Doctor's Thesis

学位論文

Cytokine dysregulation as an important factor in the pathogenesis of allergic diseases and chronic fatigue syndrome of children and adolescents
(小児期および思春期のアレルギー疾患および慢性疲労症候群の病因における重要因子としてのサイトカイン調節異常)

Rabab Ismaeil El-Mezayen
ラバブ　イスマイル　エルメザイン

Supervisor
Prof. Dr. Teruhisa Miike
指導教官
三池輝久　教授

Department of Child Development
Kumamoto University School of Medicine,
Kumamoto, Japan
熊本大学医学部小児発達学講座

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By: Rabab Ismaeil El-Mezayen
ラバブ　イスマイル　エルメザイン

Supervisor: Prof. Dr. Teruhisa Miike
(Department of Child Development, Kumamoto University, School of Medicine, Kumamoto, Japan)

Referees: 1) Prof. Tetsuro Yamamoto　Dept. of Molecular Pathology

2) Prof. Nobuo Sakaguchi　Dept. of Molecular Immunology

3) Prof. Yasuhara Nishimura　Division of Immunogenetics

4) Prof. Fumio Endo　Dept. of Pediatrics

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PREFACE

This study was based both laboratory experiments and vivd clinical evaluations and follow up that took place during my postgraduate study at Kumamoto university, School of Medicine, Kumamoto, Japan.

This thesis is divided into two chapters. The study of cytokines in vitro in patients with allergic bronchial asthma and atopic dermatitis explained in two parts in vitro study I, and in vitro study II. Included in Chapter 1.

The in vitro study of cytokines production and modulation in patients with chronic fatigue syndrome is explained in Chapter II. Each of the two chapters has its own abstract, introduction, materials and methods, results, and discussion. The References mentioned at the end as (1.1. references) of the first study, (1.2. references) of the second study and (2. references) of the study in chapter II.

I hope this thesis will simply describe the role of cytokines and its involvement in the pathogenesis of allergic disorders and chronic fatigue syndrome.

I believe that the data presented here help to further understanding to cytokines and its important role and its possible help in the treatment of allergic and immunological diseases.

SUMMARY

Cytokines are reported to be involved in the regulation of cellular interaction that comprise immune responses. IL-18 is a novel cytokine formerly called IFN-γ inducing factor, which has been thought to be an important cofactor involved in Th1 and Th2 cytokines production. The mechanism whereby IL-18 is involved in the pathogenesis of allergic disorders is still undetermined. PBMCs were obtained from patients with allergic BA, AD and controls, and then cultured with LPS or PHA. The concentrations of IL-18, IFN-γ, and IL-13 in supernatant fluids were determined by enzymatic immunoassaying. IL-18 secretion in the BA patients (gm=189 pg/ml) and AD patients (172 pg/ml) was significantly higher than that in non-allergic controls (118 pg/ml). In contrast, IFN-γ secretion in the BA patients (7.3 IU/ml) and AD (6.8 IU/ml) was significantly lower than that in non–allergic controls (20.7 IU/ml). These results imply a defect in the responsiveness to IL-18 in patients with allergic disorders. So that, a second study was undertaken to check the responsiveness to IL-18.

PBMCs were cultured with IL-18 in the presence of IL-12 or IL-2, and then the concentration of IFN-γ, IL-13 and IL-4 in the culture supernatants were determined by enzymatic immunoassaying. IFN-γ production was detected in all culture from non-allergic controls with IL-18 in the presence of IL-12, however, the results of 5 BA patients and 5 AD patients were under the detection limit for IFN-γ. In collaboration with IL-2, IL-18 was able to induce IFN-γ production by PBMCs from all non-allergic controls and all allergic patients. Synergistic induction of IL-13 production was found in cultures with IL-18+IL-2, and it was significantly increased in BA patients, suggesting the involvement of IL-18 in the pathogenesis of allergic disorders.

In addition to the role of cytokines in allergic disorders, cytokines have been suggested to play a role in the pathogenesis of chronic fatigue syndrome (CFS). PBMCs were cultured with LPS or PHA, The

concentrations of TGF-β1, IL-6, IL-10, IL-4, IFN-γ, IL-18 and TNF-α in the culture supernatants were measured by the specific immunoassaying (ELISA). In CFS patients who received the low dose intravenous -γ globulin treatment we examined the cytokines production as well as, the patients performance status score (PS).

There was significant decrease in TGF-β1 protein and mRNA production in LPS stimulated PBMC culture supernatants. Significant increase in TGF-β1 productivity and improvement of the PS in four of five CFS patients who received low dose intravenous (IV)- γ globulin treatment were observed. These results suggest that anti-inflammatory cytokine TGF-β1 might be involved in the inflammatory characteristics of CFS.

PUBLICATIONS AND NOTES

1. Rabab I. EL-Mezayen, T. Matsumoto, H. Nomiyama* and T. Miike. Increased secretion of IL-18 in vitro by peripheral blood mononuclear cells of patients with bronchial asthma and atopic dermatitis.
Clin Exp Immunol (2001) 126; 193-198.

2. Rabab El-Mezayen, T. Akemi, T. Jhoudoi, T. Matsumoto and T. Miike. Cytokines production and modulation after low dose gamma-globulin therapy in patients with chronic fatigue syndrome (CFS). (submitted), *Psychiatry research* (2003*)*.

3. Rabab El-Mezayen, T. Matsumoto and T. Miike. In vitro responsiveness to IL-18 in combination with IL-12 or IL-2 by PBMC from patients with Bronchial asthma and Atopic dermatitis. (submitted), *Clinical immunology* (2003).

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Finally, my great thanks to all patients, normal controls who participated in these studies and their parents for their kind help which is deeply appreciated.

ABBREVIATIONS

PBMC	peripheral blood mononuclear cells
LPS	lipopolysaccharide
PHA	phytohemaglutinin
PS	performance status score
CDC	the center for disease control
ELISA	enzyme linked immuno sorbent assay
IV	intra venous
CFS	chronic fatigue syndrome
BA	bronchial asthma
AD	atopic dermatitis
IL	interleukin
TNF	tumor necrosis factor
TGF	tumor growth factor
M	month
MRNA	messenger RNA
MoAb	monoclonal antibodies

General introduction

Cytokines are small-to medium-sized soluble proteins and glycoproteins secreted by one cell that can alter the behavior or properties of the cell itself or of another cell. They mediate highly potent biological effects on many cell types. They have critical roles in haematopoiesis, inflammatory responses and development and maintenance of immune responses. Importantly, cytokines act in networks or cascades. Typical properties of cytokines in these network are pleiotropy, redundancy, synergistic activity and antagonistic effects upon each other. It has been reported that stimulated lymphoid cells either express or induce the expression in other cells of heterogeneous group of soluble mediators that exhibit either effector or regulatory functions. These soluble mediators include cytokines, hormones, and neurotransmitters, which in turn affect immune function and may underlie many of the pathological manifestations seen in immunedisorders (**2.R.**35). Most cytokines produced by T cells are given the name interleukin (IL) followed by a number. In mouse models, production of cytokines by stimulated T cells has been grouped into two major patterns, T- helper 1 and T- helper 2 types, depending on whether the cytokines favor the function of macrophages and natural killer cells or of B cells, respectively. Th1-type cytokines include IL-2 and IFNγ, while IL-4, IL-5, IL-13 and IL-10 are examples of Th2-type cytokines.

One of recently identified cytokines is IL-18, a potent proinflammatory cytokine able to induce IFNgamma, GM-CSF, TNFalpha and IL-1 in immunocompetent cells, to activate killing by lymphocytes, and to up-regulate the expression of certain chemokine receptors. IL-18 is also essential to host defences against sever infections. Recent studies also demonstrated a convincing role for IL-18 in atopic responses, including atopic asthma. IL-18 induces naive T cells to develop into Th2 cells. Moreover, IL-18 also induces IL-13 and/or IL-4 production by NK cells, mast cells and basophils. Therefore, IL-18 should be seen as a unique cytokine that enhances innate immunity and both Th1- and Th2- driven

immune responses (**2.R.**37).

Allergic diseases characteristically exhibit elevated serum IgE levels. Allergic reactions are the results of the production of specific IgE antibody to common, innocuous antigens. Such antigens normally enter the body at very low doses by diffusion across mucosal surfaces, and trigger a Th2 response. Naive allergen-specific T cells are induced to develop into Th2 cells in the presence of an early burst of IL-4. The allergen-specific Th2 cells drive allergen-specific B cells to produce IgE. Although the pathophysiology of increased IgE production in such patients has been investigated, it has not been clarified completely. It has been shown that the class switch to IgE and production by B cells is regulated by the cytokines produced by Th2 cells. In particular, IL-4 which is necessary for the expression of germline C ε transcripts in B cells and induces the class switch to IgE together with additional signal such as the CD40-CD40 ligand interactions (**2.R.**36). In contrast, IFN-γ produced by Th1 cells inhibits IgE synthesis by human PBMCs *in vitro* .

Up to 40% of populations in western countries show an exaggerated tendency to mount IgE responses to a wide variety of common environmental antigens. This state is called atopy and appear to be influenced by several genetic loci. Atopic individuals have higher total levels of IgE measured in the circulation and higher eosinophils levels than their normal counterparts. They are more susceptible to allergic diseases such as hay fever, bronchial asthma and atopic dermatitis.

Chronic fatigue syndrome (CFS) and allergy cohorts are similar in terms of their immune status, and cytokines dysregulation was suggested to be a factor involved in the pathogenesis of both allergic disorders and CFS. However, CFS subjects could be discriminated by the distinct psychologic profiles among subjects with and without immune activation. It was reported that in at least large subgroup of subjects with CFS who had allergies, the concomitant influences of immune activation brought on by allergic inflammation in an individual with the appropriate psychologic

profile may interact to produce the symptoms of CFS. In addition, it has been reported that prolonged or excessive production of cytokines secondary to allergen exposure or other immune insults has been proposed as being responsible for the development of CFS (**2.R**.20). In addition, some studies of cytokines in CFS have been done in peripheral blood compartment and on the immunopathogenesis of CFS concluded that neuropsychiatric symptoms in CFS patients may be more closely related to disordered cytokines production by glial cells within the CNS which play a role in the pathogenesis of immunologically mediated fatigue in CFS.

Our studies have been done to investigate and clarify the possible involvement of cytokines and its impact on the pathogenesis and clinical manifestations of allergic BA, AD and CFS in children and adolescents. To address this issue, we studied candidate Th1 and Th2 cytokines which might be involved in the pathogenesis of these disorders.

Chapter I

Cytokine response in patient with allergic disorders

Study I: production of IL-18 *in vitro* by peripheral blood mononuclear cells of patients with bronchial asthma and atopic dermatitis

From: Rabab I. El-Mezayen, T. Matsumoto, H. Nomiyama and T. Miike. Increased secretion of IL-18 in vitro by peripheral blood mononuclear cells of patients with bronchial asthma and atopic dermatitis. *Clin Exp Immunol* (2001); **126**: 193-198.

1.1. Abstract
1.2. Introduction
1.3. Materials and Methods
1.4. Results
1.5. Discussion

1.1.1. Abstract

Objectives: This study was performed to determine whether or not IL-18, formerly called IFN-γ inducing factor, is involved in the pathogenesis of allergic disorders.

Methods: Peripheral blood mononuclear cells (PBMCs) were obtained from patients with allergic bronchial asthma (BA), patients with atopic dermatitis (AD) and controls who did not have any allergic disease, and then cultured with lipopolysaccharide (LPS) or phytohaemagglutinin (PHA). The concentrations of IL-18, IFN-γ and IL-13 in the supernatant fluids were determined by enzymatic immunoassay, and the expression of IFN-γ messenger (m) RNA in the cells was measured by colorimetric microplate assay.

Results: IL-18 secretion in the BA patients ((gm) =189 pg/ml) and AD patients (gm = 172 pg/ml) was significantly higher than that in non-allergic controls (gm = 118 pg/ml). In contrast, IFN-γ secretion in BA patients (gm = 7.3 IU/ml) and AD patients (gm = 6.8 IU/ml) was significantly lower than that in non-allergic controls (gm = 20.7 IU/ml). The amount of IL-13 in the supernatant fluids and IFN-γ mRNA in the cells were not statistically different among the BA, AD patients and non-allergic controls.

Conclusion: IL-18 is one of important cytokines that is possibly involved in the pathogenesis of allergic bronchial asthma and atopic dermatitis.

1.1.2. Introduction

It is widely accepted that helper T (Th) cells consist of two functionally different subsets, Th1 and Th2. They have very different functions: Th2 cells are most effective activators of B cells, especially in primary responses, while Th1 cells are crucial for activating macrophages. The two subsets can regulate each other, these subsets are characterized by the profile of cytokines involved: Th1 cells mainly produce IFN-γ, while Th2 cells selectively secrete IL-4, IL-5, and IL-13. There is overwhelming evidence that abnormal Th1 and Th2 functions contribute not only to the production of IgE but also to the pathogenesis of allergic disorders.

Studies on PBMCs from patients with BA and patients with AD have shown decreases in polyclonally-stimulated IFN-γ release (1-9). IL-18 also designated as IFN-γ inducing factor, is a novel cytokine that plays an important role in the Th1 cell response, primarily through its ability to induce IFN-γ production, especially in collaboration with IL-12 (10,11).

Recent animal studies, however, indicated a more complicated pleiotrophic role for IL-18 than simply induction of IFN-γ production, and IL-18 was reported to induce the production of IgE and Th2 cytokines (12-15).

Since the assessment of *in vitro* IL-18 production in allergic patients has not been reported, the present study was undertaken to examine the in vitro IL-18 secretion in different allergic disorders. Its results showed that IL-18 secretion by LPS-stimulated PBMCs *in vitro* in allergic patients was significantly higher than in non-allergic controls, IFN-γ secretion by PHA-stimulated PBMCs was significantly lower in allergic patients than in non-allergic normal controls. Reduced IFN-γ production with increased secretion of IL-18 may imply a defect in IL-18 responsiveness in allergic patients. However, recent animal studies indicated a more complicated pleiotrophic role for IL-18 than simply the induction of IFN-γ production, and IL-18 with IL-2 but not with IL-12, could be a strong cofactor for the expression of Th2 cytokines, IL-13 and IL-4, in T cells. So that, a second

study was undertaken to examine the *in vitro* inducing effect of IL-18 in combination with IL-12 or IL-2 on Th1 and Th2 cytokines production by PBMCs and to check IL-18 responsiveness in patients with different clinical categories of allergic disorders.

1.1.3. Materials and Methods

3.1. Subjects

The study groups comprised 18 patients with allergic BA without any skin disorders, 16 patients with AD without any respiratory problems, and 19 non-allergic controls without any haematological or nutritional problems. All the subjects, aged between 2 and 22 years, visited the department of Child Development, Kumamoto university School of Medicine (table1). The asthmatic patients met the diagnostic criteria of the Guidelines for the Diagnosis and Management of BA of the Japanese Society of Allergiology.

All the asthmatic patients were sensitive to house-dust mites, as judged from the skin prick test reactivity and mite-specific IgE serum levels, and had the moderate, persistent type of asthma according to the disease severity grading of the guidelines described above. They had been managed by means of inhaled cromoglycate, inhaled β-agonist, oral theophylline and/or inhaled corticosteroids. Subjects on more than 400 μ g/day of prophylactic inhaled corticosteroids were excluded to avoid any possible systemic effects of absorbed steroids on cytokines production. AD was diagnosed according to the criteria of Hanifin and Rajka and all the AD patients were affected by an exacerbated form of chronic dermatitis, i.e. more than 18% of the skin of the face, limbs and trunk was involved, and thus were graded as having the moderate or sever type of AD according to the guidelines for the SCORAD index.

None of the allergic donors was undergoing systemic corticosteroids therapy. The test was performed during the remission phase not only in the patients with BA, but also in those with AD, and all the subjects were free from infection. Heparinized blood (5ml) was obtained at the time of routine

blood examination, and this study was approved by the Ethics Committee of the Kumamoto Society for Paediatric Allergy. Informed consent for the investigation was obtained for all allergic and non-allergic subjects, the parents' consent being obtained for children.

Table .1. Profiles of subjects

	Controls	BA	AD
Number	19	18	16
Age (years)	14±6 (4-22)	13±5 (4-20)	13±5 (4-21)
Sex (M/F)	11/8	11/7	9/7
IgE (U/ml)	93 (50-174)	933 (537-1622)	1349 (741-2455)

Age (years) is shown as the mean±1 standard deviation and range. The levels of IgE are presented as the geometric means and 95% confidence intervals.

3.2. Cell culture

PBMCs were isolated by density gradient centrifugation, washed three times, and then adjusted to 1 x 10^6 cells/ml with RPMI 1640 medium containing 10% heat-inactivated fetal calf serum and 2 mM L-glutamine. The cells were then cultured in round-bottomed Falcon tubes (No. 2054; Beckton Dickinson, Lincoln Park, NJ, USA) with 50 ng/ml LPS or 5 μg/ml PHA, or without any stimulation, at 37° C under 5% CO_2. In some experiments PBMCs were cultured with LPS for 1 hour in the presence of cyclohexamide (100 μg/ml; Sigma, St. Louis, MO, USA).

The kinetics of IL-18, IFN-γ, and IL-13 secretion were similar in allergic and non-allergic subjects, with maximal secretion of IL-18 at 6 h in LPS-stimulated cultures, and maximal secretion of IFN-γ and IL-13 at 3 days in PHA-stimulated cultures. Since there was moderate secretion of IL-

18 within 1 h of stimulation with LPS, and moderate secretion of IFN-γ and IL-13 after 18 h of stimulation with PHA, the PBMC cultures were suspended at 1 h for the IL-18 assay, and at 18 h for the IFN-γ and IL-13 assays.

3.3. Measurement of IgE, IL-18, IFN-γ, IL-13 and IL-12

Plasma IgE was measured with an ELISA kit, IgE-MP Mitsui, from Mitsui-Seiyaku, Tokyo, Japan. This kit includes a MoAb as the first antibody and a peroxidase-conjugated polyclonal anti-IgE antibody as a second antibody, and is capable of detecting 0.65-1000 U/ml of IgE. The ELISA kit for IL-18 was obtained from Medical& Biological Laboratories, Nagoya, Japan, and those for IFN-γ and IL-13 were purchased from Immunotech, Marseil, France. Each kit includes two non-competing MoAbs for each cytokine. Mouse MoAb#125-2H and rat MoAb#159-12 B were used in the human IL-18 ELISA kit, and both MoAbs were confirmed to neutralize IFN-γ production by IL-18 (19).

The minimum sensitivities for IL-18, IFN-γ and IL-13 assays were 12.5 pg/ml, 0.08 IU/ml and 1.5 pg/ml, respectively. The levels of IL-12 were determined with a kit involving the multiple sandwich principle from R&D Systems, Minneapolis, MN, USA. This kit include a MoAb as the first antibody and alkaline phosphatase conjugated polyclonal anti-IL-12 as a second antibody, and a color-amplification system consisting of NADH, diaphorase and tetrazolium. The standard curve was linear from 0.5 to 40 pg/ml of IL-12 with this assay kit.

3.4. Western blot analysis for IL-18

Western blotting was performed, following the protocol recommended by Cell signaling technology, Inc. (Beverly, MA, USA). Briefly, samples of cell lysates containing 20 μ g protein were heat-denatured in the presence of 62.5 mM Tris-Hcl, pH 6.8, 2% SDS, 10% glycerol and 50 mM DTT, and then fractionated by 12.5% SDS-PAGE. The protein was then transferred to a Hybond-P membrane (Amersham Pharmacia Biotech, Inc., UK). The rabbit anti-human IL-18 antibody (H-173; Santa Cruz Biotechnology, CA, USA), which detects both 24 kDa precursor and 18 kDa mature form IL-18, was diluted to a concentration of 1:1000 before use. The bound antibody was detected with horseradish peroxidase-conjugated anti-rabbit IgG (Cell Signaling Technology) and visualized with an ECL kit (Amersham Pharmacia Biotech) according to the manufacturers' instructions.

3.5. Determination of IFN- γ mRNA expression

For RNA extraction, 1 x 10^6 PBMCs were cultured with PHA for 3 h. then mRNA was isolated using a poly (A)+ RNA isolation kit with oligo dT- latex beads (mini-Messae Marker; Novagen Inc., Madison, WI, USA). Samples were then hybridized with hIFN- γ -specific biotin-labelled capture oligonucleotide probes and digoxigenin-labelled detection probes on streptavidin-coated microplates (Colorimetric mRNA Quantification kit; R&D Systems) according to the manufacturers' instructions. The standard curve was linear from 4.7 to 300 amol/ml IFN- γ mRNA.

3.6. Statistical analysis

All samples were coded and read blind on assaying. The plasma IgE level and *in vitro* Cytokines amount data were logarithmically transformed before statistical analysis, and were expressed as gm and 95% confidence intervals (ci). Group means were compared by means of the unpaired Students' *t*–test. Correlation was determined by multiple and single regression analysis. Frequencies were compared as to their statistical

significance with the Fisher' s exact test. All statistical analysis were performed with State View statistical Package 4.5 (SAS institute, NC, USA). Probability (p) values of less than 0.05 were considered significant.

1.1.4. Results

The levels of IgE in plasma from BA patients and AD patients were extremely elevated compared with those in age, sex-matched controls who did not have any allergic disease (Table1). However, no statistical difference in the plasma IgE levels was observed between BA patients and AD patients. Spontaneous secretion of IL-18, IFN- γ and IL-13 by unstimulated PBMCs was very low or unmeasurable in all cultures. The means and standard error of the mean (s.e.m.) of IL-18 concentration in the supernatant fluids of LPS-stimulated PBMCs cultured for 1 h were 114 ± 17 pg/ml without cycloheximide and 122 ± 9 with cycloheximide 173 ± 15 pg/ml without cycloheximide and 157 ± 10 pg/ml with cycloheximide in three allergic patients. Western blot analysis was performed using extracts of PBMCs cultured with or without LPS for 1 h from three BA patients, three AD patients and three non-allergic controls.

The results showed that only mature form IL-18 was present in cultured PBMCs with LPS in BA and non-allergic controls, although mature form IL-18 was detected in both unstimulated PBMCs and LPS-stimulated PBMCs from AD patients. Representative results are (shown in Fig.1). There was moderate enhancement of the level of mature form IL-18 within 1 h of stimulation with LPS in an AD patient. (Fig.2), increased IL-18 secretion by LPS-stimulated PBMCs was observed in the BA patients (189 pg/ml, 95% ci 161 – 223 pg/ml) and AD patients (gm 172 pg/ml, 95% ci 151 – 196 pg/ml) compared with controls who did not have any allergic disease (gm 118 pg/ml, 95% ci 91 – 153 pg/ml).

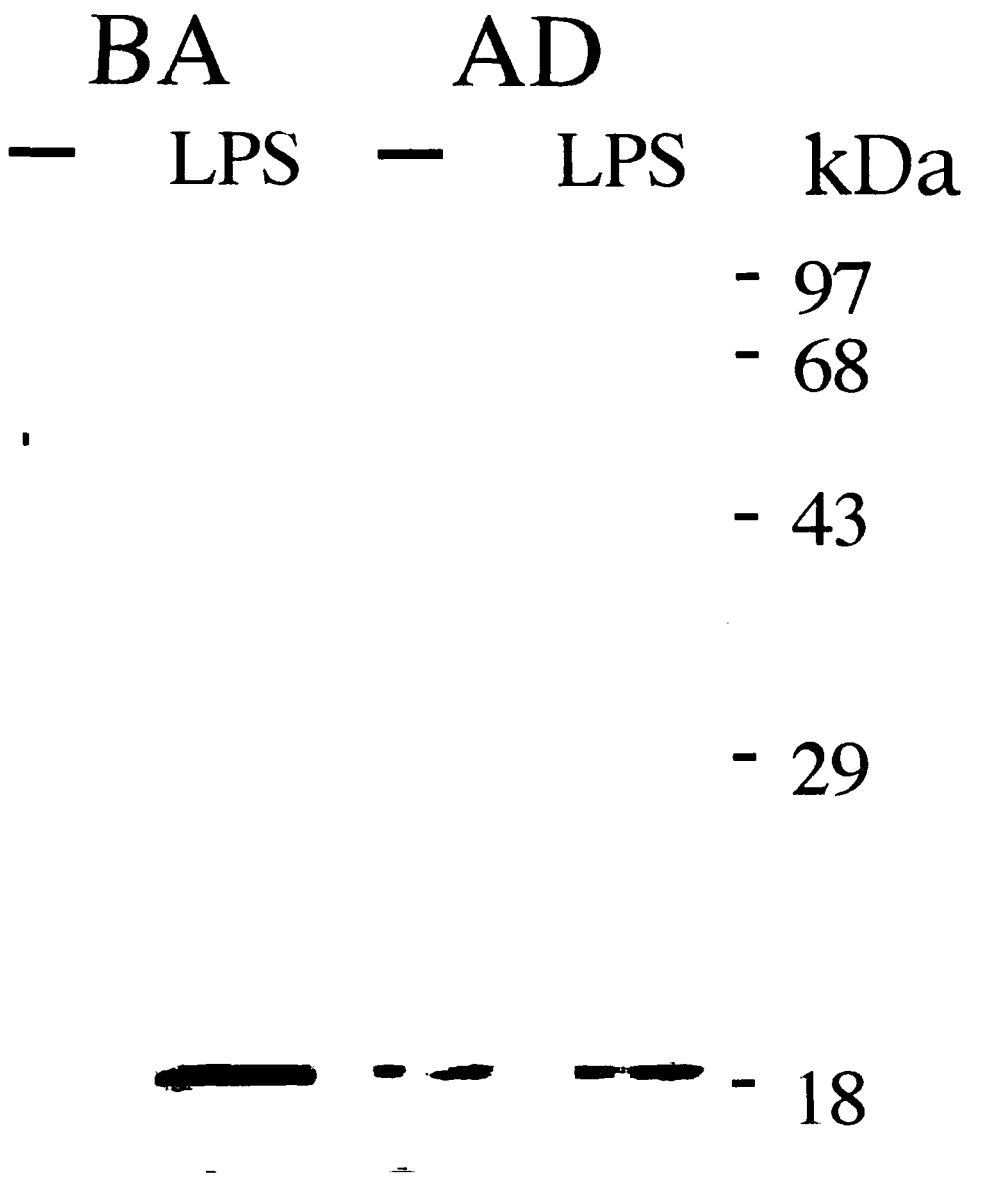


Fig.1. Detection of IL-18 in PBMCs cultured without stimulation or with LPS for 1 hour. Lysates of PBMCs were subjected to SDs/PAGE and western blotting with an anti-human IL-18 antibody (H-173).Which detects both 24 kDa precursor form IL-18 and 18 kDa mature form IL-18.

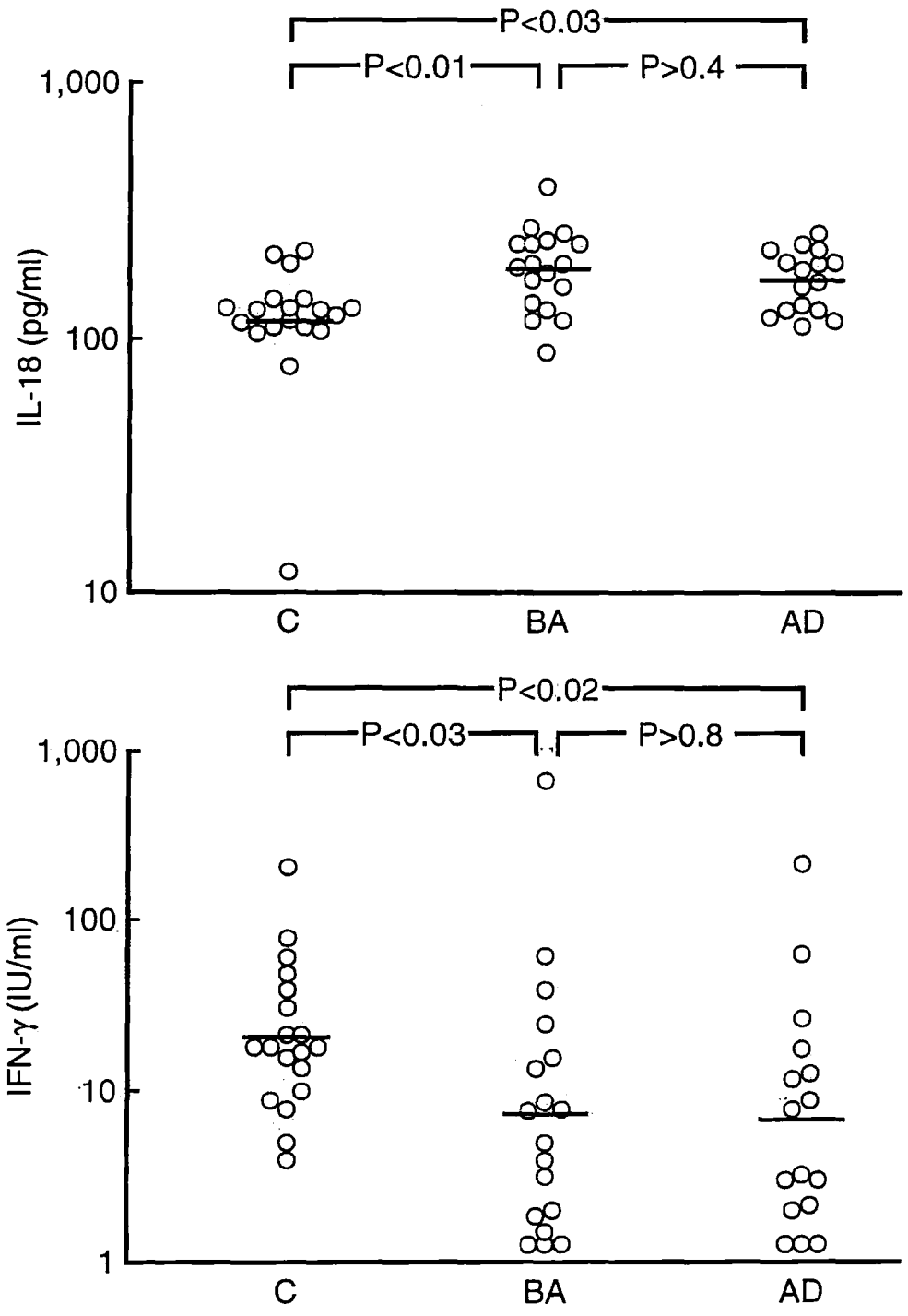


Fig.2. Secretion of (a) IL-18 by LPS-stimulated PBMC cultures at 1 h and (b) IFN-γ by PHA-stimulated PBMC cultures at 18 h was measured by enzymatic immunoassaying. Horizontal lines represent geometric means. C, non-allergic controls; BA, bronchial asthma; AD, atopic dermatitis.

In contrast, secretion of IFN-γ by PHA-stimulated PBMCs from the BA patients (gm 7.3IU/ml, 95% ci3.5 – 15.5 IU/ml) and AD patients (gm 6.8 IU/ml, 95% ci 3.3- 13.9 IU/ml) was significantly decreased compared with controls who did not have any allergic disease (gm 20.7 IU/ml, 95% ci 13.6 – 53.3 IU/ml). The amounts of IL-18 and IFN-γ in the culture supernatant fluids did not differ markedly between the BA patients and AD patients. The amounts of IL-13 secreted by PHA-stimulated PBMCs were comparable among the BA patents (gm 27.9 pg/ml, 95% ci 11.1 – 70.0 pg/ml), AD patients (gm 31.3 pg/ml, 95% ci 11.3 – 86.9 pg/ml) and non-allergic controls (gm 54.6 pg/ml 95% ci 34.8 – 85.5 pg/ml). No significant differences in IFN-γ mRNA expression in PHA-stimulated PBMCs were observed among the BA patients (mean 18.3 amol/10^6 cells, 95% ci 15.1 – 21.4 amol/10^6 cells), AD patients (20.6 amol/10^6 cells, 95% ci 14.6 – 26.5 amol/10^6 cells) and non-allergic controls (15.3 amol/10^6 cells, 95% ci 11.1 – 19.5 amol/10^6 cells).

All plasma samples gave results above the detection limits of the IL-18 assay in this study , and the mean±s.e.m. plasma IL-18 levels were 192± 17 pg/ml in BA patients, 216±29 pg/ml in AD patients and 150±18 pg/ml in non-allergic controls. Although the plasma IL-18 levels in allergic patients were more increased than those in non-allergic controls, the differences were not significant (BA patients *versus* controls, $p = 0.107$; AD patients *versus* controls, $p = 0.074$). Six plasma samples (three from 18 BA patients, two from 16 AD and one from 19 non-allergic controls) gave results above the detection limit of the IL-12 assay, and the frequencies of subjects showing results above the sensitivity of the IL-12 assay were not statistically different among the three groups.

The cytokine levels in stimulated PBMC cultures were used as independent variables, and regressed against the plasma IgE concentration as the dependent variable for multiple regression analysis. IL-18 secretion was significantly correlated with the plasma IgE concentration in BA patients ($p= 0.026$), although IL-18 secretion was not correlated with the plasma IgE concentration in AD patients ($P=0.121$) or non-allergic controls

(P =0.748). IFN-γ secretion, IL-13 secretion and IFN-γ mRNA expression were not statistically correlated with the plasma IgE concentration in the three groups, respectively. IFN-γ secretion was chosen as the dependent variable and analysis with IL-18 secretion, IL-13 secretion and IFN-γ mRNA expression as independent variables for multiple regression analysis. IL-13 secretion was significantly correlated with IFN-γ secretion in non-allergic controls (P =0.031). However, IL-13 secretion was not statistically correlated with IFN-γ secretion in BA patients (P =0.146) or AD patients (P =0.204).

Fig.3 depicts the positive correlation between the plasma IgE levels and IL-18 secretion in BA patients (r =0.556, P =0.017), and the positive correlation between IFN-γ secretion and IL-13 secretion in non-allergic controls (r=0.533, P =0.019), observed on single regression analysis, respectively, IL-18 secretion was chosen as the dependent variable and analysed with IFN-γ secretion, IL-13 secretion and IFN-γ mRNA expression as the independent variables for multiple regression analysis. IL-18 secretion was correlated with neither IFN-γ secretion , IL-13 secretion nor IFN-γ mRNA expression in the three groups.

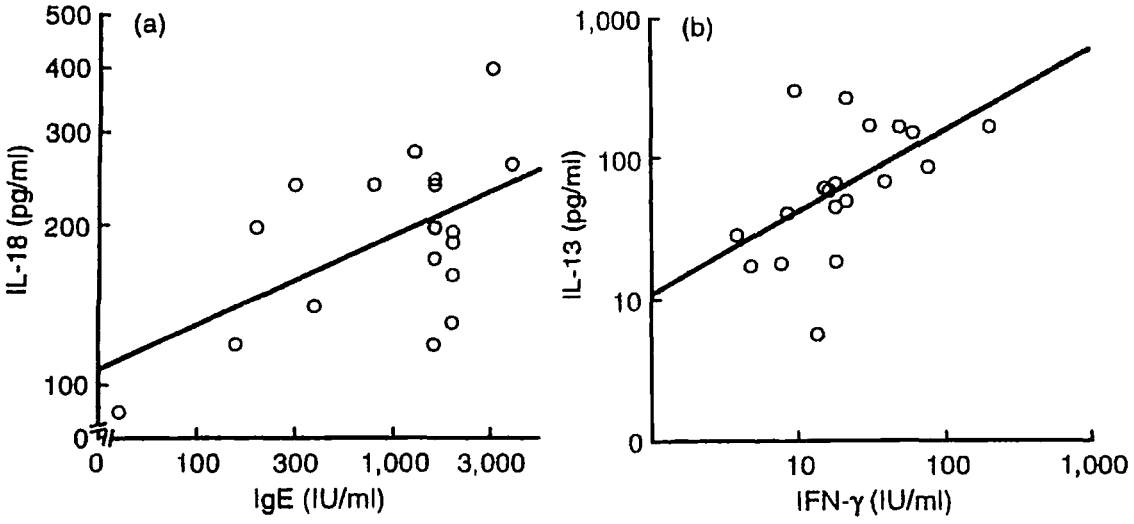


Fig.3. Correlation between plasma IgE concentration and *in vitro* IL-18 Secretion by LPS-stimulated PBMCs in BA patients (a), and that between IFN-γ secretion and IL-13 secretion in PHA-stimulated PBMCs cultures in non-allergic controls (b). The differences in the slopes between groups were assessed by single regression analysis. A significant positive correlation between IL-18 secretion and plasma IgE was found (r= 0.556, P = 0.017). Secretion of IFN-γ was significantly correlated with secretion of IL-13 (r = 0.533, P = 0.019).

1.1.5. Discussion

The original characteristics of PBMCs might be considerably altered during *in vitro* incubation with stimulants. In this study, cytokines secretion by PBMCs was measured in short-term cultures to minimize alterations in the nature of PBMCs, somewhat, reflecting the state of *in vivo* cytokines production. Spontaneous secretion of IL-18 by unstimulated PBMCs was very low or unmeasurable, and mature form IL-18 was not present in lysates unstimulated PBMCs from BA patients and non-allergic controls. Puren *et al* (20) reported that mature form IL-18 was detected in freshly obtained PBMCs from healthy donors. Mature form IL-18 was found in unstimulated PBMCs from AD patients in this study, suggesting that de-novo IL-18 synthesis occurs in these cells without any stimulation. Since the amounts of IL-18 in the supernatant fluids of 1h-stimulated PBMCs with LPS were not statistically different between cycloheximide-treated and untreated cultures, it is probable that IL-18 in the supernatant fluids might have been preformed IL-18 released by the cells.

The present study clearly showed that IL-18 secretion by stimulated PBMCs was increased in BA patients and AD patients. The amount of IL-18 did not differ markedly between the BA patients and AD patients. This implies that the increased IL-18 secretion is related to the allergic state *per se* rather than to the clinical features of allergic diseases. The effects of IL-18 in allergic disorders have been previously assessed in murine asthma models with different outcomes (14,15,21). In the study by Hofsta *et al.* (21), the combined *in vivo* administration of IL-18 and IL-12 inhibited allergen-induced airway hyperresponsiveness, lung production of eosinophilia and serum IgE, although the administration of IL-18 alone failed to modulate any of these allergic responses. However, Kumano *et al.* (14) and Wild *et al.* (15) showed that in vivo IL-18 administration alone enhanced allergen-induced eosinophilic recruitment to the lungs, Th2 cytokines production and IgE production in sensitized mice. The latter

finding in murine models are in accord with the results of the present study for BA patients.

In the recent study by Hoshino *et al.* (22), the administration of IL-18 alone *in vivo* significantly increased the serum IgE level in non-sensitized mice, and the administration of IL-18 plus IL-2 induced a 70-fold higher serum level of IgE than in control mice. Alternatively, human IL-18 may be involved in the increased IgE production in BA, since the IL-18 secretion by LPS-stimulated PBMCs was associated with the plasma IgE level in BA patients in the present study. An important question is whether the cytokines imbalance demonstrated in the *in vitro* study was the cause or the result of the disease process. All the patients were examined during the remission phase in the present study, suggesting that an imbalance of IL-18 and IFN-γ deficiency in PBMCs precedes the allergic state, rather than resulting from it, as suggested by the finding of decreased IFN-γ production in cord blood cells of neonates who subsequently developed allergic diseases (23). Therefore, defective IFN-γ secretion by PBMCs *in vitro* is likely to underlie allergic disorders.

IL-18 has been described as a potent inducer of IFN-γ (10,11), and reduced IFN-γ secretion with increased IL-18 may imply a defect in IL-18 responsiveness in allergic patients. Supporting this possibility, Jung *et al.* (24) reported that a deficiency of IFN-γ production in sever AD patients could not be reversed by IL-18 *in vitro*. There is an absolute requirement for IL-12 for development of the Th1 cell response, and it well established that in the presence of low picomolar concentration of Il-12, Il-18 becomes essential for the production of IFN-γ (25). Another interpretation is that the regulatory cytokines involved in IFN-γ production may differ between allergic and non-allergic controls. However, the frequencies of cases showing results above the detection limit of the IL-12 assay (0.5 pg/ml) were not statistically different among BA patients, AD patients and non-allergic controls.

In contrast to reduced *in vitro* IFN-γ secretion in the BA patients and AD patients, however, equivalent expression of IFN-γ mRNA in stimulated PBMCs was observed for the allergic patients compared with the normal controls.

Comparable studies have demonstrated that expression of IFN-γ mRNA is not necessarily correlated to secretion, as children with AD exhibit comparable or increased expression of IFN-γ mRNA in stimulated PBMC compared with controls, despite exhibiting reduced IFN-γ secretion (5, 9). The discrepancy between IFN-γ mRNA expression and secretion demonstrated here for allergic patients suggests that reduced secretion of IFN-γ is not related to abnormal regulation of transcription, but rather to a post-transcriptional defect of IFN-γ production. This study demonstrated a positive correlation between IFN-γ secretion and Th2 cytokine IL-13 secretion in cultures of PHA-stimulated PBMCs in non-allergic controls.

IL-18 has been reported to have the ability to stimulate the production of IFNγ- by T cells, NK cells and B cells (11, 25), and appeared recently to induce the production of IL-13 by T cells and NK cells *in vitro* (12). The present study did not reveal any correlation between IL-18 secretion by LPS-stimulated PBMC cultures and IFN-γ secretion or IL-13 secretion by PHA-stimulated PBMC cultures in non-allergic controls. IFN-γ was found not to be generally co-expressed with IL-13 in human T cells in an intracellular cytokine staining assay, and IFN-γ does not affect IL-13 production in stimulated human T-cell cultures (26). However, both IFN-γ and IL-13 are cytokines with long-lasting kinetics on secretion from T cells (26). It is tempting to speculate that IFN-γ and IL-13 play roles in ongoing immune responses in humans without any disorders.

IL-18 is considered to possess biological properties other than induction of IFN-γ, IgE or Th2 cytokines, such as up-regulation of the expression of intercellular adhesion molecule-1(ICAM-1) and fas-ligand in human

myelomonocytic KG-1 cells (27, 28). Circulating ICAM-1 levels have been reported to increase gradually with the severity of AD (29), and successful phototherapy for AD patients has been found to result from ultraviolet A radiation-induced apoptosis of skin-infiltrating activated T cells through the Fas/Fas-ligand system (30). The plasma levels of IL-18 were not different between the remission-phase allergic patients and the non-allergic controls. Therefor, further study was done to confirm the biological significance of IL-18 during the allergic inflammatory process. (see below)

Study II

IL-18 responsiveness in patients with bronchial asthma and atopic dermatitis

From: Rabab I. El-Mezayen, T. Matsumoto and T. Miike. In vitro responsiveness to IL-18 in combination with IL-12 or IL-2 by PBMC from patients with Bronchial asthma and Atopic dermatitis. (submitted), *Clinical immunology* (2003).

2.1. Abstract
2.2. Materials and Methods
2.3. Results
2.4. Discussion

1.2.1. Abstract

Background: Interleukin (IL)-18 is a proinflammatory cytokine, and is now recognized as an important regulator of both Th1 and TH2 cytokine production. An increased IL-18 secretion has been reported in patients with allergic disorders. It is predominantly produced by activated macrophages, and synergize with IL-12 to induce IFN-γ synthesis, thereby promoting Th1 cytokine response. Paradoxically, IL-18, by itself, strongly induces immunoglobulin (Ig) E and allergic inflammation, indicating a role for IL-18 in promoting Th2 response. This study was performed to investigate the inducing effect of combining IL-18 and IL-12 or IL-2 on Th1 and Th2 cytokines production by peripheral blood mononuclear cells (PBMCs) from patients with allergic diseases.

Patients and Methods: PBMC derived from Fourty-four allergic patients (23 bronchial asthma (BA) and 21 atopic dermatitis (AD) ones) and 20 healthy controls, who did not have any allergic disorders,.were cultured with IL-18 in the presence of IL-12 or IL-2. The levels of IFN-γ, IL-13 and IL-4 in the culture supernatants were determined by enzymatic immunoassay. IL-12Rβ1 expression was analysed by flow cytometry.

Results: IFN-γ production was detected in all cultures from non-allergic controls with IL-18 in the presence of IL-12, however, the results for 5 BA patients and 5 AD patients were under the detection limit for IFN-γ. In collaboration with IL-2, IL-18 was able to induce IFN-γ production by PBMCs from all non-allergic controls and all allergic patients, with the exception of one AD patient. Synergistic induction of IL-13 production was found in cultures with IL-18+IL-2, and the IL-13 induction was significantly increased in BA patients when compared with that in non-allergic controls (p=0.006). The stimulation by IL-18, even in combination with IL-2, failed to induce IL-4 production by PBMCs from both non-allergic controls and allergic patients.

Conclusion: Although the induction of IFN-γ by IL-18+IL-12 was impaired in around a quarter of the allergic patients, the impairment of IFN-γ production was completely restored by IL-2 in the presence of IL-18. IL-13 production induced by IL-18+IL-2 was increased in BA patients, suggesting the involvement of the IL-18+IL-2 mechanism in the pathogenesis of BA.

1.2.2. Materials and Methods

2.1. subjects

The study groups comprised 23 patients with allergic BA without any skin disorders, 21 patients with AD without asthmatic episodes, and 20 non-allergic controls without any haematologic, immunological or nutritional problems. All the subjects, aged between 3 to 21 years old, visited the Pediatrics clinic of Kumamoto University Hpspital (see table1).

Table1. Profiles of study subjects.

	Control	Bronchial asthma	Atopic dermatitis
Number	20	23	21
Age (years)	11.8±5.5 (3-21)	11.7±4.9 (4-19)	12.4±4.8 (3-20)
Sex (M/F)	11/9	12/11	12/9
IgE (IU/ml)	30 (16-57)	1,157 (695-1,923)	1,588 (925-2,729)

Age (years) is shown as the mean±standard deviation. The levels of IgE are presented as the geometric means and 95% confidence intervals.

The asthmatic patients met the diagnostic criteria of the Guidelines for the Diagnosis and Management of BA of the Japanese Society of Allergology (16). All the asthmatic patients were sensitive to house-dust/mites, as judged from the skin prick test reactivity and mite-specific IgE serum level, and had the moderate, persistent type of asthma according to the disease severity grading of the guidelines described above. They had been managed by means of inhaled cromoglycate, an inhaled β-agonist, oral theophylline and/or inhaled corticosteroids.

Subjects on more than 400 μ g/day of prophylactic-inhaled corticosteroids were excluded to avoid any possible systemic effects of absorbed steroids on cytokines production . AD was diagnosed according to the criteria of HαR (17), and all the AD patients were affected by an exacerbated form of chronic dermatitis, i.e.more than 18% of the skin of the face, limbs and trunk were involved, and thus were graded as having the moderate or sever type of AD according to the guidelines for the SCORAD index (18). None of the allergic patients were undergoing systemic corticosteroids therapy. The test was performed during the remission phase not only in the patients with BA, but also in those with AD, and all the subjects were free from infection. Five milliliters of heparinized peripheral blood was obtained at the time of routine blood examination, and this study was approved by the Ethics Committee of the Kumamoto Society for Pediatric Allergy. Informed consent for the investigation was obtained for all allergic and non-allergic subjects, the parents' consent being obtained for children.

2.2. Cell culture

PBMCs were isolated by density gradient centrifugation, washed three times, and then adjusted to 1×10^6 cells/ml with RPMI 1640 medium containing 10% heat-inactivated fetal calf serum and 2mM L-glutamine. The cells were then cultured in round-bottomed Falcon tubes (No. 2054; Beckton Dickinson, Lincoln Park, NJ) with IL-18 (Medicalα Biological Lab., Nagoya, Japan), IL-12 or IL-2 (Genzyme Techn, MPLS, MN), the

combination of IL-18 + IL-12 or IL-18 + IL-2, 5μg/ ml of PHA (DIFCO Lab.,Detroit, MI) or without any stimulant at 37° C under 5% CO_2.

2.3. Measurement of IgE, IFN-γ, IL-13, IL-4 and IL-10

Plasma IgE was measured with an ELISA kit obtained from ICN Phamaceuticals, NY. This kit includes a monoclonal antibody (MoAb) as the first antibody and a peroxidase-conjugated polyclonal anti-IgE antibody as the second antibody, and is capable of detecting 5-1, 000 IU / ml of IgE. The human IFN-γ, IL-13, IL-4 and IL-10 concentrations were measured with commercially available ELISA kit (BioSource International, Camarillo, CA) according to the procedure recommended by the manufacturer, respectively.

Each kit includes two non-competing MoAb for each cytokine, and does not measure the presence of other cytokines. Duplicated samples were all coded and read blind in the assay. The absorbance was measured with a Model 2550 EIA reader (BioRad, Richmond, CA). Each kit is capable of detecting 4-1,000 pg/ml of IFN-γ, 12-2,500 pg/ml of IL-13, 2-500 pg/ml of IL-4, and 1-500 pg/ml of IL-10, respectively.

2.4. Skin test

A skin prick test with the usual panel of aeroallergens and food extracts (Torii, Tokyo) were performed. Histamine hydrochloride (1 mg/ml) and 10% glycerine-saline were applied as control solutions. Wheal reactions greater than those with histamine hydrochloride were considered positive.

2.5. Statistical analysis

The plasma IgE levels and in vitro cytokine level data were expressed as geometric means (gm) and 95% confidence intervals (ci). The Mann Whitney *U*- test was used to compare the study groups for significant differences. Frequencies were compared by means of Fisher' s exact test. Correlation was determined by multiple and single regression analysis. All

statistical analysis were performed with State View Statistical Package 4.5 (SAS Institute, Cary, NC). Probability (P) values of less than 0.05 were considered significant.

1.2.3. Results

The levels of IgE in plasma from BA patients and AD patients were extremely elevated compared with those in age-and sex- matched controls who did not have any allergic diseases (table1). However, no statistical difference in the plasma IgE levels was observed between the BA patients and AD patients. Spontaneous secretion of IFN-γ, IL-13, IL-4 and IL-10 by unstimulated PBMCs was very low or unmeasurable in all cultures. IFN-γ,and IL-13 production by PBMCs stimulated with various doses of IL-18, IL-12, IL-2, IL-18+IL-12 and IL-18+ IL-2 was determined in a non-allergic controls.

The results are summarized in (table2). IL-18 alone was not capable of inducing IFN-γ *in vitro*. IFN-γ production was found after 24 hr in a PBMCs culture with a high dose of IL-12 (100 ng/ml) alone, however, low doses of IL-12 induced much more IFN-γ production *in vitro* in the presence of IL-18. IL-18 and IL-2, respectively, induced low levels of IL-13 production after 5 days by PBMCs, however, combined stimulation with IL-18+IL-2 caused the production of much more IL-13 in the cultures than IL-18 alone or IL-2 alone, showing the synergistic induction of IL-13 production. High doses of IL-2 (100-200 ng/ml) induced low levels of IFN-γ production (24-52 pg/ml) in PBMCs cultures after 5 days, however, strong synergistic production of up to 13,100 pg/ml of IFN-γ was found on stimulation with IL-18 (200 ng/ml) + IL-2 (100 ng/ml).

The amounts of IL-4 in PBMC cultures with various concentrations of IL-18, even in combination with 100 ng/ml of IL-2, were under the detection limit of the assay. (As shown in Fig. 1 A) IFN-γ production was detected in all PBMC cultures from non-allergic controls with IL-18 (200 ng/ml)+ IL-12 (20 ng/ml), however, the results for 5 BA patients and 5 AD patients

were under the detection limit for IFN-γ. The frequency of AD patients who did not respond to stimulation with IL-18+IL-12 was significantly higher statistically than in the case of non-allergic controls (P=0.048). Although 5 of the 23 BA patients did not respond to the stimulation with IL-18+ IL-12, the P value was 0.051 when compared with that for non-allergic controls. (Table 3) summarizes the clinical features of the 5 BA patients and 5 AD patients who did not respond to IL-18+IL-12 stimulation.

Although 3 AD patients showed extremely elevated serum-IgE levels of more than 5,000 IU/ml, none of them had the clinical disorders due to immunodeficiency, as judged on examination of hospital charts. The amounts of IFN-γ in the culture supernatants from the patients who responded to stimulation with IL-18+IL-12 with BA (n=18, gm 5,598 pg/ml, 95% ci 3,532-8,872 pg/ml) and with AD (n=16, gm 3,882 pg/ml, 95% ci 1,337- 11,272 pg/ml) were not different statistically when compared with in the case of non-allergic controls (n=20, gm 7,464 pg/ml, 95% ci 5,495-10,139 pg/ml).

On the contrary, IL-18+IL-2 was capable of inducing IFN-γ production by PBMCs from all non-allergic subjects and all allergic subjects (Fig.1B), with the exception of one AD patient, i.e.K.H. in (table 3). No significant differences in IFN-γ production induced by IL-18+IL-2 were observed statistically among the BA patients (gm 12,823 pg/ml, 95% ci 11,376-14,454 pg/ml), AD patients (gm 7,396 pg/ml, 95% ci 2,992-18,281 pg/ml), and non-allergic controls (gm 13,428 pg/ml, 95% ci 11.967-15.066 pg/ml).

Table 2. IL-18 synergizes with IL-12 or IL-2 to induce IFN-γ and IL-13

IFN-γ production (pg/ml)

IL-12 / IL-18	0 ng	10ng	20ng	100ng
0 ng	<4	<4	<4	217
10 ng	<4	71	62	129
50 ng	<4	814	1,295	1,488
200 ng	<4	2,961	4,119	4,271

IL-13 production (pg/ml)

IL-2 / IL-18	0 ng	10 ng	100 ng	200 ng
0 ng	<12	75	197	166
5 ng	<12	n.d*	336	n.d.
50 ng	51	171	310	298
200 ng	109	n.d.	513	433

Non-allergic PBMCs were cultured with various concentrations of IL-18, IL-12, IL-2, IL-18+IL-12 or IL-18+IL-2.

The amounts of IFN-γ in the supernatants were measured after 24 hr, and IL-13 was determined after 5 days by enzymatic immnuoassay, respectively.

ng,. nanogram per mililiter. *n.d. = not done.

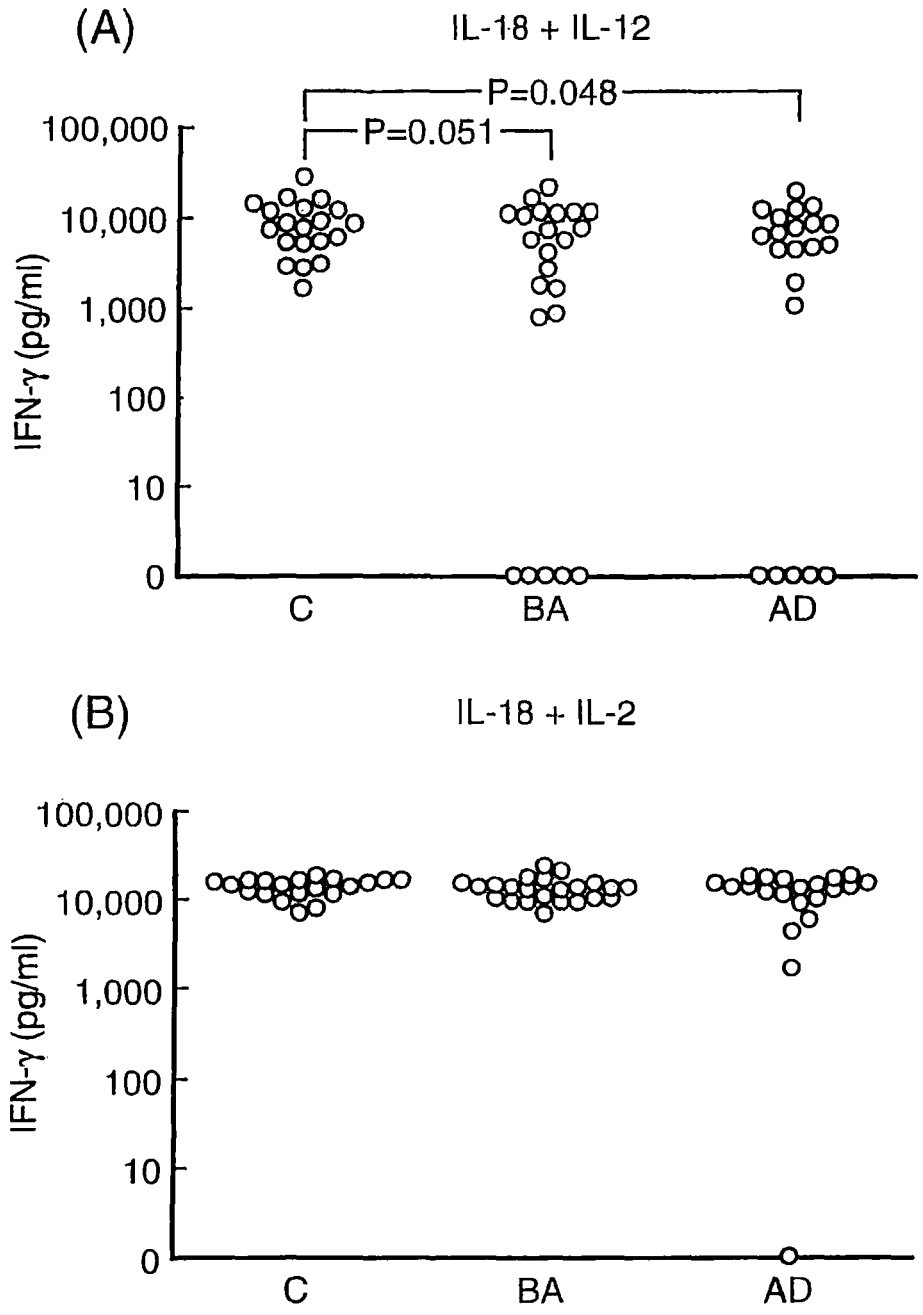


Fig.1. IFN-γ production (**A**) by IL-18+IL-12 stimulated PBMCs cultured at 24 h and (**B**) by IL-18+IL-2 stimulated PBMCs cultured at 5 days was measured by enzymatic immunoassay. C, non-allergic controls; BA, bronchial asthma; AD, atopic dermatitis.

Table 3. The clinical features of the patients in whom IFN-γ was not produced by PBMCs following stimulation with IL-18+IL-12.

Subject	Age (yr)	Sex M/F	Complications	Plasma IgE, (IU/ml)	prick test
Bronchial asthma					
O.K.	19	F	Fibroma	378,	Mites
M.M.	7	F	AR*1	498,	Mite
K.R.	18	F	AR	728,	Mite
I.S.	18	F	AR	2,609,	Mites,grass
T.F.	19	M	AR	1,594,	Mites,wheat Cedar and history of SU*2
Atopic dermatitis					
O.S.	17	M	FA*3	5,658,	Mites,Egg,Milk
I.Y.	13	F	NO	5,027,	Mites
T.A.	12	M	AR	7,512,	Mites,Dog hair
M.R.	16	F	NO	937,	Not done
K.H.*4	13	M	AR	511,	Mites

*1 Allergic Rhinitis; *2 Systemic Urticaria; *3 Food Anaphylaxis;

*4 PBMC from K.H. did not produce IFN-γ on stimulation with IL-18+IL-2

(As shown in Fig.2), increased IL-13 production after stimulation with IL-18+IL-2 was observed in BA patients (gm 743 pg/ml, 95% ci 575-959 pg/ml) compared with in non-allergic controls (gm 452 pg/ml, 366-558 pg/ml). The levels of IL-13 production in AD patients (gm 593 pg/ml, 95% ci 461-762 pg/ml) were comparable to those in BA patients and non-allergic controls. No significant differences in IL-4 production by PHA-stimulated PBMCs in 24 hr were observed among the BA patients(gm 35 pg/ml, 95% ci 25 -50 pg/ml), AD patients (gm 44 pg/ml, 95% ci 31-64 pg/ml), and non-allergic controls (gm 51 pg/ml,95% ci 32-80 pg/ml).

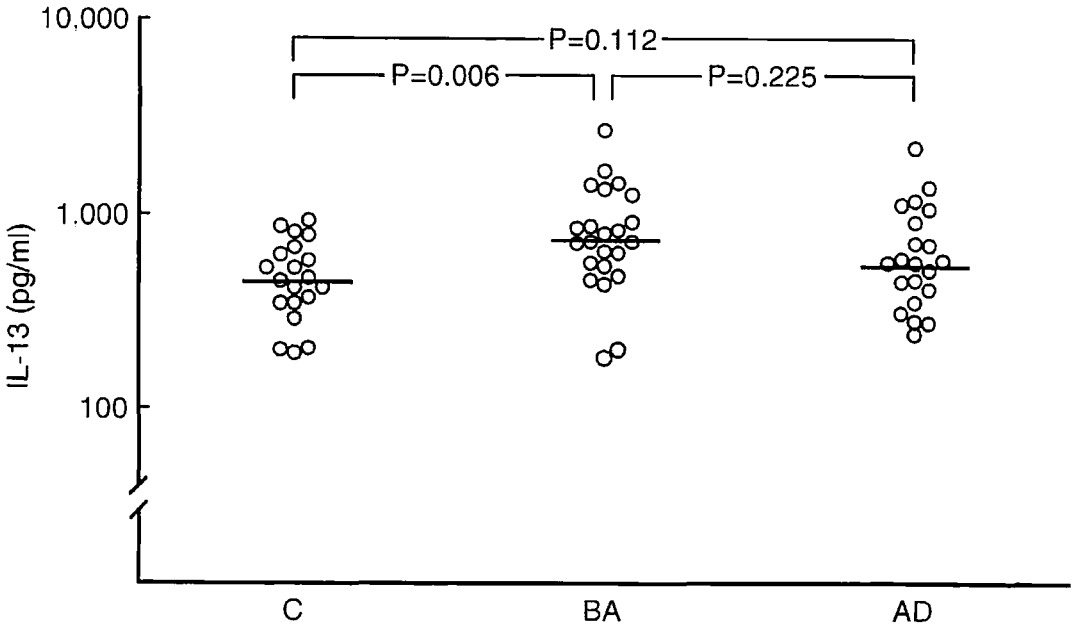


Fig.2. Production of IL-13 by IL-18+IL-2 stimulated PBMCs cultured at 5 days, measured by enzymatic immunoassay. It is significantly increased in BA patients than other two groups. C, non-allergic controls; BA, bronchial-asthma; AD, atopic dermatitis.

In addition, the amounts of IL-10 produced by PHA-stimulated PBMCs in 24 hr were comparable among the BA patients (gm 232 pg/ml,95%ci 195-275 pg/ml), AD patients (gm 274 pg/ml 95% ci 195-385 pg/ml) and non-allergic controls (gm 191 pg/ml 95% ci 109-332 pg/ml). The plasma IgE concentrations was chosen as the dependent variable and then regressed against the independent variables of the cytokines levels in stimulated PBMC cultures for multiple regression analysis. The results did not show any significant correlations between the plasma IgE concentration and the cytokine levels in each group, respectively.

Furthermore, no correlationship were observed statistically on single regression analysis between one of the cytokine level and the other cytokine levels in stimulated PBMC cultures from each group, respectively.

1.2.4. Discussion

Although IL-18 has been described as potent inducer of IFN-γ production, the present data show that IL-18 itself did not induce IFN-γ, but acted in synergy with either IL-12 or IL-2 to enhance IFN-γ production.

To date, IL-12 has been shown to augment expression of IL-18 receptors on Th1 cells, NK cells and B cells, which produce IFN-γ(1,2). IFN-γ induction was found in all cultures from non-allergic controls with IL-18+IL-12, however, the results for 5 of 23 (22%) BA patients and 5 of the 21 (24%) AD patients were under the detection limit for IFN-γ in PBMC cultures. Since PBMCs from all cases including allergic patients produced IFN-γ following stimulation with IL-18+IL-2, with the exception of one AD patient, it was suggested that impairment of IL-12 signal cascade was involved in around a quarter of the allergic patients. Recently, various allergic conditions have been reported to be associated with altered IL-12 receptors gene expression. Matsui *et al.* (11) demonstrated that IFN-γ production in response to IL-12 stimulation of PBMCs was reduced in 24 of 75 allergic patients, and 10 of these 24 allergic patients were heterozygous for truncated or missense mutations of the IL-12 receptors β 2 chain gene. In another study (12), both IL-12 production and IL-12 receptors β 2 chain gene expression in tissue biopsy specimens from patients with allergic rhinitis were found to be reduced compared with those in control patients. However, flow cytometric analysis (data not shown) of PBMC stimulated with IL-18+IL-12 showed that there are no differences in IL-12 β 1 receptor expression between the allergic patients who produced high and those who produced non-detectable IFN-γ.

In our first study (mentioned above), equivalent expression of IFN-γ mRNA in PHA-stimulated PBMCs was observed for allergic patients compared with non-allergic controls, and PHA stimulation elicited detectable IFN-γ production by PBMCs from all allergic patients (5). Therefore, other regulatory cytokines seem to reverse the impairment of IL-

12 signalling in IFN-γ production in allergic subjects. Supporting this possibility, the present study showed that IL-18+IL-2 stimulation caused the production of a lot of IFN-γ in PBMCs by almost all cases including allergic patients. IL-18 has been thought to be an important cofactor involved in Th1 cytokines production and Th1 cell development, however, it is clearly shown in this study that IL-18 is capable of inducing Th2 cytokine IL-13 production in synergy with IL-2. Recently, Hoshino *et al.* (6) described that treatment with IL-2 *in vivo* up-regulated IL-18 receptors expression on Th2 cells as well as Th1 cells, and observed the induction of IL-4, IL-10 and IL-13 production as well as IFN-γ production in mice upon stimulation with IL-18+IL-2. IFN-γ and IL-13 are not generally found to be co-expressed on human T-cells in an intracellular cytokine staining assay, and IFN-γ does not affect IL-13 production in stimulated human T-cell cultures (13).it has also , demonstrated a positive correlation between IFN-γ production and IL-13 as cytokines with long-lasting kinetics on secretion from T cells (13). Therefore, it is tempting to speculate that the synergistic induction of both IFN-γ and IL-13 production upon IL-18+IL-2 stimulation plays a role in an ongoing immune response.

Alternatively, significantly more IL-13 was produced by PBMCs after stimulation with IL-18+IL-2 from BA patients than those from non-allergic controls and AD patients. Recent animal model data suggest that IL-13 is a central cytokine in the promotion of asthma, through the stimulation of bronchial epithelial mucus secretion and smooth muscle hyperreactivity (14-16). Accumulated evidence has demonstrated exaggerated IL-13 production in the lungs of human asthmatic subjects (17, 18). Interestingly, elevation of IL-13 mRNA and protein levels appears to be more associated with asthma than with allergic conditions, because the levels are increased in the lungs of allergic and non-allergic asthmatic patients but not in those of allergic non-asthmatic patients, in comparison with in non-allergic non-asthmatic subjects (18). IL-18 has been reported to be constitutevly expressed in normal human lungs (19), and the concentration of IL-2 has been reported to be higher in the bronchoalveolar lavage of symptomatic

patients with BA than in that of normal subjects (20). Therefore, it seems likely that the IL-18+IL-2 mechanism is involved in the pathogenesis of BA in the lungs.

Chapter II

Cytokine response in patients with chronic fatigue syndrome (CFS)

From: Rabab I. El-Mezayen, A. Tomoda, T. Matsumoto, T. Jhodoi and T.Miike. Cytokines production and modulation after low dose gamma globulin therapy in patients with chronic fatigue syndrome (2003). (submitted), *psychiatry research.*

2.1. Abstract
2.2. Introduction
2.3. Materials and Methods
2.4. Results
2.5. Discussion

2.1. Abstract

Objectives: To investigate the cytokine production pattern and the effectiveness of intravenous (IV). Gamma globulin treatment in patients with chronic fatigue syndrome (CFS). In five CFS patients, we examined the cytokines production as well as the patients performance status score (PS) before and after IV γ-globulin therapy.

Methods: 23 healthy controls and 15 patients with CFS, all met the diagnostic criteria of the center for disease control (CDC), were included in the study. PBMCs were cultured with LPS or PHA. The concentration of cytokines was measured in the culture supernatants by the enzymatic immunoassay.

Results: In patients with CFS there was a significant decrease in TGF-β1 production in LPS-stimulated PBMC culture supernatants. It was decreased at the level of mRNA as well. Significant improvement of performance status score (PS) was observed in four of five patients received low dose IV γ-globulin treatment for 4 consecutive months.

Conclusion: cytokines dysregulation might be one of the factors involved in the pathogenesis of CFS. TGF-β1 might be involved in the inflammatory characteristics of CFS, it may help in the evaluation of the effectiveness of the treatment. Low dose IV γ-globulin therapy suggested to be likely of clinical benefits in the treatment of CFS.

2.2. Introduction

Chronic fatigue syndrome (CFS) is a condition of unknown aetiology, characterized by unexplained disabling fatigue, often accompanied by low-grade fever and subtle indication of immune system activation these syptoms are often exacerbated by physical activity (1). In addition, unrefreshing sleep, headach, impaired concentration and memory, as well as, depressed mood and anxiety are common the syndrome predominantly affects young adults in the peak of their productive years, the illness is associated with significant disruption to school and social life that may extend for several years resulting in significant economic and social cost to the community (2).

Although the pathogenesis of CFS is still a subject of intensive study, some studies suggest that a persistent viral infection is of etiologic importance as patients with CFS frequently report that their condition was preceded by an acute infective illness (eg., sinusitis, influenza-like illness, gastroenteritits, or glandular-fever-like illness) (6,7). A present report of a variety of immunologic abnormalities in a high proportion of patients suggests that an immunoregulatory defect may be involved (8,9). In addition, it was summarized (27) that magnetic resonance imaging has revealed punctate areas of high signal in the white matter. Single photon emission computed tomography (SPECT) signal abnormalities also are found more often in patients with chronic fatigue syndrome, abnormalities like those seen in patients with encephalopathy due to the acquired immunodeficiency syndrome (AIDS) and unlike the findings in patients with depression (28).

Furthermore, autonomic nervous system testing has revealed abnormalities of the sympathetic and parasympathetic systems that are not explained by depression or physical deconditioning (29). Studies of hypothalamic and pituitary function have also revealed neuroendocrine abnormalities not seen in healthy control subjects, and generally opposite to those found in major depression. There is often a central down-regulation

of the hypothalamic-pituitary-adrenal axis, resulting in a mild hypocortisolism (30), as well as disruption of both serotonergic and noradrenergic pathways (31, 32).

Cytokines have been suggested to play a role in the pathogenesis and clinical manifestations of CFS via their effects on CNS (10, 11). In addition, one striking feature of CFS is its sudden onset following an acute, presumably viral illness and persistence of recurrent (Flu-Like) symptoms, such symptoms have attributed to persistent production of cytokines (12, 13, 14). In addition, there is considerable evidence from different investigators, using different technologies band studying different groups of patients, of a state of chronic immune activation in many patients with chronic fatigue syndrome.

Most investigators have found increased numbers of CD8+ cytotoxic T cells with antigenic markers of activation (33) and depressed function of natural killer cells (34). The relation of these immunological findings to the symptoms reported by patients is unclear (35). One hypothesis is that a state of chronic immune activation could lead to the production of cytokines that disrupt neurotransmitter function and result in the symptoms of the chronic fatigue syndrome. To date, there is no clearly effective therapy for this disorder (3), although conflicting data from placebo-controlled studies suggest intravenous immunoglobulin may provide benefit (4, 5).

The group of patients included in this study received low dose IV. gamma globulin therapy in the form of biweekly dose 1gm. The purpose of this study is to determine the cytokines production pattern and investigate the effectiveness of IV. gamma globulin treatment and its relation to immuneactivity depending on the evaluation of cytokines production before and after receiving this treatment for consecutive four months.

Here, we present the cytokines production in culture supernatants and in the plasma of patients with CFS before and after IV γ-globulin treatment for more clarification and better understanding of cytokines dysregulation which might be one of important factors involved in the pathogenesis of

this syndrome and might help in the evaluation of the effectiveness of this treatment.

2.3. Materials and Methods

3.1. Subjects

This study comprised of 15 patients with CFS (8 males &7 F), aged 20.3± 9.47 years and 23 normal controls (14 males&9 F), aged 23±2.27 years. All patients referred to our hospital because of CFS symptoms. These patients met the diagnostic criteria of CFS of the Center For Disease Control (CDC). The period of CFS symptoms was longer than 12 months (Average, 1.3 years; range, 1 to 3 years). Their performance status scores (PS) on admission were more than 7 (average, 8.3 months; range,8 to 9).

3.2. Cell culture

PBMCs were isolated by density gradient centrifugation , washed three times, and then adjusted to 1×10^6 cells/ml with RPMI 1640 medium containing 10% heat–inactivated fetal calf serum (FCS), (FCS-free medium for TGF-β1 study), and 2 mM L-glutamine. Cells were then cultured with 5μ/ml phytohemaglutinin (PHA) or 50 ng/ml lipopolysaccharide (LPS) and incubated three days at 37℃ under 5 % CO_2 .

3.3. Cytokines measurement

(IL-1β, IL-6, TNFα): The concentrations of these cytokines in the culture supernatants of LPS-stimulated PBMCs were measured by the two steps (sandwich) enzyme immunoassay from Immunotech, Marseille, France. Samples and standars were incubated in a microtitre plate wells, coated with the first monoclonal antibody. The wells were emptied, washed and a second monoclonal antibody linked to acetylcholinestrase was added. After incubation the wells were emptied, washed and the bound enzymatic activity was measured by adding a chromogenic substrate. The intensity of

the color is proportional to the concentration the cytokines. The sensitivity of this ELISA assay is 3 pg/ml for IL-6, 5 pg/ml for TNF α, and 15 pg/ml for IL-1 β.

(TGF-β1): concentration in cell culture supernatants of LPS- stimulated PBMCs was assayed using the quantitative sandwich enzyme immunoassay technique from R&D systems, Minneapolis. TGF β1 soluble receptor type II, which binds TGF β1, has been pre-coated onto a microplate. standards and samples were piptted into the wells and any TGF β1 present is bound by the immoblized receptor. The minimum detectable dose of TGF-β1 is less than 7 pg/ml.

(IFN-γ): from PHA-stimulated PBMC culture supernatants, This assay employs the (ELISA) from R & D Systems, Minneapolis, MN. The sensitivity of this assay is 8 pg/ml.

(IL-4 and IL-18): IL-4 concentration in the culture supernatant of PHA stimulated PBMCs was measured by an ELISA from The Biosource International, Inc. The sensitivity of this assay is < 2.0 pg/ml. IL-18 concentration in the culture supernatants of LPS- stimulated PBMCs was measured by ELISA kit obtained from Medical & Biological Laboratories, Nagoya, Japan. The minimum sensitivity for this assay is 12.5 pg/ml.

3.4. TGF-β1 mRNA expression

For RNA extraction, 5×10^6 PBMCs were cultured with LPS for 3 hours, and then mRNA was isolated using Rneasy Mini Kit from (QIAGEN), then samples were hybridized with gene-specific biotin-labelled capture oligonucleotide probes and digoxigenin-labelled detection probes on streptavidin-coated microplate (Colorimetric mRNA Quantitation Kit ; R& D Systems, Minneapolis, MN) according to the maniufacturers' instructions. The standard curve was linear from 6.25 to 400 attomole/ml (amol/ml) of TGF β1 mRNA.

.3.5. Statistical Analysis

The data reported were logarithmically transformed before statistical analysis and were expressed as gm and 1sd range. The Mann Whitney *U* - test was used to compare the study groups for significant differences. Probability (*P*) values of less than 0.05 were considered statistically significant.

2.4. Results

Cytokine response and TGF- β 1 mRNA:

Before intravenous (IV) - γ globulin treatment;

TGF- β 1 protein production in the culture supernatants of LPS-stimulated PBMCs from CFS subjects (gm 4,642 &1sd range 3,033-7,102) was significantly decreased when compared with the normal controls (6,330&4,449-9,006) P=0.018 (see Fig.1).TGF-beta1 mRNA production in LPS stimulated PBMCs was significantly decreased in CFS patients (35.8 &1,9-6,6) when compared with the normal controls (92.54 & 6,55-13,1) and $P<0.001$ (Fig. 2.). However, no statistical significant differences could be detected when IL-10, IL-18, IL-4, TNF α, IFN γ, IL-6 were measured. Results are shown in table 1.

After intravenous (IV) – γ globulin treatment;

In four of five patients with CFS who received the treatment, TGF- β 1 protein production and mRNA were found to be progressively increased with receiving low dose - γ globulin treatment 1 gm/biweekly for 4 months. Importantly, the study of the patients performance status scores (PS), demonstrated that four of five patients who received the treatment showed an improvement in their performance status score (PS). (Fig. 3, A&3 B)

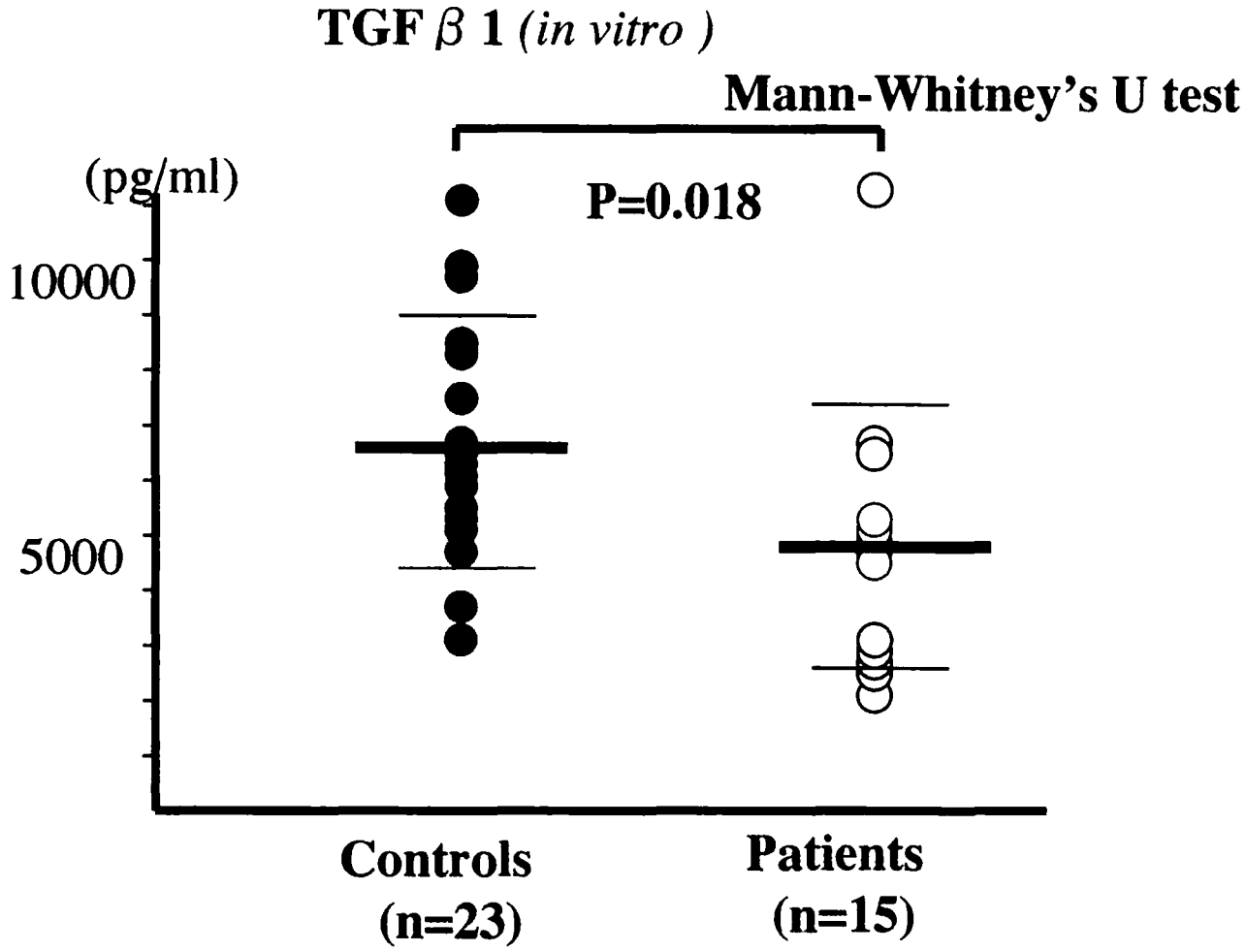


Fig.1. TGF β 1protein production in LPS-stimulated PBMC cultures is significantly deceased than normal controls.

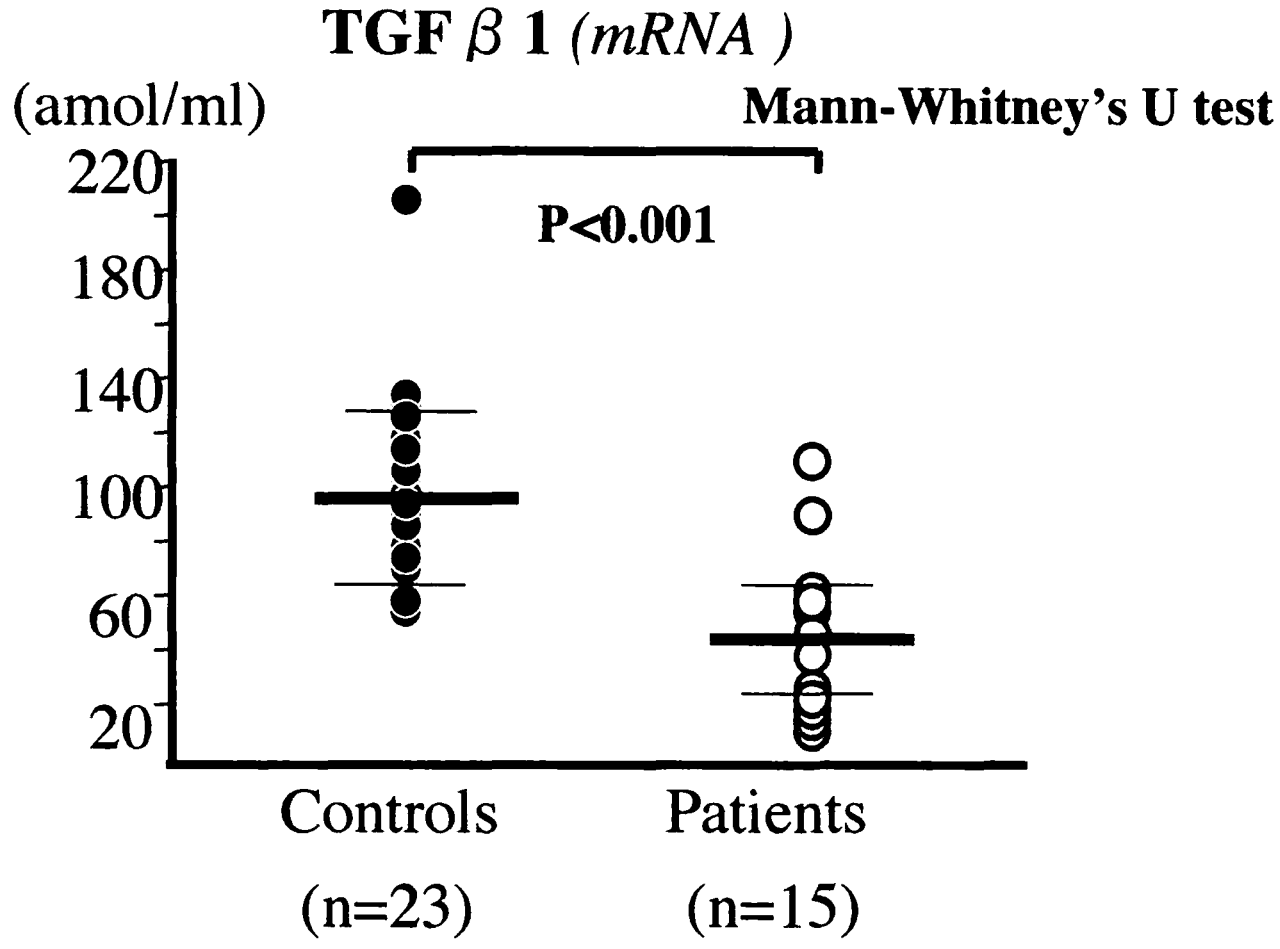
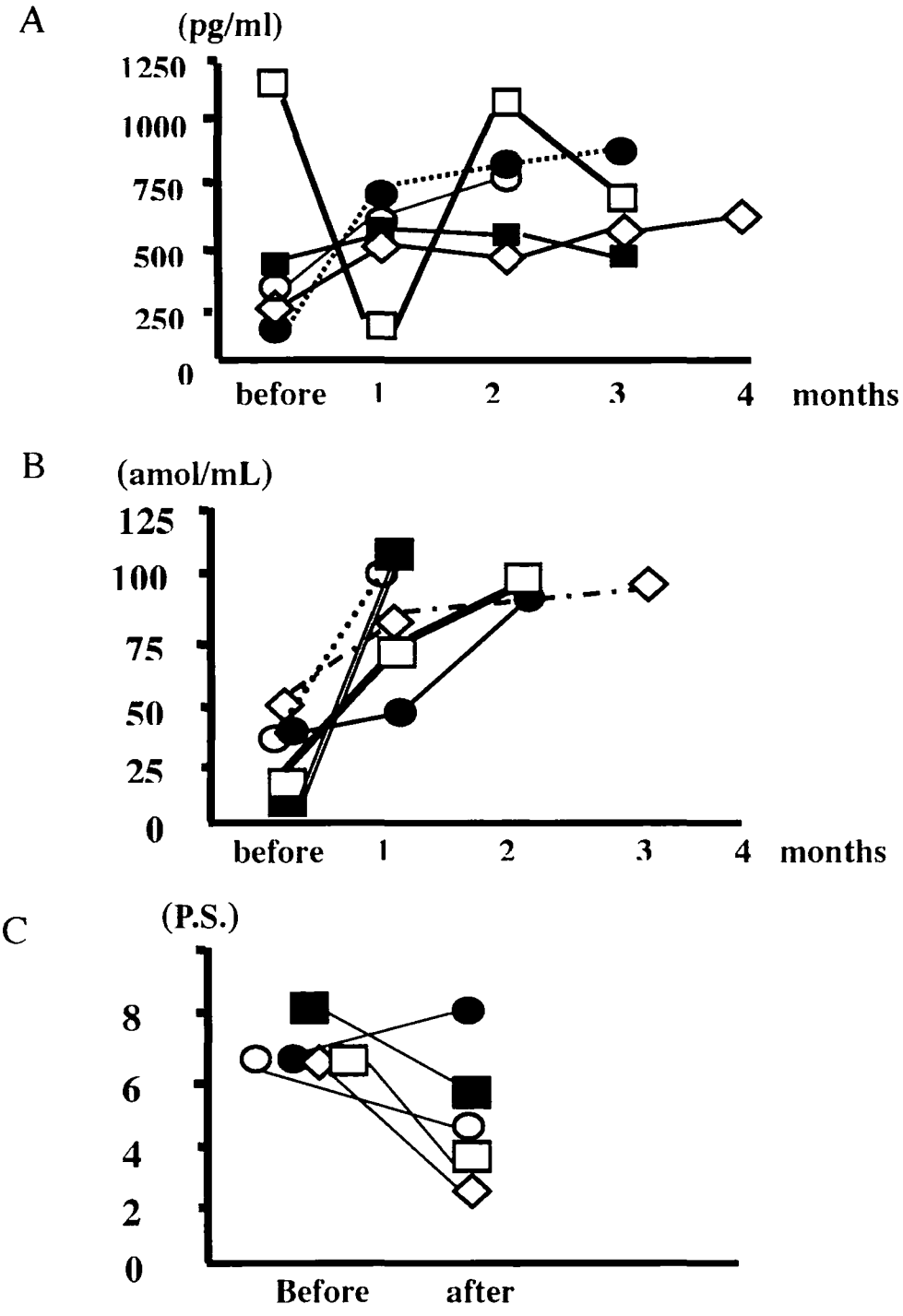


Fig.2. TGF- β 1mRNA is significantly decreased in CFS patients compared to normal controls .

Table 1. Cytokines levels in culture supernatants and plasma before intravenous (IV) γ-globulin therapy.

Cytokine (pg/ml)	Control (n= 23)	Patient (n= 14)	P
TGF-beta1(In vitro)	6, 330* (4, 449-9, 006)	4, 642 (3, 033-7, 102)	=0.018
TGF-beta1 mRNA (amol/ml)	92, 54 (6, 55-13, 1)	35, 8 (1, 9-6,6)	<0.001
TGF-beta1 (plasma)	5, 2 (0. 96-3. 46)	4, 9 (0. 26-1.1)	
IL-6 (in vitro)	11, 7 (3, 9-4, 2)	13, 5 (3, 8-4, 4)	
IL-6 (plasma)	1, 3 (0. 12-0. 35)	1, 2 (0. 16-0. 38)	
TNF-α (in vitro)	5, 48 (2, 3-3, 2)	8, 11 (2, 6-3, 2)	
IL-1-β (in vitro)	4, 16 (2, 3-2, 9)	3, 42 (2, 2-2, 8)	
IL-10 (in vitro)	44, 6 (3, 1-3, 8)	29,6 (3, 4-3, 9)	
IL-18 (in vitro)	60, 7 (2, 5-2, 6)	58, 5 (1, 6-1, 9)	
IFN-γ (in vitro)	21, 3 (1, 6-2, 9)	33, 4 (1, 7-2, 5)	
IL-4 (in vitro)	8, 2 (0.43-1.4)	16, 1 (0.45-1.9)	

*gm (Geometric means and 1sd range)



dFig.3. Effects of gamma globulin therapy on TGF beta1protein production in culture supernatant of LPS stimulated PBMC (A), TGF beta 1 mRNA expression (B), and change of Performance status scores (P.S.) (C). ●, ◇, ■, ○, and □ are Case 1, 2, 3, 4 and 5 respectively.

2.5. Discussion

The present study demonstrated the cytokines production pattern in patients with chronic fatigue syndrome (CFS) as well as, comparative evaluation of cytokines production before and after receiving low dose of intravenous (IV) γ-globulin treatment for 4 consecuitive months in five patients who completed the protocol. Abnormal regulation of cytokines activity may contribute to the pathophysiology and clinical manifestations of CFS (10,11).

The present study suggests that intravenous gamma globulin (low dose intravenous (IV) γ-globulin) therapy is likely to be of clinical benefit in treatment of patients with chronic fatigue syndrome. Given the finding of improvement of the status performance score in 4 of 5 CFS patients who showed improvement in their abilities to participate in daily activities and their degree of involvement in work or school, sports and different social activities when compared to their conditions before receiving the IV gamma-globulin treatment. These data concur with a previous report of diminished fatigue after intramuscular gamma globulin in patients with chronic mononucleosis syndrome (15).

Silverman and coworker presented in a pilot study the effects of IV gamma globulin in systemic juvenile rheumatoid arthritis (JRA), eight patients with active systemic JRA that was unresponsive to first-line agents, second-line agents, and/or corticosteroids received this therapy monthly for 6 months, the outcome measures included changes in articular and extraarticular features, steroid dosage, and laboratory parameters. Following IV gamma globulin therapy, there was significant improvement in arthritis and/or morning stiffness in 5 of 8 patients, while extra-articular features significantly improved in 7 of 8 patients (25).

The important aspect of our study is the observation that TGF-β1 might be considered an indicator for the improvement of CFS patients conditions, supportive to this, is the progressive increase in its production during

receiving the treatment together with the improvement of those patients performance status score. Therefore, TGF-β1 as a multifunctional anti-inflammatory cytokine might be involved in the immune function and the deficiency of this cytokine reported in CFS patients in the present study may contribute to the inflammation characteristics of CFS syndrome such as myalgia and muscular fatigue. This finding support the antiinflammatory and immunosupressive functions of TGFβ1. Our results are supported by a previous published report in which a longitudinal study of the first qPCR evaluation of TGFβ1 mRNA every 2 weeks in conjunction with monthly MRI, indicated that TGFβ1 mRNA level inversely correlates with MRI disease activity in multiple sclerosis (MS) (26).

TGFβ1 also been reported to have important roles in unresolved inflammation, immune suppression, fibrosing processes, angiogenesis and was also highly expressed in joints in RA and was considered to be a regulator of anti-inflammation in rheumatoid arthritis (24).

The improvement of the patients performance status score together with the progressive increase of TGF-β1 productivity reported here might be a good sign for the effectiveness of this trial as a treatment of patients with CFS.

In the current study we also investigated the *in vitro* production of anti-inflammatory cytokine IL-10 which has been reported to inhibit the production of IFN-γ and IL-4 by Th lymphocytes, IL-1β, IL-6 and TNF-α by mononuclearphagocytes (16,17,18,19). In addition, we also examined cytokines IL-4, IFN-γ, IL-1β, TNF-α and IL-18. However, these cytokines were found to be of comparable values between CFS patients and normal controls. This is consistent with the finding by Larry Borish which explained the increased production of proinflammatory cytokines IL-1β and TNF-α and decreased anti-inflammatory cytokine IL-10 concentration in CFS patients with allergy to be due to the presence of allergy, and that prolonged or excessive production of cytokines secondary to allergen exposure or other immune insults has been proposed as being responsible for the development of CFS (25). In addition, while Starus et al (21) found

no difference in the levels of IL-1 β in CFS patients, Visser and colleagues reported that, although CD4T cells from CFS patients produce less IFN- γ than cells from controls, IL-4 production and, cell proliferation are comparable. In other studies no difference in the production of IL-6 in CFS patients was observed (22,23).

Regarding these previous studies and our results, further detailed studies about cytokines are still needed to clarify the roles of each of these cytokines in the pathogenesis of CFS.

CONCLUSIONS

This study demonstrated that the secretion of IL-18 by peripheral blood mononuclear cells (PBMCs) was increased in patients with bronchial asthma (BA) and atopic dermatitis (AD). The induction of IFN-γ by IL-18+IL-12 was impaired in around a quarter of allergic patients, however, this impairment was completely restored by IL-18 in combination with IL-2. IL-13 production induced by IL-18+IL-2 was increased in BA patients. These results suggest the involvement of proinflammatory cytokine IL-18 in the pathogenesis of allergic disorders.

The cytokine study of patients with chronic fatigue syndrome (CFS) demonstrated that the production of anti-inflammatory cytokine TGF-β1 by PBMCs was decreased. And that receiving low dose γ globulin treatment increases the production of TGF-β1 and improves the patients' performance status scores. We suggest the involvement of TGF-β1 in the pathogenesis of CFS.

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