The Influence of Treatment of Pemphigus Vulgaris on the Serum Level of Cytokines

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Abstract

Background: In addition to humoral immunity associated with anti-desmoglein antibodies, cellular immunity and mediators including cytokines are involved in the pathogenesis of pemphigus vulgaris. In this study we evaluated the level of IL-2, IFN-γ, TNF-α, IL-4, and IL-10 in the sera of patients with pemphigus vulgaris before and after treatment.

Methods: A total number of 71 new patients with pemphigus vulgaris were included in the study. The above mentioned cytokines were measured in patients with a mild disease (20 bullae or less) and a severe disease (60 bullae or more) using ELISA method before and 4 weeks after treatment with 2 mg/kg/day prednisolone and 2mg/kg/day azathioprine. We also measured IL-4 and IL-10 in 69 mildly and 70 severely affected patients only at the beginning of the study. All patients had muco-cutaneous phenotype. Patients with a mild disease had mild mucosal involvement and patients with a severe disease had moderate to severe mucosal involvement. Serum levels of IL-2 and IFN-γ were also measured in 27 normal controls.

Results: In the total study population, the level of IL-2 decreased from 103.9 pg/ml to 82.79 pg/ml after treatment (p=0.05). Comparing cytokines between 2 groups (severe and mild), the level of IL-2 before treatment showed significantly lower figures in severe patients (147.27 versus 67.38, p=0.04). On the other hand, IFN-γ after treatment was significantly higher in severe patients (0.75 versus 0.42, p=0.04).

Conclusion: Mean level of IL-2 is lower in severe pemphigus vulgaris patients than that of mild disease. This finding indicates that, in pemphigus vulgaris, IL-2 level negatively correlates with the severity of the disease and widespread underlying autoimmune process. The data also suggests that the level of IFN-γ directly correlates with the severity of the disease.

Keywords: pemphigus vulgaris, IL-2, IFN-γ, TNF-α, IL-4, IL-10

Introduction

Pemphigus is a group of autoimmune bullous diseases which is developed by desmoglein 1 and 3 autoantibodies. It is defined by flaccid bullae and erosions in epidermis due to acantholysis[1].

In immunobullous diseases, there are different levels of skin inflammation which is expressed by inflammatory infiltrates. The importance of cellular and humoral mediators leading to cellular damage is different among various autoimmune bullous diseases.

It is believed that several cellular mechanisms are involved in the induction of some autoimmune diseases[2, 3]. Immunobullous diseases are inflammatory processes in which in addition to autoantibodies, other factors such as complements, proteases, and other mediators such as cytokines are involved[2]. The role of cytokines in the pathogenesis of autoimmune diseases is not completely investigated. Cytokines are soluble

proteins which facilitate intercellular communications. Among several cells which are able to produce cytokines, T lymphocytes and macrophages are more involved[4]. Imbalance in Th1 and Th2 responses and overproduction of cytokines in the absence of sufficient regulatory cells are known as effective factors in the induction of autoimmunity[5].

There are other reasons showing the involvement of cellular immunity in the pathogenesis of pemphigus vulgaris. In a study, investigators have shown that mononuclear cells especially T lymphocytes, upon activation, express IL-2 receptor on their surfaces. In that study, the serum level of IL-2 receptor during different stages of the disease was measured and it was concluded that in addition to autoantibodies, other agents such as activated T cells and monocyte/macrophages are involved in the pathogenesis of pemphigus[6].

A group of researchers have shown increased level of IL-15 and its relation to the severity of the disease in patients with bullous diseases. According to that study, the serum and bulla levels of IL-15 are increased in the active and passive phases of drug-induced bullous erosions. However, in pemphigus and bullous pemphigoid, the direct correlation between IL-15 and the severity of the disease is noted during the active phase of the disease[7]. Increased level of IL-6 and its correlation with the activity of the disease are shown in the patients with pemphigus vuglaris[8].

In a similar study on patients with Brazilian pemphigus folliaceus, investigators analyzed the level of IL-2, IL-4, IL-5, IL-10, IL-12, and IFN-γ in 25 patients with pemphigus folliaceus and 10 control subjects[9].

High prevalence of pemphigus[10,11] along with more availability of patients warrant clinical, epidemiological, and immunological studies of pemphigus in Iran. Based on the results from previous studies which indicate changes in some Th1 (IL-2) and Th2 cytokines (IL-10)[2], we decided to perform a study with larger study population in order to obtain more statistically notable changes among Th1/Th2 cytokines. These kinds of studies would no doubt cast light on the pathogenesis of the disease and might therefore delineate more suitable and specific treatment protocols.

In this study, we measured and compared the levels of IL-2, IFN-γ, and TNF-α (of Th1 cytokines) as well as IL-4 and IL-10 (of Th2 cytokines) before and after 4 weeks of treatment in a group of pemphigus vulgaris patients.

Patients and methods

Seventy one new pemphigus vulgaris patients enrolled in the study. The diagnosis of pemphigus was based on clinical and pathological features as well as direct immunofluorescence findings. Active disease was defined based on the presence of cutaneous or mucosal lesions. Patients with pemphigus folliaceus or other types of pemphigus were not included in the study. Pregnant women were also excluded. Before starting the treatment, based on the number of bullae, patients were divided into mild (with less than 20 cutaneous bullae) and severe (with 60 cutaneous bullae or more) groups. Patients with moderate severity (20 to 60 cutaneous bullae) and those with only mucosal lesions were not included in the study. The classification of patients based on severity to mild, moderate, and severe patients, had previously been applied by some investigators[12].

Severity of mucosal involvement was classified from mild to severe according to the study by Sharma VK et al.[12]. In that report, mucosal (oral) involvement was graded as zero (no mucosal involvement), grade 1 (up to 3 erosions), grade 2 (4 to 10 erosions), and grade 3 (more than 10 erosions/extensive confluent erosions/generalized desquamative gingivitis). Mucosal involvement was grade 1 in patients with a mild disease and grade 2 or 3 in patients with a severe disease.

The serum levels of IL-2, IFN-γ, TNF-α, IL-4, and IL-10 were measured using ELISA method before and 4 weeks after the treatment when the active disease subsided. Treatment was started with prednisolone (2 mg/kg/day) and azathioprine (2 mg/kg/day).

Two patients passed away before taking the second blood test due to severity of the disease and superinfection.

Moreover, the levels of IL-2 and IFN-γ were measured in 27 control subjects.

The level of cytokines was measured with ELISA method (kits from Bender MedSystems® Company, Austria). For instance, to measure IL-4 using ELISA method, the serum of patient was added to the plates previously coated with IL-4. After incubation, the plates were washed and labeled antibodies were added. Again, after incubation period the plates were washed and substrate was added. Finally, the reaction terminated by adding acid solution. Optical densities of sample and standard were measured by ELISA reader and the levels of IL-4 for each sample were defined according to the reference diagram. This method was repeated for

other cytokines. The levels of cytokines were measured in picograms per milliliter (pg/ml).

After obtaining final results, all the information was analyzed by SPSS 11.5 software using paired t-test and independent t-test. Difference with $p<0.05$ were considered significant and difference with $0.05<p<0.1$ were considered notable.

Results

The level of cytokines in patients and control subjects are shown in table 1. Among the total study population, the decrease in the level of IL-2 was statistically significant ($p<0.05$). In patients with mild disease, none of the cytokines changed significantly. However, the level of IL-2 showed a notable decrease ($p=0.06$).

Before treatment, the difference in the level of IL-2 between patients with a mild and patients with a severe disease was statistically significant ($p<0.04$). The level of IFN-γ showed a notable difference between the two groups ($p=0.07$). After treatment, the difference in the level of IFN-γ between patients with a mild and patients with a severe disease was statistically significant ($p<0.04$).

Table 1. *Mean cytokine levels in patients with pemphigus vulgaris according to the severity of disease before and after 4 weeks of treatment.*

		IL-2	IFN-γ	IL-4	IL-10	TNF-α
	Controls	198.04±459.46 (27 patients)	0.4±0.66 (27 patients)	-	-	-
Before treatment	All patients	103.90±310.98 (70 patients)	0.59±1 (71 patients)	19.79±26.77 (69 patients)	23.17±39.74 (71 patients)	0.44±0.54 (71 patients)
	Mild patients	147.27±371.25 (32 patients)	0.4±0.73 (32 patients)	21.40±25.39 (31 patients)	22.78±35.34 (32 patients)	0.41±0.45 (32 patients)
	Severe patients	67.38±248.74 (38 patients)	0.74±1.16 (39 patients)	18.48±28.12 (38 patients)	23.49±43.47 (39 patients)	0.47±0.60 (39 patients)
After treatment	All patients	82.79±276.76 (65 patients)	0.59±1.15 (66 patients)	17.60±16.68 (64 patients)	21.49±38.74 (66 patients)	0.48±0.57 (66 patients)
	Mild patients	105.42±307.76 (31 patients)	0.42±0.82 (31 patients)	19.38±20.45 (29 patients)	21.95±35.95 (31 patients)	0.49±0.66 (31 patients)
	Severe patients	62.15±248.03 (34 patients)	0.75±1.37 (35 patients)	16.13±12.89 (35 patients)	21.09±41.57 (35 patients)	0.47±0.48 (35 patients)

Before treatment, the level of IL-2 between patients with a severe disease and control subjects was statistically different ($p<0.01$). After treatment, similarly, the level of IFN-γ between patients with a severe disease and control subjects was statistically different ($p<0.01$).

Discussion

In this study, the level of IL-2 in patients with pemphigus vulgaris significantly decreased from 103.9 pg/ml to 82.79 pg/ml ($p=0.05$). IL-2 is recognized as T cell growth factor. Previously, it was believed that IL-2 is produced only by activated T cells. Recently, it is shown that dendritic cells are also capable of transient IL-2 production. IL-2R deficiency could lead to multiorgan inflammation as well as production of autoantibodies. Therefore, IL-2 and its receptor are involved in autoimmunity. In animal studies, IL-2 is associated with differentiation and post natal maturation of regulatory cells which take part in the process of self and non-self discrimination[13].

Suppression of the production of some cytokines is a crucial impact of glucocorticoids on immune system. The production of the following cytokines is decreased by corticosteroids: IL-1, IL-2, IL-3, IL-6, IL-8, TNF-α, and GM-CSF[14].

Azathioprine has immunosuppressive and myelosuppressive effects. The inhibition of the immune system, explains its usage in dermatology. Other actions of azathioprine include interaction with IL-2 synthesis and suppressive activity at the level of membrane[15]. Glucocorticosteroids, also, by the means of IL-1 monocytic synthesis suppression, would decrease IL-2 production[16]. Therefore, administration of azathioprine and corticosteroid in patients with pemphigus could decrease the serum level of IL-2 (after 4 weeks of treatment). Ultimately, inhibition of inflammatory process after 4 weeks of treatment could play a role in the decrease of this cytokine.

Also, it is shown that increased level of cortisol is accompanied by a decrease in the level of IL-2 and IL-3. Four weeks after the treatment, the patients in our study were on mean dosage of 50 mg corticosteroids (prednisolone), and with regard to chemical and functional similarity of cortisol and prednisolone[14], it is possible to suppose that the serum level of IL-2 decreased 4 weeks after the treatment compared to level before treatment[17].

Comparing patients with a mild and severe disease, the level of IL-2 before the treatment was significantly lower in patients with a severe disease.

As noted above, IL-2 plays a role in the induction of autoimmunity. In the present study, we compared mild and severe disease to obtain highlighted results. A lower level of IL-2 in patients with severe disease could be explained as follows.

Recently it is shown that the number of CD4+ CD25+ T regulatory cells in patients with pemphigus vulgaris is lower than that of normal subjects[18]. In animal models, also, it is shown that the reduction of IL-2 synthesis, by genetic mechanism, is correlated with reduced function of CD4+ CD25+ regulatory T cells which play a key role in maintaining immune homeostasis[19]. Therefore, it is concluded that a decreased level of regulatory T cells as well as lower activity of these cells, would result in a dramatic decrease in the level of IL-2 in patients with severe pemphigus vulgaris. Kermani-Arab et al. have previously shown lower levels of IL-2 in patients with severe pemphigus[20]. In our study, similarly, the level of IL-2 was lower in patients with pemphigus compared to normal subjects and its value was negatively correlated with the severity of the disease.

The serum level of IFN-γ in all patients, mild or severe disease, did not show any significant changes. Before treatment, the level of IFN-γ showed higher figures in patients with a severe disease than that of mild disease (0.74 pg/ml vs. 0.4 pg/ml, $p=0.07$).

IFN-γ, IL-1α, IL-1β, IL-6, and TNF-α are proinflammatory cytokines which are involved in biologic phenomena such as inflammation and systemic inflammatory reactions[21].

Therefore, higher levels of IFN-γ, especially before treatment (comparing severe and mild patients), could be attributed to the underlying inflammatory process in pemphigus. Our search in Pubmed did not find any similar studies. However, in a study by Baroni et al.[22], the level of IL-10, IL-8, and IFN-γ were measured in serum and blister fluid and compared to those of normal controls and it was reported that the level of IFN-γ, in contrast to all patients with pemphigus vulgaris, was not detectable in any of the normal subjects. This study, similar to our study, suggests systemic Th1 activation which corresponds with elevated level of IFN-γ in patients with a severe disease.

On the other hand, some authors, based on Th1 reaction, have proposed anti- IFN-γ for the treatment of patients with pemphigus vulgaris[23].

In our study, the level of IFN-γ was higher in patients with a severe disease than that of patients with a mild disease and controls. It also showed positive correlation (direct relationship) with the severity of the disease.

Combination therapy of azathioprine with corticosteroids did not show any impacts on the level of IFN-γ in the total study population, mild, and severe patients, and we found no similar articles in Pubmed to compare with our findings.

In this study, the mean level of IL-10 in the total study population did not show any significant changes among all study groups (total study population, mild, and severe patients) after treatment or with regard to disease severity. The mean value of IL-10 was insignificantly higher in total study population before treatment ($p>0.05$).

Among patients with a severe disease, the mean value of IL-10 showed a decrease, though insignificant, after the treatment ($p>0.05$). There are controversial findings about this issue in the medical literature. For example, in the study by Baroni et al.[22], the level of IL-10 was not detectable in serum of 15 patients and 16 controls, but in a study by Bhol et al.[24] the serum level of IL-10 was significantly higher in the active phase of the disease than that of the remission period. Although we suggest nothing contrary to these results, our findings were not statistically significant. IL-10 is an anti-inflammatory cytokine. Therefore, it is logical to observe elevated figures before treatment (during inflammation)[4].

In our study, the serum level of IL-4 decreased in all study groups (total study population, mild, and severe patients) after treatment. However, changes were not statistically significant. The decrease in the level of IL-4 after the treatment could be due to corticosteroid therapy[25].

Recently, it is shown that T cell clones specific for desmoglein 3 which are cocultured with autologous mononuclear cells of pemphigus patients secrete IL-4 and IL-10. It seems that concordant changes are expected in the level of IL-10 and IL-4. Our results support this finding[24,26].

For TNF-α, measured values for all study groups (total study population, mild, and severe patients) were between zero (not detectable) and 2.5 pg/ml. It is shown that serum TNF-α and IL-6 are elevated in patients with pemphigus vulgaris and the value increases according to the extent of disease[8].

Except for TNF-α, all cytokines in our study were favorably detectable by applied ELISA kits. All values for TNF-α were almost undetectable (close to zero). We suggest that the applied kits were not sensitive enough to detect TNF-α. By the means of supernatant cell culture and phytohaemagglutinin

(PHA) stimulation, we may obtain better results. However, most of studies on cytokines in pemphigus are done without PHA stimulation using normal serum and available commercial kits[9, 24].

As far as our search in Pubmed revealed, in the present study the largest sample size for the evaluation of cytokines in pemphigus is applied. Due to small sample sizes, in most of the previous studies, median has been used instead of mean to present statistical evaluations. With regard to high prevalence of pemphigus vulgaris in Iran, it is appropriate to measure the serum and bullae fluid level of cytokines and corresponding receptors in serum before the treatment, at the time of bullae disappearance, and after the treatment. It is recommended to evaluate IL-2 and its receptor before and after treatment every 2 weeks for 8 weeks. Furthermore, the evaluation of number, as well as, function of regulatory T cells is recommended for future studies.

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