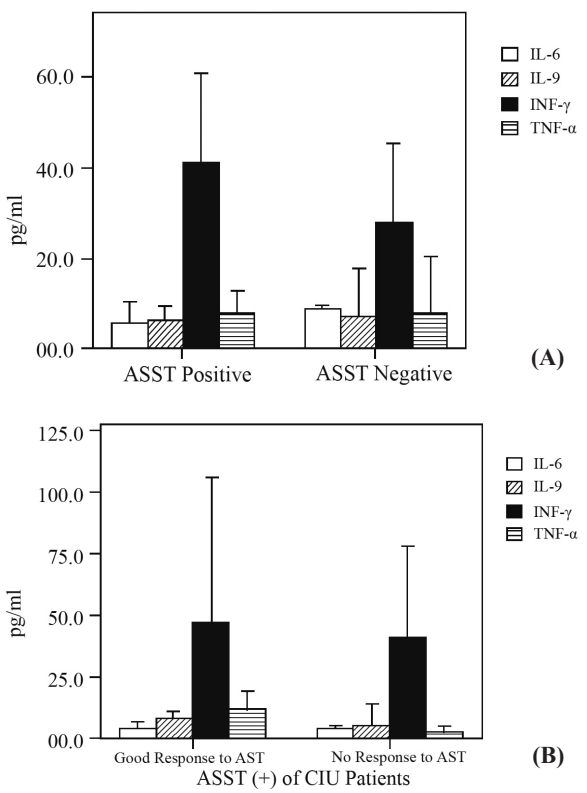


Figure 3. Response of ASST positive CIU patients to autologous serum treatment. **A)** IL-6, IL-9, INF-γ, and TNF-αof ASST positive and ASST negative CIU patients. **B)** IL-6, IL-9, INF-γ and TNF-α serum level of ASST positive CIU patients receiving AST.

Table 1. Demographic and other characteristics of the patients.

Characteristics	Control N=6	CIU N=8	P value
Age	37.83±6.96	36.75±14.05	0.85
Male	2	3	NA
Female	4	5	NA
White blood cell	6.45±0.63	9.54±2.548	0.82
Hemoglobulin	13.97±1.70	14.42±1.47	0.79
Platelet	234.50±39.80	271.84±179.83	0.79
Neutrophil	65.08±3.78	54.86±23.76	0.33
Monocyte	6.17±1.13	7.88±2.45	0.33
Eosinophil	1.97±1.516	1.44±0.95	0.79
No. of ANA≧1:80	ND	3	NA
No. of Anti-TPO Ab (+)	ND	0	NA
No. of Thyroglobulin Ab (+)	ND	1	NA

CIU: Chronic idiopathic urticaria
ND: Not detected
NA: Not applicable
(+): Positive detection

Table 2. Total IgE, ANA and Thyroblobulin of ASST positive and negative patients.

CIU patients	ASST positive N=4	ASST negative N=2	P value
Total IgE	545.00±212.10	91.45±98.35	0.85
No. of ANA≧1:80	2	1	NA
No. of Thyroglobulin Ab (+)	Not detected	1	NA

CIU: Chronic idiopathic urticaria
NA: Not applicable
(+): Positive detection

Furthermore, 4 out of 6 ASST positive CIU patients received AST for 9 consecutive weeks. Two of the subjects showed good response to the AST (symptom score decreased to below 5 and no need of emergency anti-histamine medicine) and 2 had no response. In addition, the level of TNF-αand IFN-γ were higher in patients with good response to AST (Figure 3B).

Serum level of IL-9 decreased gradually during AST in good response patient. We further analyzed serum cytokine in 2 patients with the good AST

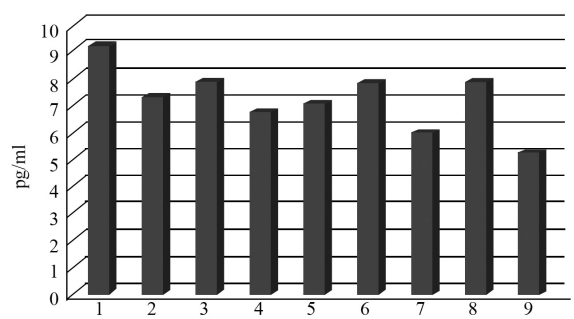


Figure 4. The serum level of IL-9 for ASST positive patient during AST. The IL-9 serum level was measured for consecutive 9 weeks.

response. Our data showed that IL-9 had decreasing trend after AST in ASST positive patients (Figure 4).

4. Discussion

Inflammation is the major event leading to the persistent skin lesion in CIU. Previous study reported that the CIU inflammatory response is induced by patients` pathogenic IgG which cross-link to IgE receptor on mast cells and basophils, leading to histamine release, follows by activation of C5a complement.[14,15,16] Since the immune-modulated response is very complicated, comprising individualistic variance, the CIU and AST-associated mechanism that leads to successful treatment has not been clarified.

CIU has been speculated to be caused by disrupted innate immunity, leading to imbalance cytokine-chemokine regulation. In this study, we proposed that successful AST is immunomodulatory whereby specific cytokine is involved. Previous study showed that hive biopsy had increased level of $CD4^+$ lymphocytes, neutrophils, eosinophils and basophils. The cytokine profile of the biopsy includes IL-4, IL-5, and IFN-γ increase.[17] However, our results revealed that IL-9 was the TH2 cytokine showing difference between CIU and control group (Figure 2B). The increase in IL-6 and C-reactive protein (CRP) in the bloodstream of CIU patients was previously reported to be a systemic inflammation.[2,18] Consistent with this study, IL-6 was increased in CIU patients based on our results, indicating that chronic inflammatory response is involved in CIU. Besides, IL-10 in CIU also showed higher level compared to control group, suggesting that CIU patients were experiencing the TH2-related chronic inflammation response.

TNF-α can trigger cell apoptosis and inflammation, and is mainly secreted by activated T cells and macrophages.[19] However, TNF-α was suppressed in CIU compared to control. Previously, it was reported that the ratio of IL-6, IL-10 and TNF-α are related to disease outcome.[20] Except IL-10, IL-6 was also reported regulate the production of TNF-α.[21] It is interesting that ASST positive patients having higher serum TNF-α showed improved response to AST (Figure 3B) and the role of TNF-α in CIU requires further investigation.

ASST is believed to be a valuable way to detect patients with or without circulating functional autoantibodies.[22] However, previous study showed that both ASST positive and ASST negative patients could get improvement after receiving AST, but underlying mechanism is not clear.[23,24] Based on our observations, INF-γ was higher in ASST positive patients and numerous studies have found that INF-γ plays essential role in development and severity of systemic autoimmunity. [7,25] In our opinion, ASST positive means higher chance for severe skin autoimmune reaction, and TNF-α can be used as predictors for successful AST. CIU patients with ASST require higher dose of antihistamimne or additional immunomodulatory treatment and should be classified as unique group of CIU.[26]

Previously, IL-9 was found to be increased in acute spontaneous urticaria patients[27]. T-helper cells Th9 are the major source of IL-9 and Th9 cells recruit mast cells during allergic inflammation[28]. Recently, it is known that Th9 cells could promote immune tolerance in certain models which protect against parasitic infections[29]. In contrast, Th9 cells trigger prominent allergic allergic inflammation, asthma, and autoimmune diseases, highlighting their pathological roles in the immune system[29]. Our observation that IL-9 is increased in CIU compared to control and decreased during AST revealed that IL-9 is involved in CIU mechanism.

In summary, the cytokine-related pathogenesis of CIU might be due to prolonged IL-6 activation which suppressed TNF-α and enhanced IL-9 production, eventually simulating mast cells to cause chronic urticaria. Furthermore, AST could release symptoms of CIU through decreased IL-9 in blood circulation.

5. Conclusion

To conclude our observation, the cytokines IL-9, IL-6 and TNF-α are involved in mechanism of CIU inflammation. The INF-γ increase potentially leads to CIU patients with ASST positive; whereas higher TNF-α level may be crucial for successful AST.

Conflicts of interest

The authors in this study declared no conflicts of interest.

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