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Running head: Chemokine profile in BP

Summary

Background. Bullous pemphigoid (BP) is an autoimmune inflammatory disease causing blister formation at the dermo-epidermal junction. Cutaneous infiltration of activated CD4+ T cells and eosinophils is an early event in blister formation during the disease process, suggesting that the trafficking of circulating leukocytes through the sites of inflammation is crucial in the pathogenesis of the disease. While the accumulated evidence suggests that some cytokines are involved in the pathogenesis, there have been few reports about serum chemokine profile in BP patients.

Objective. To determine serum profiles of various chemokine levels and clinical association in BP patients.

Methods. Concentrations of 10 chemokines including IP-10, MIG, MIP-1α, MIP-1β, RANTES, eotaxin, MCP-1, MCP-2, MCP-3, and GRO-α were measured in serum samples from 38 patients with BP, 16 with pemphigus vulgaris (PV), and 17 normal controls using a sandwich immunoassay-based multiplex protein array system.

Results. While there was no significant increase in any serum chemokine levels in PV patients, serum levels of IP-10 and MCP-1 were significantly increased in BP patients compared with healthy controls. Furthermore, serum levels of IP-10, MIG, MCP-1, and eotaxin in BP patients significantly increased with disease severity determined by the affected area.

Conclusion. These observations suggest that elaborately orchestrated network of chemokines, especially MCP-1 and IP-10, contributes to the pathomechanism of BP.

Key words: autoimmunity, bullous pemphigoid, chemokine, multiplex protein analysis system, Th1/Th2, pemphigus vulgaris

Introduction

Bullous pemphigoid (BP) is an autoimmune subepidermal blistering disease, usually occurring in the elderly, which is characterized by large, tense blisters. The production of autoantibodies directed to a 180-kDa hemidesmosomal protein (BP180) of the basement membrane zone is the initiating event of the pathomechanisms[1]. Once the autoantibodies bind to the basement membrane, a cascade of inflammatory events occurs, resulting in a subsequent blister formation at the dermo-epidermal junction. During this process, cytokines and chemokines are considered to play crucial roles in inflammatory cell recruitment, deposition, and perpetuation[2]. Therefore, clarifying the serum and local levels of cytokines and chemokines may help to understand the immune dysregulation in the pathomechanism.

It has been demonstrated that various cytokines including interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-8, interferon (IFN)-γ, and tumour necrosis factor (TNF)-α increased in sera of BP patients[3-8]. By contrast, while serum levels of several chemokines including eotaxin[9-11], "regulated upon activation, normal T-cell expressed and presumably secreted" (RANTES)[6], thymus and activation-regulated chemokine (TARC)[12], and mucosae-associated epithelial chemokine (MEC)[13] have been reported, the involvement of most chemokines in the disease remains unknown. In this study, we utilized a recently developed protein array system and assessed the serum levels of 10 chemokines: IFN-γ-inducible protein-10 (IP-10), monokine induced by IFN-γ (MIG), macrophage inflammatory protein (MIP)-1α, MIP-1β, monocyte chemoattractant protein (MCP)-1, MCP-2, MCP-3, and growth-regulated oncogene-α (GRO-α), in addition to eotaxin and RANTES.

Materials and methods

Subjects.

Serum samples obtained from 38 patients with bullous pemphigoid (BP; 17 females and 21 males; mean age, 71.0 years), 16 patients with pemphigus vulgaris (PV; 9 females and 7 males; mean age, 55.7 years) and 17 healthy individuals (8 females and 9 males; mean age, 61.7 years) were examined. BP and PV were diagnosed on the basis of clinical features, findings of skin biopsy examinations by light microscopy, direct immunofluorescence and indirect immunofluorescence test. Patients with BP and PV had not been treated with oral corticosteroids, minocycline/tetracycline, or immunosuppressive drugs. As an index of disease severity, the percentage of the body surface area covered with skin lesions (oedema, erythema, blisters, erosions) was estimated when the serum samples were obtained. Patients affected less than 20%, 20-40%, and more than 40% of their whole body surfaces were classified into be group 1 (mild), 2 (intermediate), and 3 (severe), respectively. Institutional review board approval and informed consent from all patients and controls were obtained. The samples collected were stored at -80° C until use.

Multiplex chemokine assay.

Ten chemokines simultaneously measured included IP-10, MIG, MIP-1α, MIP-1β, RANTES, eotaxin, MCP-1, MCP-2, MCP-3 and GRO-α. A sandwich immunoassay-based protein array system (Biosource, Camarillo, CA, USA), which contains dyed microspheres conjugated with a monoclonal antibody specific for a target protein, was used. Serum samples were thawed and run in duplicates. Antibody-coupled beads were incubated with the plasma sample after which they were incubated with biotinylated detection antibody before finally being incubated with streptavidin-conjugated phycoerythrin. A broad sensitivity range of standards (Biosource) ranging from 1.95 to 32,000 pg/ml were used for the quantitation of a

dynamic wide range of chemokine concentrations and provide the greatest sensitivity. Standards included all the chemokines assayed. This captured immunoassay was then read by the Bio-plex array reader (Bio-Rad Laboratories, Hercules, CA, USA) which uses Luminex fluorescent-bead-based technology (Luminex Corporation, Austin, TX, USA) with a flow-based dual laser detector with real-time digital signal processing to facilitate the analysis of up to 100 different families of colour-coded polystyrene beads and allow multiple measurements of the sample ensuing in the effective quantitation of chemokines. The concentrations of analysis in these assays were quantitated using a calibration or standard curve. A regression analysis was performed to derive an equation that was then used to predict the concentration of the unknown samples.

Statistical analysis.

Statistical differences between measured values of BP patients, those of PV patients, and those of controls were analyzed using Steel-Dwass' multiple comparison test. Trends in the measured values according to the affected area (disease severity) were analyzed by the two-sided Jonckheere-Terpstra test. Correlations between each serum chemokine level were analyzed using with Spearman's correlation coefficient by rank test. *P* values less than 0.05 were considered statistically significant.

Results

Serum levels of ten chemokines in BP and PV patients

Concentrations of ten chemokines, IP-10, MIG, MIP-1α, MIP-1β, RANTES, eotaxin, MCP-1, MCP-2, MCP-3 and GRO-α were simultaneously measured in the sera from BP patients, PV patients, and healthy controls. Serum levels of IP-10 and MCP-1 were significantly increased in BP patients compared with healthy controls ($p = 0.011$ for IP-10 and $p = 0.014$ for MCP-1, Fig. 1). Serum levels of MIG, MIP-1α, MIP-1β, and eotaxin were also slightly higher in BP patients than in controls, although the differences were not significant. By contrast, all chemokines except for eotaxin showed comparable levels between PV patients and healthy controls (Fig. 1). Serum eotaxin levels in PV patients were higher than those in controls although the difference was not significant (Fig. 1).

When each chemokine level higher than the mean + 2SD of the control serum samples were considered to be elevated, IP-10 level was elevated in 39%, MIG in 29%, MIP-1α in 18%, MIP-1β in 29%, eotaxin in 32% and MCP-1 in 36% of BP patients (Table 1). In PV patients, 36% had elevated eotaxin level, 24% had elevated MCP-1 level, and 18% had elevated IP-10 level. While increased RANTES level was observed in one control sample, all the other chemokine levels in control samples were less than the mean + 2SD levels. Collectively, BP patients showed more prominent abnormalities in chemokine levels than PV patients.

Positive correlation between serum levels of MIG and MCP-1 and the severity.

To assess the association of chemokine levels with disease severity, BP patients were classified into three groups by their affected % areas of the body surface. Group 1 (mild) included patients affected less than 20% of their whole body surface. Patients affected 20-40% and more than 40% of their whole body surfaces were classified into group 2 (intermediate) and 3 (severe), respectively. Significant disease severity-related

increases in serum levels of IP-10, MIG, MCP-1, and eotaxin ($p = 0.008$ for IP-10, $p = 0.0007$ for MIG, $p < 0.0001$ for MCP-1 and $p = 0.005$ for eotaxin by Jonckheere-Terpstra test) were present (Fig. 2). By contrast, serum levels of MIP-1α, MIP-1β, MCP-2, MCP-3, GRO-α, or RANTES showed no such tendency (data not shown). Thus, serum levels of MIG, IP-10, MCP-1, and eotaxin appear to reflect the disease severity.

Correlation between serum chemokine levels

We further analyzed the correlation between serum levels of measured chemokines. Serum IP-10 levels in BP patients correlated positively with serum levels of MIG ($r = 0.385$; $p = 0.018$), MCP-1 ($r = 0.328$; $p = 0.041$), and eotaxin ($r = 0.345$; $p = 0.031$). Serum eotaxin levels also correlated positively with serum concentration of MCP-1 ($r = 0.490$; $p = 0.001$). There was a strongly positive correlation between serum levels of MIP-1α and MIP-1β ($r = 0.979$, $p < 0.0001$). Thus, several chemokines were concomitantly elevated, which suggests that these cooperatively contribute to the disease development.

Discussion

The aim of this study was to determine the profile of various chemokines (IP-10, MIG, MIP-1α, MIP-1β, RANTES, eotaxin, MCP-1, MCP2, MCP-3 and GRO-α) in sera from patients with BP. While no chemokine elevated significantly in sera from PV patients, BP patients exhibited significantly increased serum levels of IP-10 and MCP-1 compared with healthy controls. Serum levels of IP-10, MIG, MCP-1 and eotaxin in BP patients also showed significant increase with disease severity determined by their affected area. Additionally, there were positive correlations between IP-10 and MIG, IP-10 and eotaxin, IP-10 and MCP-1, eotaxin and MCP-1, and MIP-1α and MIP-1β. Collectively, a variety of chemokines are likely to contribute to the development of BP cooperatively.

Pathogenesis of the inflammatory skin disease is often related to the T helper (Th) 1/Th2 balance. BP is considered to be dominantly mediated by Th2 responses. Consistently, Kakinuma et al. have reported that serum levels of TARC, a major Th2 chemokine, were elevated in patients with BP[12]. While there has been no literature describing Th1 chemokines of BP sera, IP-10 and MIG measured in this study are considered as chemoattractant of Th1 lymphocytes[14]. Serum IP-10 levels were significantly elevated in whole BP patients compared with controls. Also, there was a trend for MIG levels in sera from BP patients to increase in association with the affected area. By contrast, serum levels of MIP-1α or MIP-1β, which are also known as Th1 chemokines[15,16], showed neither significant differences between BP patients and controls nor trends to increase as the disease severity. Positive correlations between IP-10 and MIG levels suggest that IP-10 and MIG may work at adjacent parts of intricate inflammatory cascades in the development of BP. Also, considering that increase of serum MIG levels were limited in severe patients, MIG may especially contribute to exacerbation of BP. Alternatively, insufficient sensitivity of MIG detection in this system may interfere with significant differences between serum levels of whole

BP patients and controls. Nonetheless, serum IP-10 and MIG levels may also serve as indicators for disease severity in BP.

Interestingly, T cells positive for CXC chemokine receptor 3, which are preferentially expressed on Th1-type cells, have been demonstrated to exist beneath the bullae[17]. Furthermore, Budinger et al. have described that the autoreactive T cells, which participate in the blister formation of BP and recognize the extracellular domains of BP180, possess both Th2 and Th1 cytokines[18]. Taken together, not only Th2 cells but also Th1 cells may be important for pathogenesis of BP.

BP lesions exhibit prominent eosinophil infiltration. Eotaxin is considered to be a potent chemoattractant and activator of eosinophils [19], and main attractant of Th2 lymphocytes[20,21]. Although serum eotaxin levels were not significantly increased in total BP patients, serum eotaxin levels were associated with disease severity and also positively correlated with IP-10 and MCP-1. Correlation between eotaxin and these chemokines implies that there may be a similar process for infiltrations of eosinophils and another subset of leukocytes including Th1 lymphocytes. Some previous reports have demonstrated elevated levels of eotaxin in sera and blister fluid as well as increased expression in the skin lesion of BP patients[9-11]. The enhanced expression of eotaxin indicates that this chemokine may also play an important role in the pathomechanism of this autoimmune bullous skin disease by recruiting and activating eosinophils and lymphocytes.

MCP-1 is a chemoattractant and activator of monocytes, lymphocytes, and basophils, but lacks activity on neutrophils and eosinophils [22,23]. Serum MCP-1 levels in BP patients were elevated, exhibiting a tendency to increase according to the disease severity. Furthermore, MCP-1 levels were correlated positively with IP-10 and eotaxin, which act on other types of inflammatory cells than MCP-1 regulates. Therefore, correlation between MCP-1 and these chemokines suggests that there may be a similar process for infiltrations of different subsets of leukocytes utilizing a variety of

chemokines. While there is no literature regarding serum MCP-1 levels in BP, some studies have demonstrated that expression of CCR2, the chemokine receptor to which MCP-1 binds, increased in chronically inflamed skin lesions of both atopic dermatitis and psoriasis[24]. Therefore, MCP-1 may play an essential role in prolonged inflammation of BP lesion.

Serum RANTES levels did not show significant differences between BP patients and controls. RANTES is known as a chemoattractant for monocytes, T lymphocytes, and eosinophils[25]. Our data is consistent with the findings that D'Auria et al[6]. have reported. Furthermore, they demonstrated that the RANTES concentrations in blister fluid were less than 1/100 of those in the sera. Therefore, RANTES may be rapidly degraded in the blister fluid. Alternatively, RANTES derived from platelets may increase the serum concentration.

In summary, the current study indicates that BP patients have a complex disorder of various chemokine levels in their sera. This supports that various immune pathways are involved in the pathogenesis of BP. It was also remarkable that the chemokine profile of the sera from BP patients showed heterogeneity, which may suggest that the different profile correspond to distinct disease subset or severity, or may reflect different stage of the disease. Nonetheless, it appears that especially MCP-1 and IP-10, then MIG and eotaxin, play relatively important roles than the other chemokines in BP. Further profiling of immune mediators including chemokines will elucidate the precise mechanism of the disease development.

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Figure legends

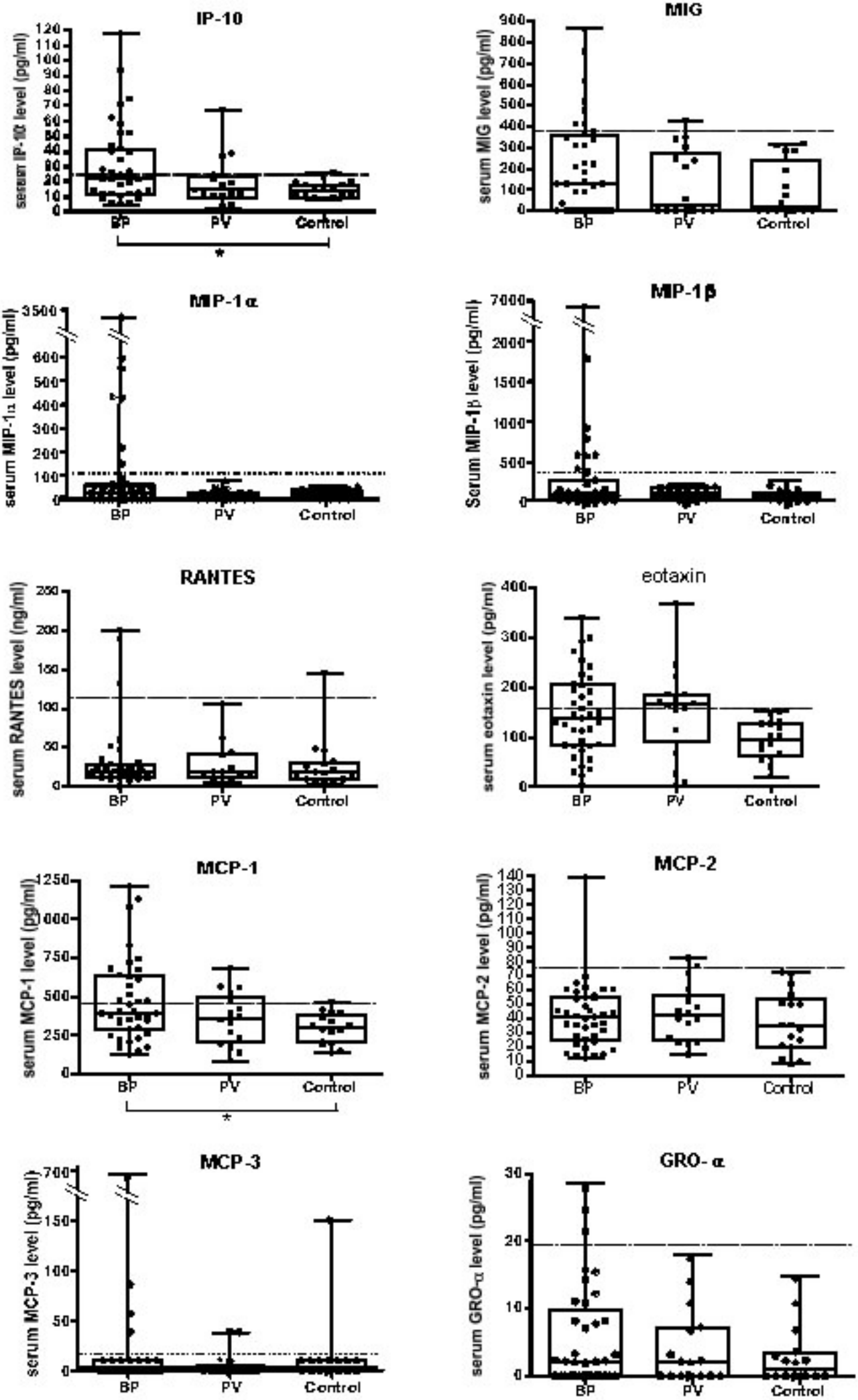
Figure 1: Comparison of serum levels of IP-10, MIG, MIP-1α, MIP-1β, RANTES, eotaxin, MCP-1, MCP-2, MCP-3 and GRO-α in patients with BP, PV, and normal controls (Control). The measured values from individual patients were plotted by dots. The boxes and whiskers indicate the median ± (or +) 25% and maximum/minimum values in each group. A broken horizontal line indicates the cut-off value (mean + 2SD of the control samples). *$p < 0.05$ by Steel-Dwass' multiple comparison test. Significant differences were also confirmed using Mann-Whitney *U*-test with Bonferroni correction.

Figure 2: Serum levels of IP-10, MIG, MCP-1, and eotaxin in BP patients classified by affected area and in controls. Patients affected less than 20%, 20-40%, and more than 40% of their body surface were classified into Groups 1 (mild), 2 (intermediate), and 3 (severe), respectively. The boxes and whiskers indicate the median ± (or +) 25% and maximum/minimum values in each group.

Table 1. Frequency of elevated samples of each chemokine.

	IP-10	**MIG**	**MIP-1α**	**MIP-1β**	**Eotaxin**	**MCP-1**	**MCP-2**	**MCP-3**	**RANTES**	**GRO-α**
BP (n = 38)	15 (39)	11 (29)	7 (18)	11 (29)	12 (32)	13 (36)	1 (3)	2 (6)	3 (8)	9 (24)
PV (n = 17)	3 (18)	3 (18)	1 (6)	1 (6)	6 (36)	4 (24)	1 (6)	0 (0)	1 (6)	3 (18)
Control (n = 17)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (6)	0 (0)

Values are numbers (percentages) of the patients who showed elevated serum levels of each chemokine (> mean + 2SD of controls).



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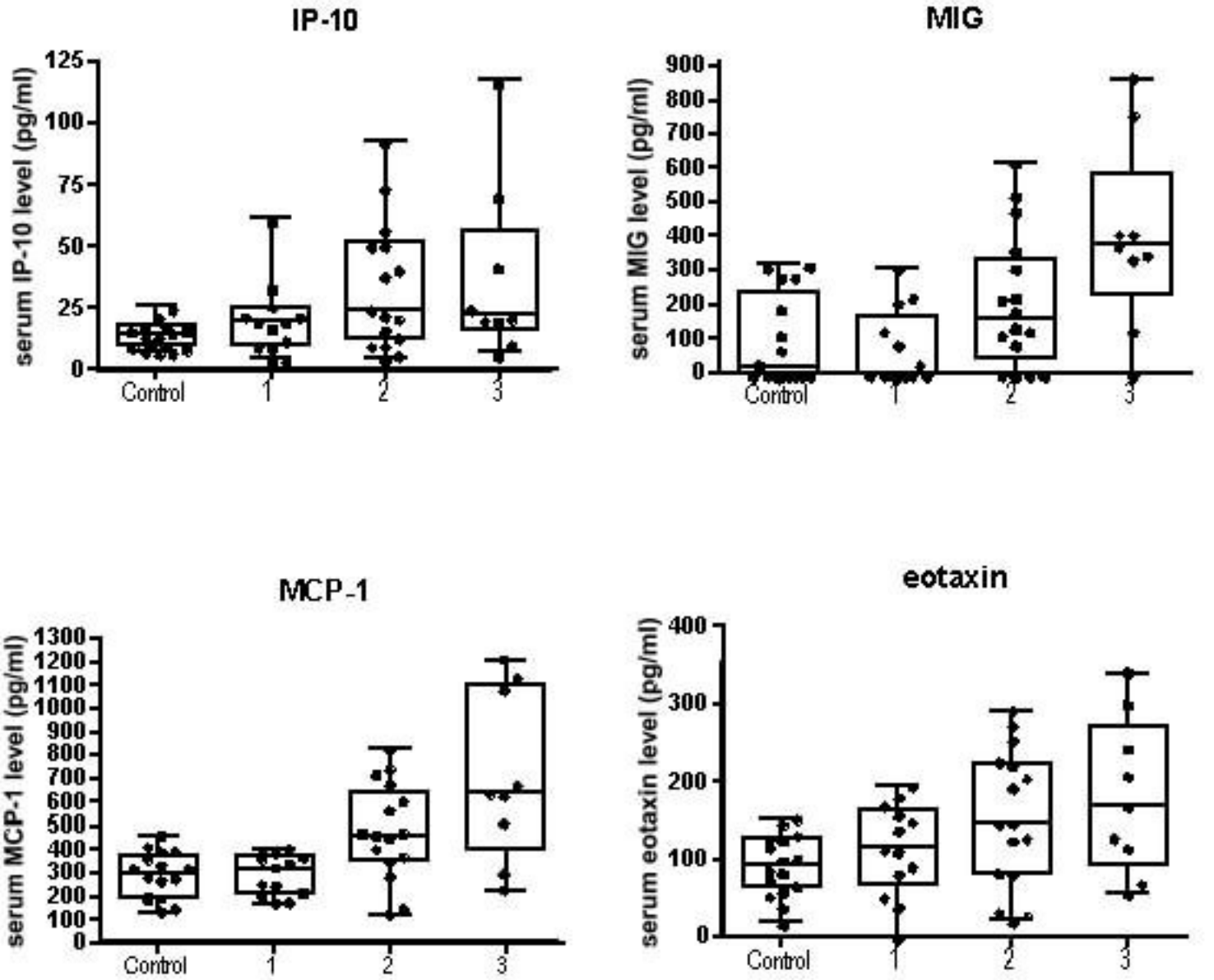

Figure 2
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