Changes in serum cytokine levels during plasmapheresis in patients with myasthenia gravis

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Background: The effect of plasmapheresis on cytokine levels in patients with myasthenia gravis (MG) has not been well established.
Methods: Cytokine levels were measured in 19 patients with MG before and after treatment with one course of double-filtration plasmapheresis (DFP). The control group comprised 6 age- and sex-matched healthy volunteers.
Results: At baseline, patients with MG had higher levels of IL-10 than normal controls. The levels of IL-2, IL-4, IL-5, and tumor necrosis factor-α were almost undetectable in MG patients. After a single session of DFP treatment, IL-10 levels were significantly increased. After three sessions, IL-10 levels were still higher than those at baseline. Elevated IL-10 level was significantly associated with use of immunosuppressant drugs, thymectomy, and good response to DFP treatment.
Conclusions: Interleukin-10 might play a crucial role in the pathogenesis and perpetuation of MG.

Introduction

Over 85% of patients with generalized myasthenia gravis (MG) have circulating antibodies to the acetylcholine receptor (AchR) [1]. Both T-helper (Th)1 and Th2 lymphocytes and cytokines probably participate in the development of MG [2]. It has been shown that patients with MG have higher serum levels of interleukin (IL)-2, IL-18, soluble IL-2 receptor (sIL-2R), sIL-6, and soluble tumor necrosis factor (TNF) receptor II than healthy individuals [3–8]. In addition, the number of peripheral blood mononuclear cells (PBMC) secreting IL-2, IL-4, interferon (IFN)-γ, and IL-10 has been shown to be significantly higher in patients with MG than in healthy individuals [9–11], although some reports have revealed data that contradict the above-mentioned findings [6,12,13].

Plasmapheresis provides rapid amelioration of clinical weakness by removing pathogenic antibodies to the AchRAb [14]. Plasmapheresis not only removes humoral factors, but may also modulate cellular immunity [15]. Research on the effect of plasmapheresis on cytokine levels, however, is limited [7,16,17]. The small number of recruited patients, the high variability of plasmapheresis protocols used, and the variation of concomitant immunosuppression makes it difficult to compare cytokine data obtained during plasmapheresis among reported studies.

The present study compared the influence of a single session of double-filtration plasmapheresis (DFP) treatment with that of an entire course on serum cytokine levels in 19 MG patients. MG score, AchRAb concentration, and cytokine levels before and after DFP treatment were analyzed.

Materials and methods

Subject characteristics

From June 2003 to July 2005, 19 MG patients, including 12 women and seven men aged 15–77 years, underwent plasmapheresis treatment because of recent worsening of clinical weakness or in preparation for thymectomy. The diagnosis of MG was based on clinical, pharmacological, serological, and electrodiagnostic criteria. Clinical status was graded according to Osserman's classification. Among the 19 patients, MG was classified as grade IIa in four patients, IIb in eight patients, and III in seven patients. The duration of illness ranged from 0.1 to 29 years. All patients received anticholinesterase therapy; 11 were also treated with prednisolone (average

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dose, 20.6 mg/day) and one was treated with prednisolone (20 mg/day) and azathioprine (75 mg/day). Twelve patients had undergone thymectomy. Histopathologic findings included thymic hyperplasia in six patients and thymoma in six patients. Six age- and sex-matched healthy volunteers from members of our department (two men and four women) were used as controls. All of the volunteers were in good health and reported feeling well on the day of DFP. This study was approved by the Ethics Committee at the Shin Kong Wu Ho-Su Memorial Hospital. Informed consent was obtained from all patients and volunteer subjects prior to inclusion in the study.

Apheresis

Double-filtration plasmapheresis was performed using a Plasmacure (Kuraray, Osaka, Japan) plasma separator and an Evaflux 4A (Kuraray) plasma fractionator in a KM 8800 automated membrane plasmapheresis monitor (Kuraray). Our standard protocol for DFP consists of filtration of one estimated plasma volume followed by isovolumetric isotonic-saline replacement at a constant flow rate to minimize the effect of volume changes. Plasma volume was calculated using the formula: (body weight (kg)/13) $\times$ (1−Hematocrit). Heparin was used as the anticoagulant. The loading dose of heparin was 2000 IU and the maintenance dose was 1000 IU/h, based on the manufacturer's recommendation. Vascular access was established with a double-lumen catheter in a central vein (10 patients), an arteriovenous shunt (one patient) or a dialysis catheter (FASFLO AVFistula, Enfield Med., Taipei, Taiwan) in an antecubital vein (eight patients and six controls). Each course of treatment consisted of a mean of 4.7 consecutive DFP sessions (SD, 0.7; range, three to five sessions) on an alternate-day basis. The total number of DFP sessions was determined by the patient's doctor. None of the patients were administered medication before the DFP session.

Clinical evaluation

Patients were evaluated based on a modified MG score before and after the entire course of plasmapheresis [14]. The score was calculated as the sum of the grades in six items including duration of outstretched time of arms and legs, vital capacity, functional grading of facial muscles, and the ability to chew and swallow. Each item was graded on a scale of 0 (normal) to 3 (severe paralysis). A MG score of 0 indicated normal status and a score of 18 indicated maximal weakness. Patients were divided into good responder and poor responder groups based on changes in MG score after DFP therapy. Good response was defined as a decrease of 2 or more points in the MG scale and poor response was defined as an increase of 2 or more points in the scale.

Blood collection

Blood samples were collected in anticoagulant-free tubes. They were immediately centrifuged at 3000 revolutions per minute for 10 min and stored at −80°C until assayed. The serum concentrations of AchRAb, IL-2, IL-4, IL-5, IL-10, TNF-α, and IFN-γ were measured at the indicated time-points. Serum samples were prospectively obtained before and after the first DFP session in all patients and controls. In the MG group, an additional sampling was done after the third session to evaluate the changes in cytokine levels after serial sessions of DFP treatment.

Measurement of serum cytokines and AchRAb

Flow cytometry was performed with the Cytometric Bead Array kit for Human Th1/ Th2 cytokines (BD Biosciences, San Diego, CA, USA) to measure the serum levels of IL-2, IL-4, IL-5, IL-10, TNF-α, and IFN-γ using the provided reagents and standards according to the manufacturer's instructions. The levels of AchRAb were detected using a radioimmunoprecipitation kit (RSR Limited, Cardiff, UK).

Statistical analysis

Descriptive values of variables are expressed as the mean $\pm$ SEM. Comparison of data before DFP in the MG group with that in the control group was performed using the Student's *t*-test. The paired *t*-test was used to evaluate the differences in cytokine levels after plasmapheresis. Differences in cytokine levels between patients and controls were assessed using the Student's *t*-test. The correlation of cytokine levels with age, AchRAb titer, MG score at baseline, and changes in MG score after DFP treatment was analyzed using a regression model. The associations between cytokine levels and clinical parameters including sex, thymectomy, thymic pathology, immunosuppressant use, clinical grade, and clinical response to DFP treatment were analyzed using the Wilcoxon rank-sum test. The confidence limit was predetermined at an alpha level of 0.05.

Results

After plasmapheresis, the mean MG score decreased from 6.9 (range, 1–17) to 4.4 (range, 0–13). The mean

serum concentration of AchRAb fell significantly from 275.9 nmol/l to 56% of the original level after plasmapheresis treatment. The serum levels of cytokines are summarized in Table 1 and Fig. 1. At baseline, patients with MG had higher levels of IL-10 ($P = 0.0076$) than normal controls. Baseline serum levels of IL-2, IL-4, IL-5, and TNF-α were almost undetectable in MG patients. After a single session of DFP treatment in MG patients, the mean IL-10 level significantly increased ($P = 0.0204$), while the level of IL-4 showed a decreasing trend. There were no significant changes in cytokine levels after DFP treatment in the normal control group.

Among the 15 patients who completed three sessions of DFP treatment, the mean IL-10 level was still higher after three sessions of treatment than at baseline ($P = 0.0906$); however, the extent of the increase in IL-10 after three sessions of DFP treatment was less prominent than after a single session of treatment ($P = 0.0687$) (Table 2). Serum levels of IL-10 in MG patients decreased significantly during the course of DFP.

Table 3 summarizes the correlation between IL-10 level and clinical parameters. The analysis revealed that elevated IL-10 level at baseline was significantly related to the use of immunosuppressive agents ($P = 0.0362$), thymectomy ($P = 0.0088$) and good clinical response to DFP treatment ($P = 0.0699$). A higher IL-10 level at baseline was correlated with a higher MG score at baseline ($R^2 = 0.22$; $P = 0.0427$). After DFP treatment, IL-10 levels were still higher in patients who received immunosuppressive agents ($P = 0.0012$) and in those who demonstrated good clinical response to DFP treatment ($P = 0.0280$). Patients with higher IL-10 levels after DFP treatment had a tendency to have a better clinical response to DFP treatment as measured by a greater reduction in MG score ($R^2 = 0.17$; $P = 0.0845$). There was no correlation between the clinical variables and the other five cytokines studied.

Plasmapheresis treatment was well tolerated by both patients and control subjects. There were no major plasmapheresis-related complications such as hypo-

Table 1 Serum levels of interleukin (IL)-2, IL-4, IL-5, IL-10, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ in patients with myasthenia gravis (MG) and in normal controls before and after a single session of double-filtration plasmapheresis (DFP)

	Normal controls ($n = 6$)		MG patients ($n = 19$)	
	Pre-DFP	Post-DFP	Pre-DFP	Post-DFP
IL-2 (pg/ml)	0 ± 0	0 ± 0	0.04 ± 0.03	0.02 ± 0.02
IL-4 (pg/ml)	0 ± 0	0 ± 0	0.20 ± 0.14	0 ± 0
IL-5 (pg/ml)	0 ± 0	0 ± 0	0.91 ± 0.74	0.28 ± 0.13
IL-10 (pg/ml)	0 ± 0	0 ± 0	2.65 ± 0.88*	22.65 ± 9.25**
TNF-α (pg/ml)	0.30 ± 0.30	0 ± 0	0.30 ± 0.19	0.02 ± 0.01
IFN-γ (pg/ml)	0 ± 0	0 ± 0	2.49 ± 1.64	1.11 ± 0.76

The reference ranges of all cytokines measured are <1.0 pg/ml. The results represent the mean ± SEM for each cytokine.
*$P = 0.0074$; as compared with pre-DFP in normal controls.
**$P = 0.0420$; as compared with pre-DFP in MG patients.

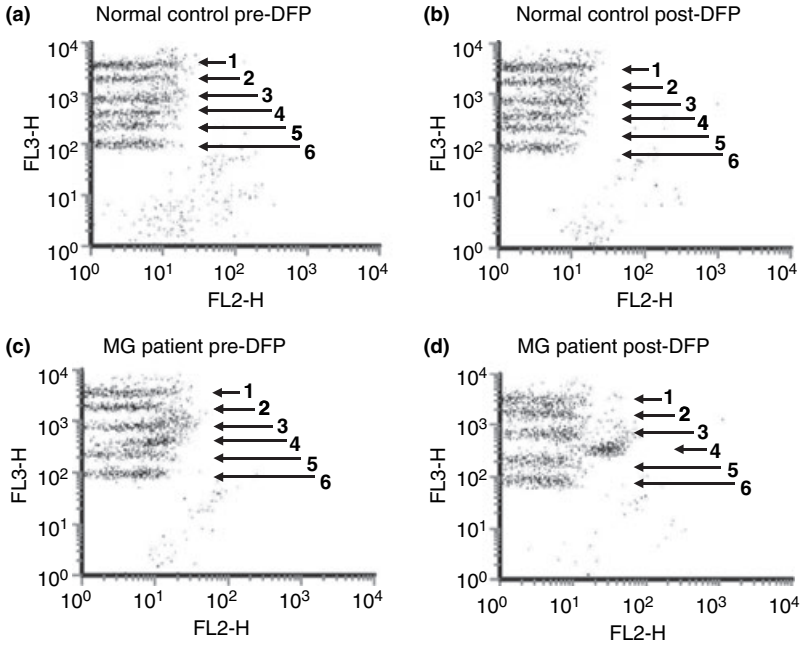

Figure 1 The sera of normal controls [pre-DFP(a); post-DFP(b)] and myasthenia gravis (MG) patients [pre-DFP (c); post-DFP (d)] were collected and then subjected to analysis of cytokines using a Cytometric Bead Array kit and a flow cytometer as described in the Materials and methods. Interleukin (IL)-2, IL-4, IL-5, IL-10, tumor-necrosis factor (TNF)-α, and interferon (IFN)-γ are indicated by arrows numbered from 1 to 6, respectively. DFP, double-filtration plasmapheresis.

Table 2 Comparison of the serum levels of interleukin (IL)-2, IL-4, IL-5, IL-10, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ in patients with myasthenia gravis treated by double-filtration plasmapheresis (DFP)

	Pre-DFP (n = 8)	Post-1st session of DFP (n = 15)	Post-3rd session of DFP (n = 15)	P-value[a]	P-value[b]	P-value[c]
IL-2 (pg/ml)	0.09 ± 0.06	0.02 ± 0.02	0.11 ± 0.09	0.1478	0.4210	0.1702
IL-4 (pg/ml)	0.47 ± 0.33	0 ± 0	0.07 ± 0.05	0.0992	0.1357	0.0836
IL-5 (pg/ml)	2.05 ± 1.73	0.18 ± 0.13	0.23 ± 0.16	0.1583	0.1649	0.4002
IL-10 (pg/ml)	2.98 ± 1.57	26.74 ± 10.47	9.22 ± 4.19	0.0204	0.0906	0.0687
TNF-α (pg/ml)	0.47 ± 0.41	0.21 ± 0.20	0.09 ± 0.07	0.2952	0.1954	0.2803
IFN-γ (pg/ml)	5.87 ± 3.69	1.80 ± 0.99	2.52 ± 1.73	0.1592	0.2152	0.3606

The results represent the mean ± SEM for each cytokine. Post-1st DFP: after the first session of DFP treatment. Post-3rd DFP: after the third session of DFP treatment.

[a]Comparison between the levels of cytokines before DFP (pre-DFP) and after the first session of DFP treatment (post-1st DFP).

[b]Comparison between the levels of cytokines before DFP (pre-DFP) and after the third session of DFP treatment (post-3rd DFP).

[c]Comparison between the levels of cytokines after the first session (post-1st DFP) and after the third session of DFP treatment (post-3rd DFP).

Table 3 Associations between interleukin (IL)-10 levels and clinical variables in patients with myasthenia gravis treated by double-filtration plasmapheresis

	Variable 1	Variable 2	P-value[a]
IL-10 at baseline (pg/ml)	Good responder (n = 9)	Poor responder (n = 10)	0.0699
	4.42 ± 1.59	1.06 ± 0.56	
	Use of IS (n = 11)	No use of IS (n = 8)	0.0362
	4.11 ± 1.34	0.65 ± 0.44	
	Thymectomy (n = 11)	No thymectomy (n = 8)	0.0088
	4.29 ± 1.30	0.40 ± 0.40	
IL-10 after DFP (pg/ml)	Good responder (n = 9)	Poor responder (n = 10)	0.0280
	40.86 ± 17.42	6.27 ± 4.41	
	Use of IS (n = 11)	No use of IS (n = 8)	0.0012
	38.51 ± 14.36	0.85 ± 0.45	

The results represent the mean ± SEM for IL-10.

IS, immunosuppressive agents; DFP, double-filtration plasmapheresis.

[a]Analyzed using the Wilcoxon rank-sum test.

tension or infection. Plasma filter-induced hemolysis was detected in 15% of all sessions, although this minor complication did not have a significant impact.

Discussion

In this study, IL-10 level was significantly higher in MG patients than in controls and correlated well with clinical severity. Our finding that IL-2 level in MG patients did not differ from that in healthy controls is contrary to the findings of two studies that IL-2 level was higher in patients than in healthy individuals [3,4]. In contrast, increased serum concentrations of IL-18 [5], IL-2 [3,4], sIL-2R [4,6,7], sIL-6 [8], and sTNF receptor II [7], but not those of IFN-γ or TNF-α [6] have been reported in studies of MG patients. IL-18 [5] and sIL-2R levels [6] correlate well with the generalized type of MG as well as with the clinical severity of the disease.

Matusevicius *et al*. found that the number of AChR-reactive IL-10 mRNA-expressing PBMC was higher in MG patients than in controls with non-inflammatory neurological diseases [18]. Huang *et al*. reported that the number of IL-10-secreting cells tended to be higher in patients with generalized MG than in patients with ocular MG. The authors proposed that those patients with ocular MG represented a clinical subgroup of MG with localized and less severe symptoms [11]. In an experimental autoimmune MG model, clinical features became worse and serum levels of AchRAb increased in rats after intraperitoneal administration of IL-10. These findings support that IL-10 plays a role in the pathogenesis of MG.

Limited research has been conducted on the effect of plasmapheresis on the changes in cytokine levels in MG patients [7,16,17]. In a study of 20 MG patients assigned to treatment with plasma exchange (PE), Tesar *et al*. found that neither a single treatment nor an entire course of PE significantly influenced the serum levels of sIL-2R and sTNF-R II [7]. A single session of PE had no influence on serum levels of sIL-2R and sTNF-R II despite the high levels of both soluble cytokine receptors in the plasma filtrate [7]. This finding can probably be

attributed to filter-induced stimulation of proinflammatory cytokine production or to limited filtration of some of these cytokines because they were bound to circulating soluble receptors. Goto *et al.* reported that plasmapheresis reduced the Th1/Th2 cytokine ratio and suggested that it also induced a shift in Th1/Th2 balance in the peripheral blood of patients with neuropathy [17]. The same group of researchers, however, also reported contradictory findings that patients with Miller Fisher syndrome treated by immunoadsorption plasmapheresis shifted from Th2-dominant status to Th1-dominant status [19]. The small number of recruited patients, the high variability of plasmapheresis protocols used, and the variation of concomitant immunosuppression, therefore, makes it difficult to compare cytokine data obtained during plasmapheresis among reported studies.

In conclusion, cytokines might play an indirect role in neuromuscular transmission in MG patients [2]. Our findings suggest that IL-10 levels are enhanced in sera of MG patients. Furthermore, we found that increased concentrations of IL-10 correlate with clinical severity and response to DFP treatment. Thus, IL-10 might play a crucial role in the pathogenesis and perpetuation of MG. The clinical implications of the activation of the Th2 pathway by plasmapheresis in MG patients remain to be elucidated.

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