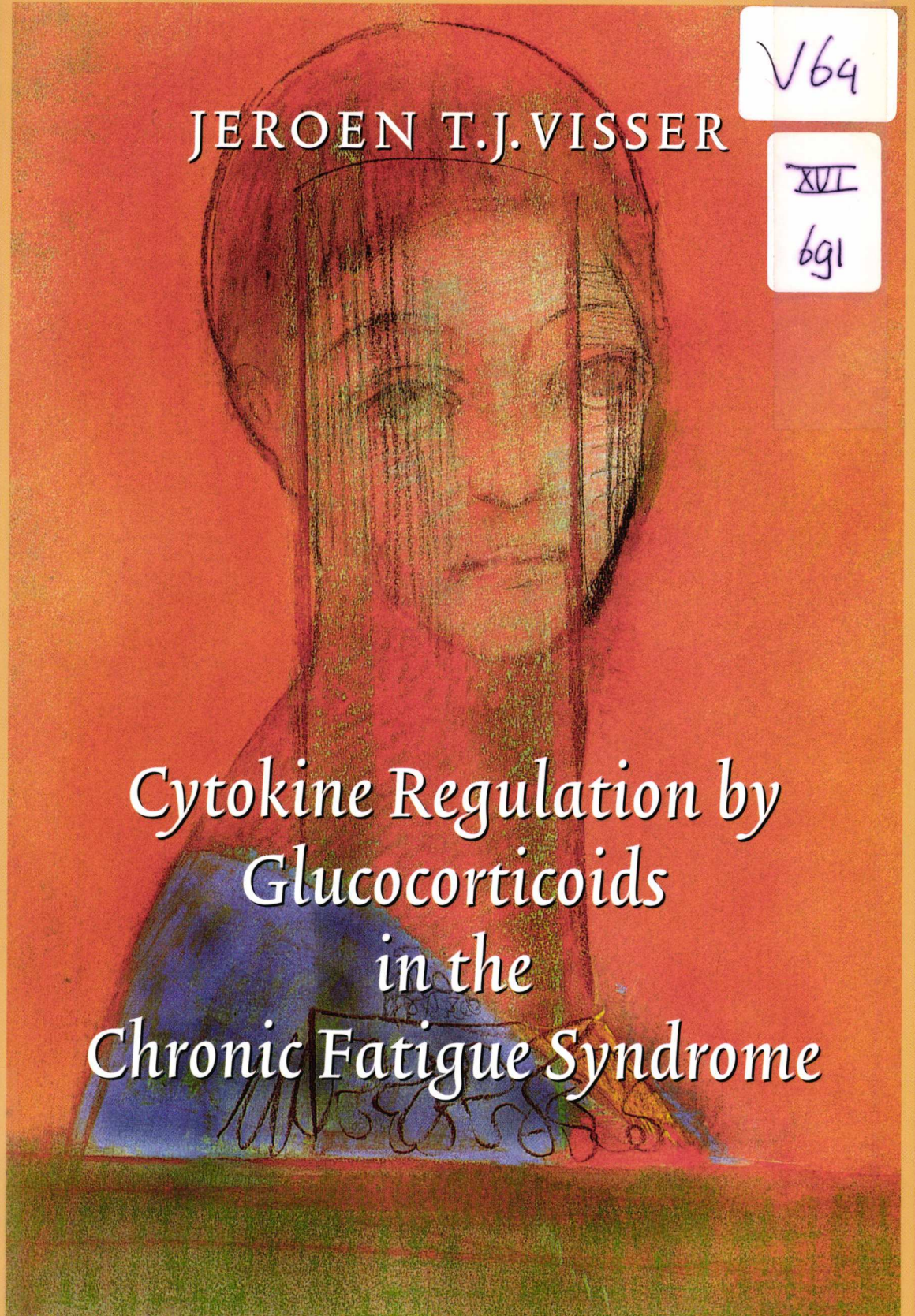
JEROEN T.J. VISSER



Cytokine Regulation by Glucocorticoids in the Chronic Fatigue Syndrome

Cytokine Regulation by Glucocorticoids in the Chronic Fatigue Syndrome

Some say that Knowledge is something that you will never have
Some say that knowledge is something sat in your lap
But I must admit, just when I think I am King
I just begin

Kate Bush
The Dreaming; 1981.

Voor mijn ouders en
Natalie

STELLINGEN

behorende bij het proefschrift:

Cytokine regulation by Glucocorticoids in the Chronic Fatigue Syndrome

1 Het stimulerende effect van glucocorticoïden op de Th2 respons kan voor een deel verklaard worden door stimulatie van IL-10 en suppressie van IL-12 productie. *(Dit proefschrift)*

2 IL-10 en glucocorticoïden versterken elkaar in de remming van IL-12. *(Dit proefschrift)*

3 Bestudering van de responsiviteit op glucocorticoïden biedt aanknopingspunten voor de ontwikkeling van een meer objectieve diagnostische test voor het Chronisch Vermoeidheid Syndroom.
(Dit proefschrift)

4 Voor de mate van de respons op glucocorticoïden, is naast affiniteit en dichtheid van glucocorticoïd receptoren, de interactie met co-activatoren en transcriptie factoren een bepalende factor.
(Dit proefschrift, Bamberger et al. Endocrine reviews 17 (3): 245-61, 1996)

5 IL-10 bevordert het optreden van insulitis, maar remt in de effector fase de ontwikkeling van diabetes. *(Mueller et al. J Autoimmun 9: 151-159, 1996, Lee et al. J Exp Med 183: 2663-2668, 1996)*

6 Kortdurende therapie met een hoge dosis glucocorticoïden heeft meer voor- dan nadelen. *(Prof. Dr. Paul Nieuwenhuis, pers. com.)*

7 Het karakter van een immuunrespons is afhankelijk van de biologische beschikbaarheid van corticosteroïden.
(Prof. Dr. Graham Rook, pers. com.)

8 Gezien het feit dat de nestgrootte van zebravinken bepalend is voor de mate van hun immuunrespons, kunnen kinderen beschouwd worden als hinderen voor de immuunrespons van de ouders. *(Bos N, Proc R Soc Lond 264:1021-1029, 1997)*

9 Door olympisch kampioen schanssspringen te worden in 1984 in de klassieke stijl en in 1994 in de moderne Boklev stijl is Jens Weissflog de beste schansspringer aller tijden.

10 De dood van generaal Custer tijdens de slag bij Little Big Horn in 1876 voorkwam dat hij president werd van de Verenigde Staten, maar bezorgde de Sioux leiders Sitting Bull en Crazy Horse wereldfaam.

Jeroen Visser, 20 januari 2000.

Cytokine Regulation by Glucocorticoids in the Chronic Fatigue Syndrome

PROEFSCHRIFT

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus Dr. W.A. Wagenaar,
hoogleraar in de faculteit der Sociale Wetenschappen,
volgens besluit van het College voor Promoties
te verdedigen op donderdag 20 januari 2000
te klokke 14.15 uur

door

Jeroen Theodorus Jacobus Visser
geboren te Wognum
in 1967

PROMOTIECOMMISSIE

promotor

Prof. Dr. E.R. de Kloet

co-promotor

Dr. L. Nagelkerken, TNO Preventie en Gezondheid

referent

Prof. Dr. J.W.M. van der Meer, Katholieke Universiteit Nijmegen

overige leden

Prof. Dr. M.L. Kapsenberg, Universiteit van Amsterdam

Prof. Dr. C. Heijnen, Universiteit Utrecht

Prof. Dr. D. D. Breimer

Prof. Dr. R.R.P. de Vries

Dit onderzoek werd uitgevoerd bij TNO Preventie en Gezondheid, afdeling Immunologische en Infectieziekten te Leiden in samenwerking met de divisie Medische Farmacologie, Leiden Amsterdam Center of Drug Research te Leiden.

Het onderzoek beschreven in dit proefschrift werd mogelijk gemaakt door een subsidie van Zorg Onderzoek Nederland, Grant nr: 002829080.

Contents

Abbreviations

CFS	Chronic Fatigue Syndrome
GC	glucocorticoid
DEX	dexamethasone
HC	hydrocortisone
ACTH	adrenocorticotrophin
CRH	corticotrophin releasing hormone
HPA	hypathalamic pituitary adrenal
GR	glucocorticoid receptor
MR	mineralocorticoid receptor
Bmax	maximum binding capacity
Kd	dissociation constant
LPS	lipopolysaccharide
PBMC	peripheral blood mononuclear cells
APC	antigen presenting cell
Th	T helper
CD	cluster of designation
IL	interleukin
IFN	interferon
TNF	tumor necrosis factor
PCR	polymerase chain reaction
PMA	Phorbol 12-myristate 13-acetate
PHA	Phytoheamaglutanin
PKC	protein kinase C
AP-1	Activator protein one
NFkB	Nuclear factor kappa B
IkB	Inhibitor kappa B

Preface

The Chronic Fatigue Syndrome (CFS) is a disease of unknown origin characterized by severe disabling fatigue lasting more than six months, which results in a reduction of daily activity of more than 50%. Several reports have demonstrated alterations in the immune system and disturbances in the HPA-axis of CFS-patients.

In view of the importance of HPA-axis integrity in regulating immune responses, the hypothesis was tested that disturbances found in the immune response of CFS-patients can be a result of an altered responsiveness to glucocorticoids. For this purpose, it was investigated how lymphocytes from CFS-patients respond to glucocorticoids.

The findings presented in this thesis demonstrate that lymphocytes from CFS-patients display an altered cytokine profile and have an increased sensitivity to glucocorticoids. This increased sensitivity could not be attributed to an increased affinity or density of glucocorticoid receptors. Moreover, in this thesis it is demonstrated that the Th2 inducing potential of glucocorticoids involves the upregulation of IL-10 and the downregulation of IL-12.

The difference in cytokine production between patients and controls was affected by the heterogeneity of the patient population and the lymphocyte subset under investigation. However, the increased sensitivity for glucocorticoids appeared a robust phenomenon, which was much less affected by these factors.

Therefore, studying glucocorticoid responsiveness in CFS-patients is recommended to be a more promising approach for the development of diagnostic tools and therapy.

CHAPTER ONE

General Introduction

The Chronic Fatigue Syndrome

The chronic fatigue syndrome (CFS) is a disease of unknown origin characterized by severe disabling fatigue with a duration of more than six months and a reduction in normal activity of at least 50% [1, 2, 3]. The syndrome started to attract world wide attention in the middle of the nineteen eighties and selection criteria were developed to provide consensus for diagnostic guidelines to select patients for scientific research. After several years of debate and the development of different sets of criteria [1, 2], Fukuda et al proposed a definitive set of criteria in 1994 [3], which are nowadays used in scientific research for the inclusion of CFS-patients. However, these criteria are not suitable for diagnostic purposes.

Several causes have been held responsible for the onset and maintenance of the syndrome, including viral infections, a disturbed hypothalamus-pituitary-adrenal-gland (HPA) axis and an altered immune function [4, 5]. Infectious agents can cause fatigue complaints known as the postviral fatigue syndrome [6] and several groups in the past have suggested an infectious etiology for the disease [5, 6]. However, although some CFS-patients show elevated titers to the early antigen of the Epstein Barr virus and show seropositivity for Coxsackie B virus, subsequent studies demonstrated that these findings cannot be generalized for all CFS-patients [5-8]. The hypothesis that infectious agents are involved was further supported by observations that immune responses are altered in CFS-patients as compared to healthy controls. Many reports describe differences in immune parameters like altered composition and activation of lymphocyte subsets in CFS-patients, decreased natural killer (NK) cell function and alterations in cytokine production [9-21], which will be discussed in more detail below. The idea that CFS might be caused by an infectious trigger or other event, which subsequently leads to a chronic activation and imbalance of the immune system, became a major hypothesis in the etiology of CFS [5-7]. This hypothesis was in part based on the fact that clinical treatment with recombinant cytokines such as IL-1, IL-2 and

interferons give rise to symptoms like mild fever and severe fatigue complaints which are much alike those observed in CFS-patients [21-26].

It is well known that there is crosstalk between the neuroendocrine system and the immune system. For example cytokines like IL-1, TNF-α and interferons are potent activators of the HPA-axis [26-30]. Also hormones such as glucocorticoids and neuropeptides like vasoactive intestinal protein (VIP) and substance P have been shown to possess immuno-modulatory capacities [29-32]. So besides the immune system also the neuroendocrine system should be taken into account in the etiology of CFS.

Infection and Immunity in the Chronic Fatigue Syndrome

The role of bacterial and viral infections in CFS

As mentioned above infectious agents can cause fatigue syndromes [6]. Another indication for the involvement of infectious agents in CFS is the suddenness of its onset in the majority of the patients. Especially much attention has been given to viruses and bacteria that are known for their persistence like Epstein Barr virus, several types of retroviruses, entero-viruses and Borrelia [5-7, 33].

Although several reports show seropositivity of individual CFS-patients for infectious agents like Epstein Barr virus, Cocksackie B virus and Bornea virus [6, 34-36], the exposure of the patients to these infectious agents is not increased as compared to the controls and no strong positive correlation could be made with the infectious agent [8, 36-38]. Another piece of evidence against the involvement of infectious agents in the etiology of CFS is the lack of clear symptoms of inflammation or tissue damage. Furthermore, treatment of patients with anti-viral drugs gives some improvement of the symptoms but no complete recovery [39-41].

Nevertheless, it is likely, that a subset of CFS-patients originates from people originally infected with an infectious agent, diagnosed as having post viral fatigue syndrome and after several years of consistent fatigue complaints are diagnosed as having CFS. In order to identify such a subset of CFS-patients, longitudinal studies should be performed.

Although until now no infectious agent can be correlated with the etiology of CFS, recurrent viral or bacterial infections might be responsible for maintenance of the fatigue complaints. There is some evidence that the state of immune activation in CFS-patients enables the reactivation of several otherwise latent viruses, such as human herpes virus 6 (HHV-6) or EBV, whose replication can then contribute to or sustain symptoms of the

syndrome [34-36]. Observations supporting this possibility are alterations in the anti-viral 2',5'-oligoadenylate synthetase / Rnase L pathway in PBMC of CFS patients [42, 43].

Immunological abnormalities in CFS

As mentioned above the idea that CFS might be caused by an infectious trigger or other event, which subsequently leads to a chronic activation and dysbalance of the immune system, became a major hypothesis in the etiology of CFS. This hypothesis is supported by the fact that treatment with recombinant cytokines such as IL-1, IL-2 and interferons gives rise to CFS-like symptoms such as mild fever, concentration disturbances and fatigue [22-30].

At first it was investigated whether alterations in the immune status of CFS-patients could be detected that could be caused by an infectious event. Furthermore, the functionality and activation state of several lymphocyte subsets important for the defense against viral infections was studied. The aim of this research was the detection of a defect in the immune system responsible for recurrent infections. Furthermore, an imbalance in the cytokine profile or other immunological parameters might be useful for the development of an objective diagnostic marker for the syndrome and possible therapy.

Several authors have demonstrated alterations in immunological parameters in CFS-patients. These abnormalities include a decreased NK cell activity [10, 11], a reduced mitogenic response of lymphocytes [12-14] and alterations in cytokine production [12-21]. Cytokines like IL-1, IL-6 and TNF-α which are strongly augmented by viral or bacterial infections were shown to be normal or increased in CFS-patients [15-18]. The cytokine IFN-γ important for an optimal induction of cellular immunity, which is important to limit the magnitude of viral infections has been shown to be normal or decreased in CFS-patients [13-15, 17]. The observed differences in cytokine production in CFS-patients may in part be related to an altered composition of the peripheral blood mononuclear cells (PBMC) of the studied patients. In several reports CFS-patients showed increased numbers of

activated cytotoxic T cells as evidenced by the increased expression of the activation markers CD38 and HLA-DR. Also the activation marker CD5 was demonstrated to be increased on B cells. Furthermore several reports demonstrated increased numbers of memory T cells and reduced numbers of naive T cells [12-21].

The observed immunological disturbances in CFS-patients suggest a slight reduction of the cellular immunity as evident from a reduced functionality of NK cells [10, 11]. Furthermore, a reduced IFN-gamma production [13-15], one report by Lloyd et al [9] regarding a reduced delayed type hypersensitivity (DTH) response, an increased incidence of allergic reactions and reactivation of viral infections [44, 45] supports this hypothesis.

The observed differences in the immunological parameters are not consistent and show a lot of diversity. An explanation for this inconsistency could be variations in the group of patients under investigation. A study with a well-defined patient group and a well-matched control group showed no differences in the composition of the PBMC, although slight differences in cytokine production were seen [17]. When the patients were subgrouped according to disease onset or on the basis of how well they were feeling on the day of testing, more pronounced differences were seen [17].

Taken together differences in immune parameters in CFS-patients demonstrated so far are subtle and this appears to be dependent on the parameter under investigation, the heterogeneity of the patient population and the quality of the control group.

Neuroendocrinology in CFS

Endrocrinological studies performed by Demitrack et al., Dinan et al. and Scot et al. [46-48], demonstrated an impaired functioning of the HPA-axis in CFS-patients. CFS-patients show significantly reduced levels of urinary free cortisol and reduced production of ACTH upon stimulation with CRH. Furthermore a blunted response in cortisol production to ACTH has been demonstrated [46-48]. These alterations are quite different as from those found in depression, which are rather characterized by hyperfunctioning of the HPA-axis resulting in high levels of cortisol [49, 50]. Accordingly, most of the people suffering from depression show reduced dexamethasone suppression, probably because of a reduced functioning of the glucocorticoid receptors (GR) [50-51].

Treatment of CFS-patients with fluoxetine – a potent antidepressant – has not much effect on the symptoms of depression and fatigue in CFS [52, 53]. These findings suggest that the mechanisms underlying the presentation of depressive symptoms in CFS-patients are different from those in patients with a major depressive disorder.

Post-traumatic stress disorder (PTSD) patients and fibromyalgia patients, syndromes with which CFS is often compared, show some resemblance in HPA-axis activity with CFS-patients. Like CFS-patients, PTSD and fibromyalgia patients show low 24-h urinary cortisol levels [54 - 56]. Furthermore, PTSD patients show enhanced cortisol suppression in response to a dexamethasone challenge and have increased GR levels in PBMC [56].

Not much is known about the functioning of the autonomic nervous system in CFS. Experiments performed by De Becker et al [57] suggest a modest sympatic hyperactivity in CFS-patients after exposure to stress.

Interaction between the immune system and the neuroendocrine system

Altered functionality of the HPA-axis is associated with immune dysfunction and autoimmunity

A major system involved in the crosstalk between the immune system and the neuro-endocrine system is the HPA-axis. As explained above, the immune system can influence the functionality of the HPA-axis by the production of cytokines [26-30]. Furthermore the hormones belonging to the HPA-axis have potent immunomodulatory properties. Although ACTH and CRH have been shown to activate macrophages and can recruit monocytes to the site of inflammation [58, 59], the most potent immunomodulatory hormones are the glucocorticoids (GC) [60-62]. It has been shown that the integrity of the HPA-axis plays an important role in the development of experimental autoimmune diseases like allergic encephalomyelitis (EAE) or arthritis in certain rat strains [63-66]. For instance, Lewis rats, which are susceptible for EAE show an impaired production of GC upon stressful events [63, 65]. On the other hand, the relatively resistant PVG rat becomes sensitive to EAE induction after adrenalectomy and this sensitivity could be reversed by corticosterone replacement therapy [64]. Accordingly the capacity of animals to increase GC levels is an important factor contributing to their sensitivity for streptococcal cell wall induced arthritis [66]. Interestingly, IFN-β therapy in Lewis rats with EAE induces high levels of corticosterone, which may play an additional role in the resistance to the induction of EAE [67].

The hypothesis that the integrity of the HPA-axis is important for the sensitivity to autoimmune diseases is further supported by observations in human autoimmune diseases. Patients suffering from rheumatoid arthritis display decreased levels of GC, as a result of an impaired functioning of the HPA-axis [68]. Furthermore, an altered functionality of the HPA-axis was reported in multiple sclerosis patients [69].

Glucocorticoids are beneficial for the Th2 response

GC are potent immunomodulatory hormones, which are widely applied because of their immunosuppressive characteristics. GC can influence the functionality of the cells of the immune system, by inducing apoptosis, inhibition of proliferation, interfering with cytokine production and specific modulation of the capacity of antigen presenting cells to activate T cells [60-62].

The balance between Th1 and Th2 cells -two important subsets of T helper cells- is important for the character of the immune response to pathogens (Figure 1). Th1 cells, that produce high levels of IFN-γ, promote cellular immunity via the activation of cytotoxic functions of several effector cells such as NK cells, macrophages and cytotoxic T cells. Th2 cells on the other hand produce high levels of IL-4, IL-5 and IL-13 and are supportive for humoral immunity, by providing help for B cells in the production of antibodies [70-72].

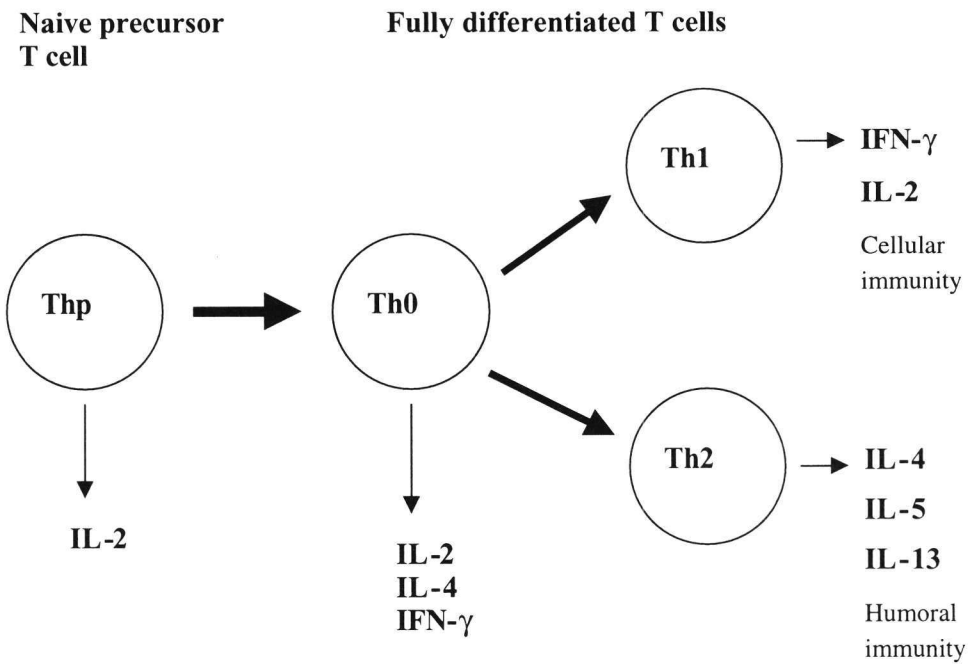


Figure 1. Differentiation of Naive precursor T cells towards fully matured Th1 or Th2 cells.

The differentiation of naive T cells into either Th1 or Th2 cells is regulated by several environmental factors. Dominant factors regulating this differentiation are the nature of the antigen, the type of antigen presenting cells and soluble factors including cytokines and hormones in the microenvironment of the differentiating T cell [70-77]. The development of naive T cells into either Th1 or Th2 cells is dependent on a variety of cytokines. The presence of IL-4 during a developing immune response has been shown to favor Th2 responses [73]. On the other hand IL-12 has been demonstrated to be a crucial factor in the development of Th1 responses [74-77]. Therefore, the type of antigen presenting cell (APC) and its capacity to produce IL-12, may be one of the major determinants in the differentiation of naive T cells towards Th1 or Th2 cells.

It has been demonstrated that human monocytes may be heterogeneous, evidenced by the fact that $CD14^+/CD16^+$ cells do not express mRNA for IL-10 in response to LPS as compared to $CD14^+/CD16^-$ cells [78]. This observation is of importance in view of the fact that IL-10 suppresses IL-12 [79]. The development of Th1 and Th2 cells may also depend on the activation state of APC, e.g. their ability to secrete prostaglandin E_2 which was found to suppress IL-12 production and inhibit Th1 cells [80, 81]. In as much as costimulatory molecules B7-1, B7-2 and CD40 are involved in the regulation of Th1 and Th2 responses [82], also the expression of these molecules on APC is a major determinant in the regulation of T cell responses.

In 1994 it was hypothesized by Rook et al [61] as is shown in Figure 2, that GC selectively suppress Th1 responses and stimulate Th2 responses. Several observations have substantiated this hypothesis and indicate that GC may use several mechanisms for this Th2 skewing effect.

First of all GC have selective effects on cytokines. Especially the proinflammatory cytokines and Th1 inducing cytokines are strongly suppressed by GC, while the anti-inflammatory and Th2 inducing cytokines are much less affected.

In the mouse this was demonstrated by showing that dexamethasone suppressed IL-2 and IFN-γ, but not IL-4, products of Th1 and Th2 cells respectively [83]. Using rat $CD4^+$ T cells, it was found that GC favor Th2 development [84]. Also in humans GC have selective effects on $CD4^+$ T cell subsets [85] which appears to depend also on the activation pathway [86]. Addition of GC during restimulation of primed human naive $CD4^+$ T cells, stimulates IL-4 and IL-10 production and suppresses IL-5 and IFN-γ production [86].

Accordingly, GC suppressed the synthesis of antigen specific IgE production *in vitro*, but enhanced the synthesis of total IgE and IgG4 [87-90].

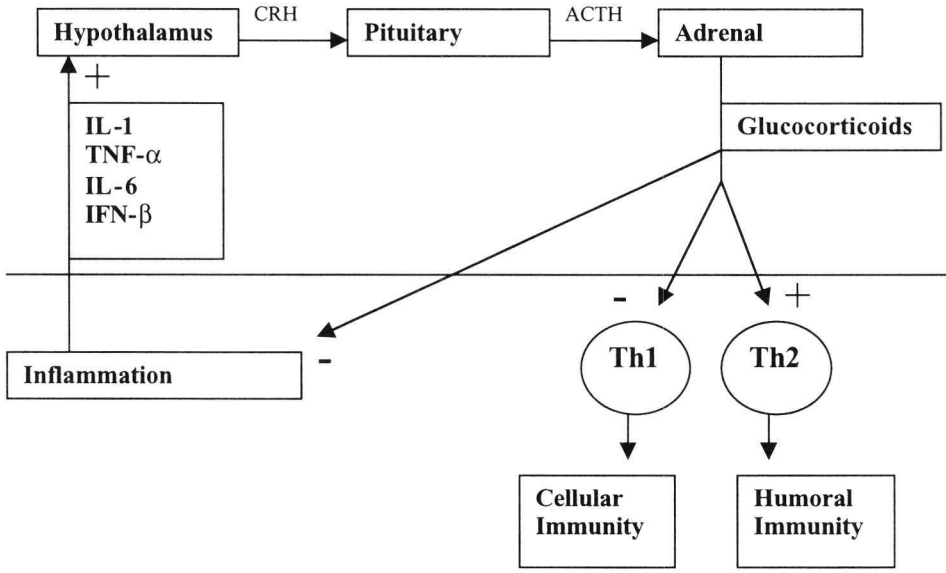


Figure 2. Effects of glucocorticoids on Th1 and Th2 responses.

Cytokines produced upon an inflammatory reaction are capable in activating the HPA-axis leading to the production of GC by the adrenal gland. These GC suppress the production of these proinflammatory cytokines and are favorable for Th2 responses. Figure adapted from Rook et al Imm Today 1994; 15 (7): 301-3.

GC were demonstrated to be detrimental for the expression of costimulatory molecules on APC like macrophages and dendtritic cells [91, 92]. This downregulation of costimulatory molecules by GC might render the APC to become tolerogenic, because loss of this costimulatory signal causes anergy in T cells.

The effect of GC on APC however appears to be dependent on their activation state. It was demonstrated by several authors, that GC are not that much affecting the antigen presentation potential of fully activated monocytes or fully matured dendritic cells, but rather induce their Th2 skewing potential, most likely via the downregulation of IL-12 [93-95].

Molecular mechanisms in cytokine modulation by glucocorticoids

GC exert their immunomodulatory properties via selective effects on transcription factors involved in the regulation of cytokine genes. Another mechanism is the binding of the activated glucocorticoid receptors (GR) to specific glucocorticoid responsive elements (GRE) in the promoter regions of regulatory genes which in turn lead to activation or silencing of the gene [96]. It has been demonstrated by several authors that the activated GR complex is inhibitory for the activity of Activator Protein 1 (AP-1) via protein-protein interaction with c-jun or c-fos, which are the two subunits of AP-1 [97-99]. Other important transcription factors for the induction of immune responses, i.e. NFkB and CREB, are also downregulated by GC via protein-protein interaction [99]. Importantly, GC were also demonstrated to upregulate IkB, a protein that maintains NFkB in its inactive state in the cytosol [100, 101], by an as yet unidentified mechanism, since a GRE has not been demonstrated in the promoter region of the IkB gene. Thus GC exploit several mechanisms in controlling immune responses (Figure 3).

These mechanisms provide several possibilities for specific regulation of immune responses by GC. For example, cytokines like TNF-α and IL-2 are very sensitive for suppression by GC, because they contain responsive elements for AP-1, CREB and NFkB in their promotor sequence [98, 102]. The cytokine IL-6 which is less sensitive for suppression by GC [102], contains besides responsive elements for AP-1, a GRE and binding sites for transcription factors that are less sensitive for GC suppression [103].

GC are only capable in exerting their effects via binding to the GR. Alterations in the functionality of these receptors will interfere with the responsiveness of cells to GC. Changes in the affinity and density of the GR have been shown to interfere with the responses to GC *in vitro* and *in vivo* [96, 104-106]. On the other hand an altered sensitivity has also been observed while the density and affinity of the receptors are apparently normal [106, 107].

Such observations may be due to an impaired interaction between transcription factors and the occupied GR.

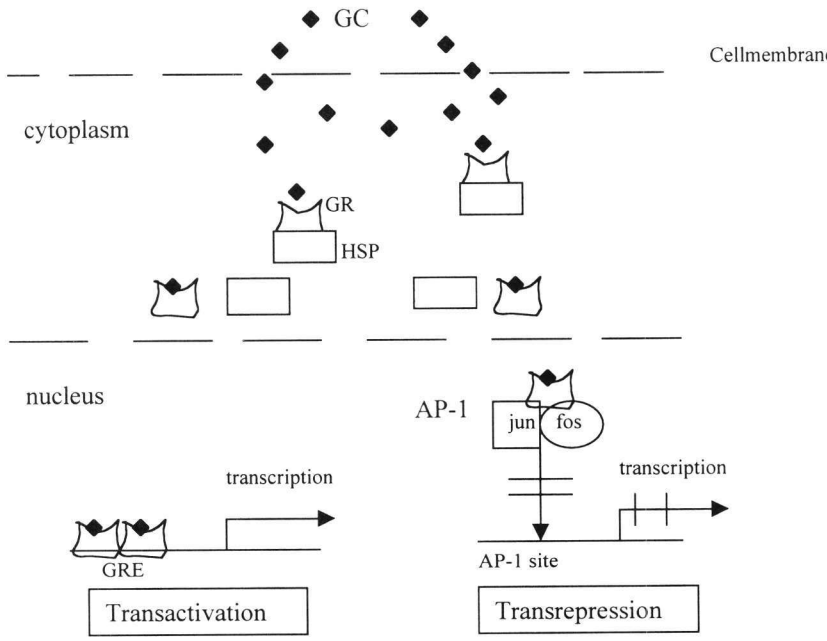


Figure 3. Regulation of gene transcription by glucocorticoids.

GC passively diffuse through the membrane and bind to their receptors in the cytosol. After formation of a GR-GC complex, this complex can act as a transcription factor by binding to a GRE in a promoter region of genes and can enhance or silence transcription, a mechanism known as transactivation. On the other hand can the activated GR bind to transcription factors via protein-protein interaction preventing the binding of this transcription factor to its responsive elements. This phenomenon is termed transrepression.

Taken together responsiveness for GC is dependent on the affinity and density of the GR, their interaction with transcription factors and the presence of GRE in promoter regions of regulatory genes.

Scope of the thesis

Although it is well established that various hormones related to the HPA-axis have immunomodulatory properties, which are not exclusively immunosuppressive in nature, it is unknown to what extent the immunological aberrations in CFS are due to an altered activity of the HPA-axis. In this thesis I will focus the attention on glucocorticoids (GC), steroid hormones that are known for their potent immunomodulatory capacities. In particular the modulation of cytokines by GC has been investigated in CFS-patients in a comparison with healthy controls, in order to establish whether the alterations found in the immune response of CFS-patients could be attributed to an altered sensitivity to GC.

In **chapter 2** it was investigated if purified T helper cells of CFS patients as compared to healthy controls matched for age and sex, display an altered functionality. Studying these cells instead of unseparated peripheral blood mononuclear cells (PBMC) provides a more accurate approach to the activity of Th1 cells which regulate cellular immunity by the secretion of cytokines like IFN-γ, as compared to Th2 cells which regulate humoral immunity by cytokines like IL-4. As was described above a dysfunctioning of the HPA-axis resulting in constitutively low levels of cortisol has been observed in CFS-patients. Because the balance between Th1 and Th2 cells, is suggested to be regulated by GC, it was studied to what extent immunological alterations in CFS were accompanied by an altered sensitivity of $CD4^+$ T cells to GC. Several factors are important for the regulation of the differentiation of naive CD4 positive cells into Th1 or Th2 cells like the type of antigen, the type of antigen presenting cell and soluble factors like cytokines and hormones in the microenvironment of the differentiating T cell. Two important cytokines in this differentiation process have been shown to be IL-10 and IL-12. Especially IL-12 is essential for the generation of Th1 responses because of its inducing effect on IFN-γ. IL-10 has been shown to be detrimental for Th1 responses, by suppression of IL-12.

In **chapter 3** it was investigated whether the Th2 skewing effects of GC may be mediated via selective effects on IL-10 versus IL-12. The relative sensitivity of these cytokines for GC was studied in whole blood cultures and the specificity of these effects was explored by using antagonists for the glucocorticoid receptor (GR).

In **chapter 4** the production of IL-10 and IL-12 was investigated in whole blood cultures of CFS-patients to investigate whether an altered balance between these two cytokines is explanatory for the observed alterations in the immune response in CFS-patients. Accordingly the regulation of these cytokines by GC was studied. Since the heterogeneity of the patient population can influence the results, we subgrouped the CFS-patients by gender and duration of the disease, as being the most objective criteria. A slightly impaired HPA-axis in CFS-patients might be the result of an altered GR function. Accordingly, a chronic state of hypocortisolism in CFS might influence the affinity and distribution of the GR in cells of CFS-patients in order to maintain homeostasis.

In **chapter 5** the affinity and distribution of GR in PBMC of CFS-patients was studied. Simultaneously the PBMC were cultured to investigate their response to GC *in vitro*, with regard to proliferation and cytokine production. This approach makes it possible to study whether an altered response to GC in CFS-patients is related to disturbances at the level of the GR.

In **chapter 6** the hypothesis that immune dysfunction characteristic for CFS, is related to an altered sensitivity to GC is discussed in the context of the findings as presented in the chapters 2 to 5. In addition the molecular mechanisms involved in cytokine modulation by GC are discussed in order to explain immune modulation by GC in CFS-patients.

In conclusion, the relevance of cytokine profiles as found in CFS-patients to be explanatory for the etiology of CFS and the usefulness of the studied immunological parameters for the development of objective diagnostic criteria and therapy, will be discussed.

References

1. Holmes GP, Kaplan JE, Gantz NM, et al. Chronic fatigue syndrome: a working case definition. Ann Intern Med 1988; 108: 387-9.

2. Sharpe M, Archard L. Banatvala J et al. Chronic Fatigue Syndrome: guidelines for research. J Roy Soc Med 1991; 84: 118-21.

3. Fukuda K, Strauss SE, Hickie I, et al. The chronic fatigue syndrome: a comprehensive approach to its definition and study. Ann Intern Med 1994; 121: 953-9.

4. Bearn J, Wessely S. Neurobiological aspects of the chronic fatigue syndrome. Eur J Clin Invest 1994; 24: 79-90.

5. Strauss SE, Komaroff AL, Wedner HJ. Chronic Fatigue syndrome: Point and counterpoint. J Infect Dis 1994; 170: 1-6.

6. Komaroff AL. Chronic Fatigue Syndromes: relationship to chronic viral infections. J Virol Methods 1988; (1-4): 3-10.

7. Mawle AC, Reyes M, Scmid DS. Is chronic Fatigue Syndrome an infectious disease? Infect Agents Dis 1994; 2: 333-41.

8. Swanink CMA, Vercoulen JHMM, Bleijenberg G, Fennis JFM, Galema JMD, van der Meer. Chronic fatigue syndrome: a clinical and laboratory study with a well matched control group. J Intern Med 1995; 237: 499-506.

9. Lloyd A, Hickie I, Hickie C, Dwyer J, Wakefield D. Cell mediated immunity in patients with chronic fatigue syndrome, healthy control subjects and patients with major depression. Clin Exp Immunol 1992; 87: 76-9.

10. Aoki T, Miyakoshi H, Usuda Y, Herberman RB. Low NK syndrome and its relationship to chronic fatigue syndrome. Clin Immunol Pathology 1993; 69 (no 3): 253-65.

11. Caliguiri M, Murray C, Buchwald D, et al. Phenotypic and functional deficiency of natural killer cells in patients with chronic fatigue syndrome. J Immunol 1987; 139: 3306-13.

12. Barker E, Fujimura SF, Fadem MB, Landay AL, Levy JA, Immunologic abnormalities associated with chronic fatigue syndrome. Clin Infect Dis 1994; 18 (suppl): s157-9.

13. Strauss SE, , Fritz S, Dale JK, Gould B, Strober W. Lymfocyte phenotype and function in the chronic fatigue syndrome. J Clin Immunol 1993; 13: 30-40.

14. Klimas NG, Salvato FR, Morgan R, Fletcher MA. Immunologic abnormalities in chronic fatigue syndrome. J Clin Microbiol 1990; 28: 1404-10.

15. Chao CC, Janoff EN, Hu S, et al. Altered cytokine release in peripheral blood mononuclear cell cultures from patients with the chronic fatigue syndrome. Cytokine 1991; 3: 292-8.

16. Swanink CMA, Vercoulen JHMM, Galama JMD, et al. Lymphocyte subsets, apoptosis, and cytokines in patients with chronic fatigue syndrome. J Infect Dis 1996; 173: 460-3.

17. Mawle AC, Nisenbaum R, Dobbins JG, et al. Immune responses associated with chronic fatigue syndrome: A case-control study. J Infect Dis 1997; 175: 136-41.

18. Hassan IS, Bannister BA, Akbar A, Weir W, Bofill M. A study of the immunology of the chronic fatigue syndrome: correlation of immunologic parameters to health dysfunction. Clin Immunol Immunopathol 1998; 87 (1): 60-7.

19. Tirelli U, Marotta G, Improta S, Pinto A. Immunological abnormalities in patients with chronic fatigue syndrome. Scand J Immunol 1994; 40: 601-08.

20. Landay AL, Jessop C, Lennette ET, Levy JA. Chronic fatigue syndrome: clinical condition associated with immune activation. Lancet 1991; 338: 707-12.

21. Borish L, Schmaling K, DiClementi JD, Streib J, Negri J, Jones JF. Chronic fatigue syndrome: Identification of distinct subgroups on the basis of allergy and psychologic variables. J Allergy Clin Immunol 1998; 102: 222-230.

22. Curti BD, Smith JW. Interleukin-1 in the treatment of cancer. Pharmacol Ther 1995; 65: 291-302.

23. Dillman RO. The clinical experience with interleukin-2 in cancer therapy. Cancer Biother 1994; 3: 183-209.

24. Wadler S. The role of interferons in the treatment of solid tumors. Cancer 1992; 15: 949-58.

25. Nolten WE, Goldstein D, Lindstrom M, McKenna MV, Carlson IH, Trump DL, Schiller J, Borden EC, Ehrlich EN. Effects of cytokines on the pituitary-adrenal axis in cancer patients. J Interferon Res 1993; 13 (5): 349-57.

26. Ohno Y, Fujimoto M, Nishimura A, Aoki N. Change of peripheral levels of pituitary hormones and cytokines after injection of interferon (IFN)-beta in patients with chronic hepatitis C. J Clin Endocrinol Metab 1998; 83 (10): 3681-7.

27. Reder AT, Lowy MT. Interferon-beta treatment does not elevate cortisol in multiple sclerosis. J Interferon Res 1992; 12 (3): 195-8.

28. Van Dam AM, De Vries HE, Kuiper J, Zijlstra FJ, De Boer AG, Tilders FJ, Berkenbosch F. Interleukin-1 receptors on rat brain endothelial cells: a role in neuroimmune interaction? FASEB J 1996; 10 (2): 351-6.

29. Blalock JE. The syntax of immune-neuroendocrine communication. Immunol Today 1994; 15: (No 11): 504-11.

30. Wilder, RL Neuroendocrine-immune system interactions and autoimmunity. Annu Rev Immunol 1995; 13: 307-38.

31. Delgado M, Pozo D, Martinez C, Leceta J, Calvo JR, Ganea D, Gomariz RP. Vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide inhibit endotoxin-induced TNF-alpha production by macrophages: in vitro and in vivo studies. J Immunol 1999; 162: 2358-67.

32. Levite M. Neuropeptides, by direct interaction with T cells, induce cytokine secretion and break the commitment to a distinct T helper phenotype. Proc Natl Acad Sci USA 1998; 95 (21): 12544-9.

33. Komaroff AL. Buchwald DS. Chronic Fatigue syndrome: an update. Ann Rev Med 1998; 49: 1-13.

34. Josephs SF, Henry B, Balachandran N, Strayer D, Peterson D, Komaroff AL, Ablashi DV. HHV-6 reactivation in Chronic Fatigue Syndrome. Lancet 1991; 337 (8753): 1346-7.

35. Manian FA. Simultaneous measurement of antibodies to Epstein-Barr virus, human herpesvirus-6, herpes simplex virus types 1 and 2, and 14 enteroviruses in chronic fatigue syndrome: is there evidence of activation of a nonspecific polyclonal immune response? Clin Infect Dis 1994; 19 (3): 448-53.

36. Di Luca D, Zorzenon M, Mirandola P, Colle R, Botta GA, Cassai E. Human herpes virus 6 and human herpesvirus 7 in chronic fatigue syndrome. J Clin Microbiol 1995; 33 (6): 1660-61.

37. Viral serologies in patients with chronic fatigue and chronic fatigue syndrome. Buchwald D, Ashley RL, Pearlman T, Kith P, Komaroff AL. J Med Virol 1996; 50(1): 25-30.

38. Wallace HL 2nd, Natelson B, Gause W, Hay J. Human Herpes Viruses in chronic Fatigue syndrome. Clin Diagn Lab Immunol 1999; 6(2): 216-23.

39. Straus SE, Dale JK, Tobi M, et al. Acyclovir treatment of the chronic fatigue syndrome. Lack of efficacy in a placebo-controlled trial. N Engl J Med 1988; 319 (26): 1692-8.

40. Strayer DR. Carter WA, McCarron TJ. Durability of therapeutic benefit with Ampligen treatment of chronic fatigue syndrome as measured by the karnofsky performance score. Fourth International AACFS Research and Clinical Conference on Chronic Fatigue Syndrome. Cambridge, Massachusetts, USA, October 10-12, 1998.

41. Lapp CW, Voyles CS, Davis P, Langford S. An overview of Ampligen and experience in a small cohort. Fourth International AACFS Research and Clinical Conference on Chronic Fatigue Syndrome. Cambridge, Massachusetts, USA, October 10-12, 1998.

42. Suhadolnik RJ, Reichenbach NL, Hitzges P, et al. Upregulation of the 2-5A synthetase / Rnase L antiviral pathway associated with chronic fatigue syndrome. Clin Infect Dis 1994; 18: S96-S104.

43. Suhadolnik RJ, Peterson DL, O'Brien K, et al. Biochemical evidence for a novel low molecular weight 2-5A-dependent Rnase L in Chronic Fatigue Syndrome. J Interfer and Cyt Research 1997; 17: 377-85.

44. Hilgers A, Frank J. Chronic Fatigue syndrome: immune dysfunction, role of pathogens and toxic agents and neurological and cardial changes. Wien Med Wochenschr 1994; 144 (16): 399-406.

45. Straus SE, Dale JK, Whright R, Metcalfe DD. Allergy and the chronic fatigue syndrome. J allergy Clin Immunol 1988; 81 (5): 791-5.

46. Demitrack MA, Dale JK, Strauss SE, et al. Evidence for impaired activation of the hypothalamic-pituitery-adrenal axis in patients with chronic fatigue syndrome. J Clin Endocrinol Metab 1991; 73:1224-34.

47. Dinan TG, Majeed T, Lavelle E, Scott LV, Berti C, Behan P. Serotonin mediated activation of the hypothalamic-pituitary-adrenal-axis in chronic fatigue syndrome. Psychoneuroendocrinol 1997; 22: 261-68.

48. Scott LV, Medbak S, Dinan TG. The low dose ACTH test in chronic fatigue syndrome and health. Clin Endocrinol 1998; 48: 733-37.

49. Holsboer F, Von Bardeleben U, Gerken A, Stalla GK, Muller O. Blunted corticotropin and normal cortisol response to human corticotropin-releasing factor in depression. New England J Med 1984; 311: 1127.

50. Holsboer F, Barden N. Antidepressants and hypothalamic-pituitary adrenocortical regulation. Endocr Rev 1996; 17 (2): 187-205.

51. Modell S, Yassouridis A, Huber J, Holsboer F. Corticosteroid receptor function is decreased in depressed patients. J Neuroendocrinol 1997; 65 (3): 216-22.

52. Vercoulen JH, Swanink CM, Zitman FG, et al. Randomised, double-blind, placebo-controlled study of fluoxetine in chronic fatigue syndrome. Lancet 1996; 347: 858-61.

53. Wearden AJ, Moriss RK, Mullis R, et al. Randomised, double-blind, placebo-controlled treatment trial of fluoxetine and graded exercise for chronic fatigue syndrome. Br J Psychiatry 1998; 172: 485-90.

54. Yehuda R, Southwick SM, Nussbaum G, Giller EL, Mason JW. Low urinary cortisol excretion in patients with post-traumatic stress disorder. J Nerv Mental Dis 1990; 178: 366-69.

55. Griep EN, Boersma JW, de Kloet ER. Altered reactivity of the hypothalamic-pituitary-adrenal axis in the primary fibromyalgia syndrome. J Rheumatol 1993; 20: 469-74.

56. Yehuda R, Boisoneau D, Lowy MT, Giller EL. Dose-response changes in plasma cortisol and lymphocyte glucocorticoid receptors following dexamethasone administration in combat veterans with and without posttraumatic stress disorder. Arch Gen Psych 1995; 52: 583-93.

57. De Becker P, Dendale P, De Meirleir K, Campine I, Vandenborne K, Hagers Y. Autonomic testing in patients with chronic fatigue syndrome. Am J Med 1998; 105 (3A): 22S-26S.

58. Hughes TK, Smith EM. Corticothropin (ACTH) induction of tumor necrosis factor alpha by monocytes. J Biol Regul Homeost Agents 1989; 3: (4): 163-6.

59. Mastorakos G, Bouzas EA, Silver PB, et al. Immune corticotropine releasing hormone is present in the eyes of and promotes experimental autoimmune uveoretinitis in rodents. Endocrinology 1995; 136: 4650-58.

60. Marx J. How the glucocorticoids suppress immunity. Science 1995; 270: 232-3.

61. Rook GAW, Hernandez-Pando R, Lightman S. Hormones, peripherally activated prohormones and regulation of the Th1/Th2 balance. Immunol Today 1994; 15: 301-3.

62. Wilckens T, de Rijk R. Glucocorticoids and immune function: unknown dimensions and new frontiers. Immunol Today 1997; 18 (9): 418-24.

63. MacPhee I, Antoni F, Mason D: Spontaneous recovery of rats from experimental allergic encephalomyelitis is dependent on regulation of the immune system by endogenous adrenal corticosteroids. J Exp Med 1989; 169: 431.

64. Mason D: Genetic variation in the stress response: susceptibility to experimental allergic encephalomyelitis and implications for human inflammatory disease. Immunol Today 1991; 12: 57.

65. Mason D, MacPhee I, Antoni F: The role of the neuroendocrine system in determining genetic susceptibility to experimental allergic encephalomyelitis in the rat. Immunology 1990; 70: 1.

66. Sternberg EM, Young WS, Bernardini R, Calogero AE, Chrousos GP, Gold PW, Wilder RL. A central nervous system defect in biosynthesis of corticotropin-releasing hormone is associated with susceptibility to streptococcal cell wall-induced arthritis in Lewis rats. Proc Natl Acad Sci USA 1989; 86 (12): 4771-5.

67. Ruuls SR, de Labie MC, Weber KS, Botman CA, Groenestein RJ, Dijkstra CD, Olsson T, van der Meide PH. The length of treatment determines whether IFN-beta prevents or aggravates experimental autoimmune encephalomyelitis in Lewis rats. J Immunol 1996; 157 (12): 5721-31.

68. Chikanza IC, Petrou P, Kingsley G, Chrousos G, Panayi GS: Defective hypothalamic response to immune and inflammatory stimuli in patients with rheumatoid arthritis. Arthritis Rheum 1992; 35: 1281.

69. Michelson D, Stone L, Galliven E, Magiakou MA, Chrousos GP, Sternberg EM, Gold PW. Multiple sclerosis is associated with alterations in hypothalamic-pituitary-adrenal axis function. J Clin Endocrinol Metab 1994; 79: (3): 848-53.

70. Mosmann TR, Coffman RL: Th1 and Th2 cells: Different Patterns of lymphokine secretion lead to different functional properties. Annu Rev Immunol 1989; 7: 145.

71. Romagnani S: Lymphokine production by human T cells in disease states. Annu Rev Immunol 1994; 7: 145.

72. O'Garra A, Murphy K:. T-cell subsets in autoimmunity. Current Opin Immunol 1993; 5: 880.

73. Paul WE, Seder RA: Lymphocyte responses and cytokines. Cell 1994; 76: 241.

74. Kalinsky P, Hilkens CMU, Snijders A, Snijdewint FGM, Kapsenberg ML. IL-12 deficient dendritic cells, generated in the presence of prostaglandin E2, promote type 2 cytokine production in maturing naive T helper cells. J Immunol 1997; 159: 28-35.

75. Hsieh CS, Macatonia SE, Tripp CS, Wolf SF, O'Garra A, Murphy KM: Development of Th1 $CD4^+$ T cells through IL-12 produced by *Listeria*-induced macrophages. Science 1993; 260: 547.

76. Trinchieri G: Interleukin-12: A proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen specific adaptive immunity. Annu Rev Immunol 1995; 13: 251.

77. Hilkens CMU, Kalinsky P, de Boer M, Kapsenberg ML. Human dendritic cells require exogenous Interleukin-12 inducing factors to direct the development of naive T helper cells toward the Th1 phenotype. Blood 1997; 90: 1920-26.

78. Frankenberger M, Sternsdorf T, Pechumer H, Pforte A, Loms Ziegler-Heitbrock HW: Differential cytokine expression in human blood monocyte subpopulations. Blood 1996; 87: 373.

79. D'Andrea A, Aste-Amezaga M, Valiante NM, Ma X, Kubin M, Trinchieri G: Interleukin 10 inhibits human lymphocyte interferon-γ production by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells. J Exp Med 1993; 178: 1041.

80. Van der Pouw Kraan T, Boeije L, Smeenk R, Wijdenes J, Aarden L: Prostaglandin-E2 is a potent inhibitor of human interleukin-12 production. J Exp Med 1995; 181: 775.

81. Hilkens CM, Snijders A, Vermeulen H, van der Meide PH, Wierenga EA, Kapsenberg ML: Accessory cell derived IL-12 and prostaglandin-E2 determine the IFN-γ level of activated human $CD4^+$ T cells. J Immunol 1996; 156: 1722.

82. Croft M, Dubey C. Accessory molecules and costimulation requirements for $CD4^+$ T cell response. Crit Rev Immunol 1997; 17 (1): 89-118.

83. Daynes RA, Araneo BA. Contrasting effects of glucocorticoids on the capacity of T cells to produce growth factors Interleukin-2 and Interleukin-4. Eur J Immunol 1989; 19: 2319-25.

84. Ramirez F, Fowell DJ, Puklavec M, Simmonds S, Mason D: Glucocorticoids promote a Th2 cytokine response by $CD4^+$ T cells in vitro. J Immunol 1996; 156: 2406.

85. Snijdewint FGM, Kapsenberg ML, Wauben-Penris PJJ, Bos JD. Corticosteroids class-dependently inhibit Th1- and Th2-type cytokine production. ImmunoPharmacol 1995; 29: 93-101.

86. Brinkman V, Kristofic C. Regulation by corticosteroids of Th1 and Th2 cytokine production in human $CD4^+$ effector T cells generated from $CD45RO^-$ and $CD45RO^+$ subsets. J Immunol 1995; 155: 3322-28.

87. Bohle B, Willheim M, Baier K, Stadler B, Spitzauer S, Scheiner O, Ebner C: Hydrocortisone enhances total IgE levels-but not the synthesis of allergen specific IgE-in a monocyte-dependent manner. Clin Exp Immunol 1994; 101: 474.

88. Wu CY, Sarfati M, Heusser C, Fournier S, Rubio-Trujillo M, Peleman R, Delespesse G: Glucocorticoids increase the synthesis of immunoglobulin E by interleukin-4 stimulated human lymphocytes. J Clin Invest 1991; 87: 870.

89. Nüsslein HG, Weber G, Kalden JR: Synthetic glucocorticoids potentiate IgE synthesis. Allergy 1994; 49: 365.

90. Akdis CA, Blesken T, Akdis M, Alkan SS, Heusser CH, Blaser K. Glucocorticoids inhibit human antigen-specific and enhance total IgE and IgG4 production due to differential effects on T and B cells in vitro. Eur J Immunol 1997; 27: 2351-7.

91. Moser M, de Smedt T, Sornasse T, et al. Glucocorticoids down-regulate dendritic cell function in vitro and in vivo. Eur J Immunol 1995; 25: 2818-24.

92. Girndt M, Sester U, Kaul H, Hunger F, Kohler H. Glucocorticoids inhibit activation dependent expression of costimulatory molecule B7-1 in human monocytes. Transplantation 1998; 66(3): 370-5.

93. Blotta MH, de Kruyff RH, Umetsu DT: Corticosteroids inhibit IL-12 production in human monocytes and enhance their capacity to induce IL-4 synthesis in $CD4^+$ lymphocytes. J Immunol 1997; 158: 5589.

94. DeKruyff R, Fang HY, Umetsu D. Corticosteroids enhance the capacity of macrophages to induce Th2 cytokine synthesis in $CD4^+$ lymphocytes by inhibiting IL-12 production. J Immunol 1998; 160: 2231.

95. Vieira PL, Kalinski P, Wierenga EA, Kapsenberg ML, de Jong EC. Glucocorticoids inhibit bioactive IL-12p70 production by in vitro generated human dendritic cells without affecting their T cell stimulatory potential. J. Immunol. 1998; 161: 5245-51.

96. Bamberger CM, Schulte HM, Chrousos GP. Molecular determinants of glucocorticoid receptor function and tissue sensitivity to glucocorticoids. Endocrine Reviews 1996; 17 (3): 245-61.

97. Yang-Yen HF, Chambard JC, Sun YL, Smeal T, Schmidt TJ, Drouin J, Karin M. Transcriptional interference between c-Jun and the glucocorticoid receptor: mutual inhibition of DNA-binding due to direct protein-protein interaction. Cell 1990; 62: 1205-15.

98. Vacca A, Felli MP, Farina AR, et al. Glucocorticoid receptor-mediated suppression of the Interleukin-2 gene expression through impairment of the cooperativity between nuclear factor activated t cells and AP-1 enhancer elements. J Exp Med 1992; 175: 637-46.

99. Adcock IM, Brown CR, Gelder CM, Shirasaki H, Peters MJ, Barnes PJ. Effects of glucocorticoids on transcription factor activation in human peripheral blood mononuclear cells. Am J Physiol. 1995; 268 (2 Pt 1): C331-8.

100. Scheinman RI, Cogswell PC, Lofquist AK, Baldwin AS jr. Role of transcriptional activation of IkBα in mediation of immunosuppression by glucocorticoids. Science 1995; 270: 283-86.

101. Auphan N, Di Donato JA, Rosette C, Helmberg A, Karin M. Immunosuppression by glucocorticoids: Inhibition of NF-kB activity through induction of IkB synthesis. Science 1995; 270: 286.

102. Yao J, Mackman N, Edgington TS, Fan ST. Lipopolysacharide induction of the Tumor Necrosis Factor-α promoter in human monocytic cells. J Biol Chem 1997; 272 (28): 17795-801.

103. Ray A, LaForge KS, Sehgal PB. On the mechanism for efficient repression of the interleukin-6 promoter by glucocorticoids: enhancer, TATA box, and RNA start site (Inr motif) occlusion. Moll Cell Biol 1990; 11: 5736-46.

104. Kam JC, Szefler SJ, Surs W, Sher ER, Leung DY. Combination of IL-2 and IL-4 reduces glucocorticoid receptor-binding affinity and T cells response to glucocorticoids. J Immunol 1993; 151: 3460.

105. Sher ER, Leung DYM, Surs W, Kam JC, Zieg G, Kamada AK, Szefler SJ. Steroid-resistant Asthma. Cellular mechanisms contributing to inadequate response to glucocorticoid therapy. J Clin Invest 1994; 93: 33-9.

106. Barnes PJ, Greening AP, Crompton GK. Glucocorticoid resistance in asthma. Am J Respir Crit Care Med 1995; 152: s125-s142.

107. Huizenga NATM, Koper JW, de Lange P, et al. J Clin Endocrinol Metab 1998; 83: 144-51.

CHAPTER TWO

CD4 T lymphocytes from patients with Chronic Fatigue syndrome have decreased interferon-γ production and increased sensitivity to dexamethasone

CD4 T Lymphocytes from Patients with Chronic Fatigue Syndrome Have Decreased Interferon-γ Production and Increased Sensitivity to Dexamethasone

Jeroen Visser, Bep Blauw, Boudewijn Hinloopen, Emile Brommer, E. Ronald de Kloet, Cornelis Kluft, and Lex Nagelkerken

Division of Immunological and Infectious Diseases and Division of Vascular and Connective Tissue Research, TNO Prevention and Health, and Division of Medical Pharmacology, Leiden Amsterdam Center for Drug Research, Leiden, The Netherlands

A disturbed hypothalamus–pituitary–adrenal gland axis and alterations at the immune system level have been observed in patients with chronic fatigue syndrome (CFS). Glucocorticoids are known to modulate T cell responses; therefore, purified CD4 T cells from CFS patients were studied to determine whether they have an altered sensitivity to dexamethasone (DEX). CD4 T cells from CFS patients produced less interferon-γ than did cells from controls; by contrast, interleukin-4 production and cell proliferation were comparable. With CD4 T cells from CFS patients (compared with cells from controls), a 10- to 20-fold lower DEX concentration was needed to achieve 50% inhibition of interleukin-4 production and proliferation, indicating an increased sensitivity to DEX in CFS patients. Surprisingly, interferon-γ production in patients and controls was equally sensitive to DEX. A differential sensitivity of cytokines or CD4 T cell subsets to glucocorticoids might explain an altered immunologic function in CFS patients.

Chronic fatigue syndrome (CFS) is a disease of unknown origin characterized by severe disabling fatigue with a duration of >6 months and a reduction in normal activity of at least 50% [1]. Several causes have been held responsible for the disease, including viral infections, a disturbed hypothalamus–pituitary–adrenal gland (HPA) axis and an altered immune function [1, 2]. However, there is no convincing evidence for a role of viral or other infections; for example, titers of antibodies specific for Epstein-Barr virus–related antigens do not differ between patients and controls [3]. The observation that CFS patients have lower levels of free cortisol in their urine and reduced evening plasma cortisol levels in conjunction with elevated levels of plasma adrenocorticotropic hormone [4] is in support of a disfunctioning HPA axis. As far as the immune system is concerned, several functional abnormalities have been reported in CFS, including a decreased NK cell activity, a reduced mitogenic response of lymphocytes, and alterations in cytokine production [2, 5, 6]. Although it is well established that various hormones related to the HPA axis have immunomodulatory properties, which are not exclusively immunosuppressive in nature [7], it is unknown to what extent immunologic aberrations in CFS are due to an altered activity of the HPA axis.

To our knowledge, this study is the first to compare properties of purified CD4 T cells from CFS patients with those of cells from healthy controls who were matched for age and sex. The study of these cells instead of unseparated peripheral blood mononuclear cells (PBMC) provides a more accurate approach to the comparison of Th1 cell activity with Th2 cell activity. Th1 cells regulate cellular immunity by the secretion of cytokines such as interferon-γ (IFN-γ), whereas Th2 cells regulate humoral immunity by cytokines such as interleukin (IL)-4. Of interest, the balance between Th1 and Th2 cells, which appears to be important in the development of various diseases [8], is suggested to be regulated by glucocorticoids [9]. Therefore, we studied to what extent immunologic alterations in CFS were accompanied by an altered sensitivity of CD4 T cells to dexamethasone (DEX).

Materials and Methods

Subjects. Eighteen white CFS patients fulfilling the criteria of Fukuda et al. [1] and living within the postal code area of Leiden were recruited from the Dutch Myalgic Encephalomyelitis Patient Association. To exclude any known causes of fatigue, an internist contacted each patient's general practitioner and any other specialist who had been consulted. Known causes of fatigue were determined by anamnesis, physical examination, and laboratory investigation, including thyroid function tests and tests for autoantibodies or paraproteins. The duration of symptoms ranged from 1–3 years. Each patient was accompanied by a race- and sex-matched healthy control. Ages for controls differed by not more than 4 years from those of CFS patients. The mean age was 36.7 ± 13 years for the CFS group and 40.5 ± 13 years for the controls. Patients and controls were not using medication; vitamins and homeopathics were allowed.

Received 14 April 1997; revised 17 September 1997.

Presented in part: Joint Congress of the British and Netherlands Societies for Immunology, Brighton, UK, 6–8 December 1995 (abstract 9.41); Immunology 1995;86(suppl 1):63.

Informed consent was obtained from study participants.

Financial support: Foundation for Research into the Etiology and Treatment of the Chronic Fatigue Syndrome, Beverwijk, The Netherlands.

Reprints or correspondence: Dr. Lex Nagelkerken, Division of Immunological and Infectious Diseases, TNO Prevention and Health, P.O. BOX 2215, 2301 CE, Leiden, The Netherlands.

The Journal of Infectious Diseases 1998;177:451–4

0022-1899/98/7702-0025$02.00

Table 1. Functional responses of peripheral blood mononuclear cells (PBMC) and CD4 T cells from CFS patients compared with responses of cells from age- and sex-matched controls.

	PBMC*				Negatively selected CD4 T cells*				Positively sorted CD4 T cells†			
	CFS	Controls	*n*	*P*‡	CFS	Controls	*n*	*P*‡	CFS	Controls	*n*	*P*‡
Proliferation (cpm ± SD)	4335 ± 1062	4261 ± 796	16	NS	4293 ± 1837	4522 ± 2868	16	NS	4381 ± 3373	5095 ± 3152	14	NS
IL-4 production (pg/mL ± SD)	590 ± 416	536 ± 371	11	NS	841 ± 507	934 ± 563	14	NS	532 ± 98	516 ± 136	13	NS
IFN-γ production (U/mL ± SD)	3032 ± 2578	4007 ± 2514	11	NS	1258 ± 1266	3276 ± 2787	9	.011	392 ± 194	899 ± 311	12	.034

* Stimulated with combination of anti-CD2 and anti-CD28.
† Stimulated with phytohemagglutinin.
‡ Wilcoxon rank sum test for matched pairs.

At study entry, blood was obtained for additional examinations. Differential blood counts did not reveal abnormalities. By use of flow cytometry, it was established that the percentages of CD14 monocytes, CD4 T cells, CD8 T cells, and CD19 B cells did not differ from control values. As a highly sensitive approach to exclude the presence of inflammatory processes, we established plasma levels of C-reactive protein by ELISA, using polyclonal antibodies (DAKO, Copenhagen): Values for patients were similar to those for controls (median, 0.5 and 0.7 mg/L, respectively). In routine practice, a cutoff of 5 mg/L is used to detect inflammation. No other signs or symptoms of disease developed within the 6-month study period. Only 1 patient was able to resume work within this time.

Isolation of cells. PBMC were isolated from EDTA-blood by Histopaque-1077 (Sigma, St.Louis) density centrifugation and cryopreserved to enable batchwise analysis of patients and controls at a later time. CD4 T cells were enriched to a purity of at least 85% by the depletion of CD8 T cells, B cells, NK cells, and monocytes, as described previously [10]. Additional experiments were done with CD4 T cells obtained by positive selection (>95% purity), using anti-CD4 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions; the percentage of CD14 monocytes in this fraction was 4% on average.

Cell cultures. Cultures of PBMC and CD4 T cells were done in Iscove's Modified Dulbecco's Medium (Gibco, Paisley, UK) supplemented with 10% fetal calf serum (Sebak, Aidenbach, Germany), 100 U/mL penicillin, 100 μg/mL streptomycin, 2 m*M* L-glutamine, and 50 μ*M* β-mercaptoethanol. Cells were cultured at a density of 40,000 cells/well in a volume of 200 μL in flat-bottomed 96-well microtiter plates (Costar, Cambridge, MA) and stimulated with 0.5 μg/mL phytohemagglutinin (Murex, Dartford, UK) or with a cocktail of two anti-CD2 and one anti-CD28 antibodies [10]. DEX (Sigma) was added to a final concentration of 10^{-6}, 10^{-7}, or 10^{-8} *M*.

Supernatants of CD4 T cell cultures were harvested at day 3. Proliferation was measured at day 4 by the addition 18.5 KBq (0.5 μCi) methyl-[^{3}H]thymidine with a specific activity of 74 GBq/mmol (Radiochemical Centre, Amersham, UK) during the last 6 h of culture. Supernatants of PBMC cultures were harvested on day 4, and proliferation was measured on day 5. The culture conditions described above and time points to measure cytokines and proliferation were previously established to represent suboptimal conditions for cryopreserved cells; they were used to allow the detection of stimulatory and inhibitory effects of DEX. Supernatants were stored at −20°C until assay. Labeled cells were harvested onto glassfiber filters (Canberra Packard, Meriden, CT), which were counted by use of a beta-counter (Matrix 96; Canberra Packard).

IL-4 and IFN-γ assays. Levels of IFN-γ and IL-4 were determined by ELISA as previously described [11]. For the IFN-γ ELISA, we used mouse anti-human IFN-γ (clone MD-2) as capture antibody, biotinylated mouse anti-human IFN-γ (clone MD-1) as a detecting antibody, and human recombinant IFN-γ as a reference standard (reagents were provided by Peter van der Meide, Biomedical Primate Research Centre, Rijswijk, The Netherlands). For the IL-4 ELISA, we used mouse anti-human IL-4 (clone 8D4-8) as a capture antibody, biotinylated rat anti-human IL-4 (clone MP4-25D2) as detecting antibody, and human recombinant IL-4 as a reference standard (reagents were purchased from Pharmingen, San Diego).

Statistical analysis. Statistical analysis was done using the Wilcoxon rank sum test for matched pairs. Differences with a confidence level of ≥95% were considered statistically significant ($P < .05$).

Results

Decreased Th1 activity in CD4 T cells from CFS patients. It has been shown, on the basis of cultures of unseparated PBMC or whole blood cells, that CFS patients have decreased levels of IFN-γ, TNF-α, and IL-1 production in response to phytohemagglutinin or lipopolysaccharide [2, 5]. IFN-γ can be released by CD4 T, CD8 T, or NK cells. To establish whether CFS is accompanied by an altered activity of Th1 or Th2 cells within the immunoregulatory $CD4^+$ T cell compartment, we compared cytokine production by PBMC with that by enriched CD4 T cells. To ensure optimal T cell stimulation, we stimulated cells with a combination of anti-CD2 and anti-CD28 antibodies. As shown in table 1, on average, anti-CD2– and anti-CD28–stimulated PBMC from CFS patients had re-

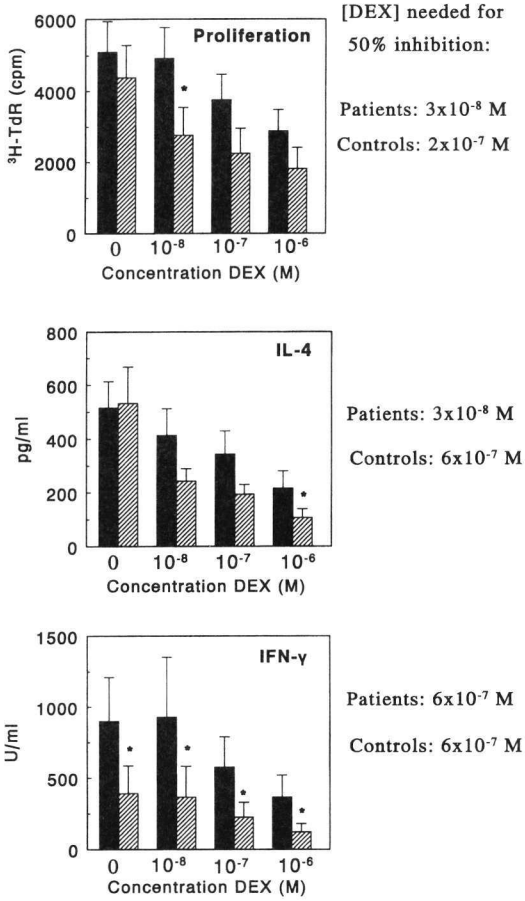


Figure 1. Positively selected CD4 T cells from CFS patients have increased sensitivity to dexamethasone. Cells were stimulated with phytohemagglutinin. Measurements of cytokine production and proliferation were done at culture days 3 and 4, respectively. Results are means ± SE of proliferation for 14 patients (hatched bars) and controls (black bars), of interleukin-4 production for 13 patients and controls, and of interferon-γ production for 12 patients and controls. * $P <$ .05, Wilcoxon rank sum test for matched pairs. DEX, dexamethasone.

duced production of IFN-γ; however, this difference appeared not to be significant. Furthermore, no differences were detected in proliferation or IL-4 production. However, when the functional properties of the enriched CD4 T cells were studied, it was found that the cells from CFS patients versus controls had significantly ($P <$ .02) decreased levels of IFN-γ production.

CD4 T cells obtained by negative selection combine the advantage of not carrying a possibly modulatory antibody with the disadvantage of a relatively low purity (although >85%). This still leaves the possibility that an unidentified contaminating cell population could contribute to cytokine production. Therefore, we examined whether our results could be confirmed using positively sorted CD4 T cells. Moreover, we used phytohemagglutinin as a suboptimal monocyte-dependent system that is highly sensitive to DEX (see below), in contrast to the anti-CD2/anti-CD28 system [10]. As shown in table 1, phytohemagglutinin-stimulated positively sorted CD4 T cells from CFS patients produced significantly less IFN-γ than did cells from age- and sex-matched controls ($P <$ .05). Again, no significant difference was observed with regard to proliferation or IL-4 production.

CD4 T cells from CFS patients have increased sensitivity to DEX. CFS patients have been shown to have a disturbed HPA axis resulting in low levels of cortisol [4]. Since a disturbed HPA axis may be reflected in the sensitivity of lymphocytes for DEX [11], we examined the possibility that CFS patients also have an altered sensitivity to glucocorticoids. As shown in figure 1 (top), the proliferative response of positively selected CD4 T cells from CFS patients is more sensitive to DEX than are responses of cells from matched controls. On average, 2 × 10^{-8} *M* DEX was needed for 50% inhibition of proliferation of CD4 T cells from CFS patients. At least a 10-fold higher DEX concentration was required for a comparable effect in controls. Likewise, IL-4 production by CD4 T cells from CFS patients was more sensitive to DEX (figure 1, middle). On average, 3 × 10^{-8} *M* DEX was needed to achieve 50% inhibition of IL-4 production by CD4 T cells from the patients, whereas a 20-fold higher concentration was required for a comparable effect in the controls.

Figure 1 (bottom) shows that CD4 T cells from CFS patients produced less IFN-γ than did control cells. Surprisingly, IFN-γ production by CD4 T cells from both groups was equally sensitive to DEX: 50% inhibition of the initial IFN-γ production was achieved by the addition of 6 × 10^{-7} *M* DEX. No correlations were found between DEX sensitivities and the extent of IL-4 or IFN-γ production (data not shown).

Discussion

In the past few years, it has become clear that various immunologic diseases are related to an altered activity of Th1 or Th2 cells [8]. To establish whether previously reported immunologic aberrations in CFS are related to an altered activity of one of these subsets, we studied the characteristics of purified CD4 T cells. Both negatively selected CD4 T cells (>85% pure) and positively selected CD4 T cells (>95% pure) from CFS patients had a reduced IFN-γ production compared with that for controls. This difference was selective for IFN-γ production; IL-4 production and cell proliferation were normal.

The difference in cytokine production between the CFS group and the control group was not related to a difference in the composition of the CD4 T cell compartment. Our patient group did not differ from the control group with respect to the fraction of naive and memory CD4 T cells (patients: 50.5% ±

12.8% CD45RA vs. 55.1% ± 18.9% CD45RO; controls: 51.8% ± 9.9% CD45RA vs. 50.1% ± 12.8% CD45RO cells; data not shown). It is therefore likely that the reduced IFN-γ production reflects a selective reduction in the activity of Th1 cells in CFS rather than a shift from Th1 to Th2. This is consistent with a report demonstrating a decrease in delayed-type hypersensitivity (which is mediated by Th1 cells) in CFS [12].

Since CFS patients have a dysfunction of their HPA axis that results in low levels of cortisol [4] and in view of the idea that glucocorticoids may selectively suppress Th1 cells [9], it would have been expected that patients would produce more IFN-γ in vitro due to less suppression in vivo. Our observation of an even lower IFN-γ production in CFS might be due to suppression of IFN-γ in vivo as a consequence of an increased sensitivity of the CD4 T cells for glucocorticoids (e.g., as a consequence of an increased number or affinity of glucocorticoid receptors). Although such an increased sensitivity could indeed be demonstrated using proliferation and IL-4 production as a read-out, this was not found for IFN-γ. This is possibly due to the fact that in patients (as opposed to controls) this cytokine is already suppressed in vivo and that relatively high DEX concentrations are needed in vitro to inhibit IFN-γ even further. Alternatively, a different mechanism might be involved. It is well established that the production of IFN-γ and the induction of Th1 responses is dependent on IL-12 [13] and that IL-10 can inhibit the production of IFN-γ through the inhibition of IL-12 [14]. Preliminary data we obtained in whole blood cultures indicate that IL-10 levels are increased in CFS, suggesting that this cytokine may also contribute to a lower IFN-γ production in CFS patients.

It is expected that more insight into the cause of CFS can be obtained by determining to what extent an altered HPA axis is responsible for the reported immunologic aberrations in CFS.

Acknowledgments

We thank Hans van Noort, Kees Lucas, and Roel de Rijk for critically reviewing the manuscript.

References

1. Fukuda K, Strauss SE, Hickie I, et al. The chronic fatigue syndrome: a comprehensive approach to its definition and study. Ann Intern Med **1994**;121:953–9.
2. Bearn J, Wessely S. Neurobiological aspects of the chronic fatigue syndrome. Eur J Clin Invest **1994**;24:79–90.
3. Swanink CMA, Vercoulen JHMM, Bleijenberg G, Fennis JFM, Galema JMD, van der Meer JWM. Chronic fatigue syndrome: a clinical and laboratory study with a well matched control group. J Intern Med **1995**; 237:499–506.
4. Demitrack MA, Dale JK, Strauss SE, et al. Evidence for impaired activation of the hypothalamic-pituitary-adrenal axis in patients with chronic fatigue syndrome. J Clin Endocrinol Metab **1991**;73:1224–34.
5. Klimas NG, Salvato FR, Morgan R, Fletcher MA. Immunologic abnormalities in chronic fatigue syndrome. J Clin Microbiol **1990**;28:1404–10.
6. Mawle AC, Nisenbaum R, Dobbins JG, et al. Immune responses associated with chronic fatigue syndrome: a case-control study. J Infect Dis **1997**; 175:136–41.
7. Wilder RL. Neuroendocrine–immune system interactions and autoimmunity. Annu Rev Immunol **1995**;13:307–38.
8. Romagnani S. Lymphokine production by human T cells in disease states. Annu Rev Immunol **1994**;7:145–53.
9. Rook GAW, Hernandez-Pando R, Lightman S. Hormones, peripherally activated prohormones and regulation of the Th1/Th2 balance. Immunol Today **1994**;15:301–3.
10. Nijhuis EWP, Hinloopen B, van Lier RAW, Nagelkerken L. Differential sensitivity of human naive and memory CD4 T cells for dexamethasone. Int Immunol **1995**;7:591–5.
11. Nijhuis EWP, Oostervink F, Hinloopen B, Rozing J, Nagelkerken L. Differences in dexamethasone sensitivity between lymphocytes from patients with Alzheimer's disease and patients with multi-infarct dementia. Brain Behav Immun **1996**;10:115–25.
12. Lloyd A, Hickie I, Hickie C, Dwyer J, Wakefield D. Cell-mediated immunity in patients with chronic fatigue syndrome, healthy control subjects and patients with major depression. Clin Exp Immunol **1992**;87:76–9.
13. Trinchieri G. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. Annu Rev Immunol **1995**;13:251–76.
14. D'Andrea A, Aste-Amezaga M, Valiante NM, Ma X, Kubin M, Trinchieri G. Interleukin 10 inhibits human lymphocyte interferon-γ production by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells. J Exp Med **1993**;178:1041–8.

CHAPTER THREE

Differential regulation of Interleukin-10 (IL-10) and IL-12 by glucocorticoids in vitro

Differential Regulation of Interleukin-10 (IL-10) and IL-12 by Glucocorticoids In Vitro

By Jeroen Visser, Anette van Boxel-Dezaire, Dion Methorst, Tibor Brunt, E. Ronald de Kloet, and Lex Nagelkerken

Antigen-presenting cells are thought to modulate the development of Th1 and Th2 cells by the secretion of interleukin-10 (IL-10) and IL-12. Because glucocorticoids (GC) favor the development of Th2 responses, we determined whether dexamethasone (DEX) and hydrocortisone (HC) have differential effects on lipopolysaccharide-induced IL-10 and IL-12 production in whole-blood cultures. Significant inhibition of IL-12(p40) and IL-12(p70) was found with 10^{-8} mol/L and 10^{-9} mol/L DEX respectively, whereas IL-10 was relatively insensitive or even stimulated. Accordingly, the expression of IL-12(p40) and IL-12(p35) mRNA was more sensitive to DEX than IL-10 mRNA. The glucocorticoid receptor (GR) antagonist RU486 enhanced IL-12 production and largely abrogated the inhibition of IL-12 by GC, indicating that this suppression was mainly GR-mediated. High concentrations of RU486 were inhibitory for IL-10, suggesting that GC may exert a positive effect on IL-10. In the presence of neutralizing anti–IL-10 antibodies, DEX was still capable of IL-12 suppression whereas RU486 still enhanced IL-12 production, indicating that GC do not modulate IL-12 via IL-10 exclusively. Taken together these results indicate that GC may favor Th2 development by differential regulation of IL-10 and IL-12.

IT IS WELL-ESTABLISHED that at least two types of T-helper cells are involved in immunoregulation. Th1 cells are supposed to dominate in the regulation of cellular immunity, whereas Th2 cells regulate humoral immunity.[1] During a normal immune response both Th1 and Th2 cell types are involved in a crossregulatory fashion. It is suggested that an imbalance between these subsets contributes to the development of disease: a strong Th2 response is thought to play a role in allergic diseases and antibody-mediated autoimmune diseases, whereas a dominating Th1 response might contribute to the development of cell-mediated autoimmune diseases.[2,3] The development of Th precursor cells into either Th1 or Th2 cells is dependent on a variety of cytokines. The presence of IL-4 during a developing immune response has been shown to favor Th2 responses.[4] On the other hand, IL-12 has been shown to be a crucial factor in the development of Th1 responses.[5,6] Therefore, the type of antigen-presenting cell (APC) may be one of the major determinants in the differentiation of naive $CD4^+$ T cells toward Th1 or Th2 cells. Recently it has been shown that human monocytes may be heterogeneous, evidenced by the fact that $CD14^+/CD16^+$ cells do not express mRNA for IL-10 in response to lipopolysaccharide (LPS) compared with $CD14^+/CD16^-$ cells.[7] This observation is of importance in view of the fact that IL-10 suppresses IL-12.[8] The development of Th1 and Th2 cells may also depend on the activation state of APC, eg, the ability to secrete prostaglandin E_2, which was found to suppress IL-12 production and inhibit Th1 cells.[9,10]

Most likely, glucocorticoids (GC) also play an important role in directing $CD4^+$ T-cell responses. In the mouse it has been shown that dexamethasone (DEX) preferentially suppressed IL-2 and not IL-4, products of Th1 and Th2 cells, respectively.[11] Using rat $CD4^+$ T cells, it was found that GC favor Th2 development.[12] Also, in humans GC have selective effects on $CD4^+$ T-cell subsets[13] which appear to depend on the activation pathway.[14] Addition of GC during restimulation of primed human naive $CD4^+$ T cells stimulates IL-4 and IL-10 production and suppresses IL-5 and interferon-γ (IFN-γ) production.[15] Accordingly, the synthesis of polyclonal IgE is increased in the presence of GC in vitro.[16-18]

The selective effect of GC on the Th1-Th2 balance is supported by the in vivo observation that GC play an important role in the development of experimental allergic encephalomyelitis (EAE).[19] Lewis rats, which are susceptible for EAE, show an impaired production of GC upon stressful events.[20] Moreover, because the relatively resistant PVG rat becomes sensitive to EAE induction after adrenalectomy,[21] it is likely that the development of autoimmunity may be related to the integrity of the hypothalamus-pituitary-adrenal (HPA)-axis. This possibility is supported by the observation that patients suffering from reumatoid arthritis display decreased levels of GC as a result of an impaired functioning of the HPA-axis.[22]

Because the functional characteristics of APC may determine the nature of a developing immune response and since GC seem to favor the development of Th2 responses, the aim of our study was to determine whether GC would have a differential effect on the production of IL-10 and IL-12. Our studies show that IL-10 and IL-12 display a different sensitivity to GC and that different mechanisms are involved in the regulation of these cytokines by GC.

MATERIALS AND METHODS

Antibodies and Reagents

Anti–IL-12 monoclonal antibodies (MoAbs) C11.79, C8.6, and 20C2 were kindly provided by Dr T van der Pouw Kraan (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands), J. Wormmeester (Laboratory of Cell Biology and Histology, Academic Medical Center, University of Amsterdam, The Netherlands), and Dr D.H. Presky (Hoffmann-La Roche, Nutley, NJ). These antibodies recognize both IL-12(p40) and the bioactive heterodimer p70, consisting of p40 and p35, as described previously.[23]

From the Division of Immunological and Infectious Diseases, TNO Prevention and Health, Leiden; and the Division of Medical Pharmacology, Leiden Amsterdam Center for Drug Research, Leiden, The Netherlands.

Submitted April 28, 1997; accepted January 23, 1998.

Address reprint requests to Lex Nagelkerken, PhD, Division of Immunological and Infectious Diseases, TNO Prevention and Health, PO Box 2215, 2301 CE, Leiden, The Netherlands.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

0006-4971/98/9111-0002$3.00/0

Anti–IL-10 MoAbs (JES3-9D7 and biotinylated JES3-12G8), anti-human tumor necrosis factor-α (TNF-α) MoAbs (MoAb1 and biotinylated MoAb11), and neutralizing anti-human IL-10 MoAb (JES3-9D7) were purchased from Pharmingen (San Diego, CA).

Recombinant human IL-12 was purchased from R&D systems (Abington, UK), recombinant human IL-10 was kindly provided by Dr S. Narula (Schering Plough Research Institute, Kenilworth, NJ), and recombinant human TNF-α was obtained from Pharmingen. *Escherichia coli* (serotype 0127:B8)-derived LPS was obtained from Sigma (St Louis, MO). The glucocorticoid-receptor (GR) antagonist RU486 (Roussel-UCLAF, Romaineville, France) and the mineralocorticoid-receptor (MR) antagonist spironolactone (Roussel-UCLAF) were a kind gift of Dr Win Sutanto (Division of Medical Pharmacology, LACDR, Leiden, The Netherlands). Recombinant IFN-γ was a kind gift of Peter van der Meide (BPRC, Rijswijk, The Netherlands). Dexamethasone, hydrocortisone, and aldosterone were purchased from Sigma. The following antibodies were used for cell sorting and assessment of cell purity: anti-CD3-FITC (Becton Dickinson, Mountain View, CA), anti–CD19-FITC (Becton Dickinson), and anti–CD14-PE (CLB, Amsterdam, The Netherlands).

Cell Cultures

Whole-blood cultures. Whole blood was obtained from healthy volunteers by venapuncture and collected in heparinized blood collecting tubes (Becton Dickinson). The blood was 1:5 diluted in Iscoves Modified Dulbecco's Medium (IMDM) supplemented with glutamax (GIBCO, Paisley, UK), 10% fetal calf serum (FCS; Sebak, Gmbh, Aidenbach, Germany), 50 μmol/L β-mercaptoethanol, 100 U/mL penicillin, and 100 μg/mL streptomycin. Fifty microliters of the diluted blood was cultured in 96-well flat-bottomed microtiterplates (Costar, Cambridge, MA) in a final volume of 200 μL per well. Cells were stimulated with 250 ng/mL LPS to induce cytokine production; IL-12(p70) was induced by stimulation with 250 ng/mL LPS in the presence of 1,000 U/mL IFN-γ.

A dose-related response to DEX or HC was studied by the addition of these hormones to the culture wells in a final concentration ranging from 10^{-10} mol/L to 10^{-5} mol/L. Stock solutions of 10 mmol/L HC, 20 mmol/L DEX, 0.1 mol/L RU486, and 0.1 mol/L spironolactone were prepared in dimethylsulfoxide (DMSO; Merck, Darmstadt, Germany) and stored at −20°C in 0.2-mL aliquots. Stock solution of aldosterone (2.86×10^{-4} mol/L) was prepared in culture medium and stored at −20°C in 0.5-mL aliquots.

In some experiments endogenous IL-10 was neutralized by the addition of 5 μg/mL anti–IL-10 antibodies. The binding of DEX and HC to the GR was blocked by simultaneous addition of 50 μmol/L or 1 μmol/L RU486 to the culture wells. The binding of HC to the MR was blocked by simultaneous addition of 1 μmol/L spironolactone to the culture wells. The whole blood was put into culture within 2 hours after venapuncture. For assessment of cytokine levels the supernatants of the cultures were obtained after 24 and 48 hours of culture and stored at −20°C.

Cultures of peripheral blood mononuclear cells (PBMC) and purified subsets. PBMC were isolated by density centrifugation in Histopaque 1.077 (Sigma). For the isolation of subsets two experiments were performed using buffycoats from healthy donors, in which B cells and monocytes were simultaneously labeled with anti–CD19-FITC and anti–CD14-PE, and T cells were labeled with anti–CD3-FITC. Cells of interest were sorted with the use of a FACS Vantage (Becton Dickinson).

Cells were cultured in 24-well flat-bottom tissue culture plates (Costar) at a density of 1×10^6 PBMC/well, 1×10^5 B cells/well, 2×10^5 T cells/well, or 1×10^5 monocytes/well under conditions as described for whole-blood cultures.

Cytokine Assays

For the IL-12(p40) enzyme-linked immunosorbent assay (ELISA), MoAb C11.79 (2 μg/mL in 50 mmol/L $NaHCO_3$, pH 9.5, 50 μL/well) was coated overnight at 4°C on round-bottom microtiterplates with high-binding capacity (Greiner, Nürtingen, Germany). For the IL-12(p70) ELISA plates were coated with MoAb 20C2 (2 μg/mL in 50 mmol/L $NaHCO_3$, pH 9.5, 50 μL/well). As for all subsequent washing steps, the plates were washed six times with phosphate-buffered saline (PBS) containing 0.05% Tween-20. Subsequently the plates were blocked for 1.5 hours with 200 μL PBS containing 0.2% gelatin and 0.05% Tween-20 (PTG). After washing, 50 μL diluted biotinylated MoAb C8.6 in PTG (final concentration, 0.25 μg/mL) was added per well together with 50 μL of the undiluted samples and simultaneously incubated for 2 hours. After washing, the plates were incubated for 1 hour with 75 μL/well poly-streptavidin-horseradish peroxidase (CLB) 1:10,000 diluted in PTG. Finally, after washing, the plates were developed with 100 μL/well 0.1 mol/L 3,5,3′,5′-tetramethyl-benzidine (TMB; Merck) in 0.11 mol/L sodium acetate pH 5.5 containing 0.003% H_2O_2. The reactions were terminated by the addition of 50 μL of 2 mol/L H_2SO_4 to each well. The plates were read at 450 nm in a Biorad 3500 platereader (Biorad, Richmond, CA). Recombinant human IL-12 diluted in culture medium was used as a standard, and the standard curves ranged from 4,000 pg/mL to 15 pg/mL.

The IL-10 ELISA was performed in an identical fashion. The plates were coated with JES3-9D7 MoAb (0.5 μg/mL) and biotinylated JES3-12G8 MoAb was used in a concentration of 2 μg/mL. Recombinant human IL-10 diluted in culture medium was used as a standard. The standard curves ranged from 2,500 pg/mL to 10 pg/mL.

For the TNF-α ELISA the plates were coated with 1 μg/mL MoAb1; biotinylated MoAb11 was used in a concentration of 1 μg/mL for detection. The supernatants were tested in a fivefold dilution in culture medium. Recombinant TNF-α diluted in culture medium was used as a standard. The standard curves ranged from 5,000 pg/mL to 19 pg/mL.

Cortisol Measurement

Blood from several individual donors was collected, immediately put on ice and allowed to coagulate. The tubes were spun down for 30 minutes (3,000 rpm, 4°C); serum was collected and immediately stored at −20°C. Cortisol was measured using the fluorescent polarization immunoassay on the TDx from Abbott (Amstelveen, The Netherlands).

RNA Quantitation Using Semi-quantitative Polymerase Chain Reaction (PCR)

Whole blood was stimulated with 250 ng/mL LPS, in the absence or presence of 10^{-6} mol/L DEX as described above. After 4 or 20 hours of culture—conditions that were found to be optimal for IL-12(p35/p40) and IL-10, respectively—erythrocytes were lysed and mRNA was extracted from the white blood cells using RNAzol B, according to the instructions of the manufacturer (Biotecx Laboratories, Houston, TX). Two micrograms of total mRNA was reverse transcribed using a Reverse Transcription System kit (Promega, Madison, WI) using condi-

tions ensuring optimal cDNA synthesis. cDNA as a readout of the mRNA was quantitated in a PCR using the PQB-3 vector[24] as an external standard, which contains primer sequences for IL-10 and β-actin. This vector as well as the PQA-1 vector were kindly provided by Dr D. Shire (Sanofi, Labège, France). To enable quantitation of IL-12(p40) and IL-12(p35) cDNA, two complementary 40-mer sequences each encompassing a 20-mer sequence of IL-12(p40) and of IL-12(p35) were cloned into the *Hin*dIII [for IL-12(p40) and (p35) sense primers] and *Bam*HI sites [for IL-12(p40) and (p35) antisense primers] of the PQA-1 vector.[24] By means of a parallelism test, PQB-3 and PQA-1/IL-12 were verified to be amplified equally efficient as cDNA from mRNA encoding cytokines or β-actin, when amplified with IL-10, β-actin, IL-12(p40), or (p35) specific primers. cDNA was quantitated in a semi-quantitative fashion by simultaneously amplifying the cDNA in triplicate and the stepfold diluted vector as an external standard in duplicate. Amplification was performed in 50-μL reactions containing 12.5 pmol sense and antisense primer, 0.25 mmol/L dNTPs (GIBCO-BRL, Gaithersburg, MD), 1 U of Taq DNA polymerase (GIBCO-BRL), and PCR buffer II with 2.5 mmol/L $MgCl_2$ (Perkin Elmer, Branchbury, NJ) for IL-12(p40) or (p35) primers and PCR buffer containing 50 mmol/L KCl, 10 mmol/L TRIS/HCl pH 8.3, 2 mmol/L $MgCl_2$, and 60 ng/mL bovine serum albumin (BSA) for IL-10 and β-actin primers. For the amplification the following sense and anti-sense primers (Isogen Bioscience, Maarssen, The Netherlands) were used (given from 5′ → 3′): IL-10 sense: ATGCTTCGAGATCTCCGAGA; IL-10 antisense: AAATCGATGACAGCGCCGTA; IL-12(p40) sense: GGAGTACTCCACATTCCTAC; IL-12(p40) antisense: CCATGGCAACTTGAGAGCTG; IL-12(p35) sense: CAGCAACATGCTCCAGAAGG; IL-12(p35) antisense: CCTAGTTCTTAATCCACATC; β-actin sense: GGGTCAGAAGGATTCCTATG; and β-actin antisense: GGTCTCAAACATGATCTGGG. Cycling conditions were 30 seconds of denaturation at 96°C, 1 minute of annealing at 55°C, and 1 minute of elongation at 72°C during 30 cycles for β-actin and 35 cycles for the cytokines.

PCR products were stained on 1% agarose gels with ethidium bromide or SYBR Green I (Biozym, Landgraaf, The Netherlands). Densities of the amplified vector (known amount in femtograms) and of the amplified cDNA (unknown amount) were analyzed using the Bio-1D digital imaging system version 6 (Vilber Lourmat, Marne La Vallée, France). The comparison of these densities enabled the subsequent calculation of amplified β-actin or cytokine cDNA in femtograms. Results are expressed as a ratio of quantified cytokine product (in femtograms) over β-actin product (in femtograms).

Data Processing and Statistics

The curvefitting option in the Biorad microplatemanager software was applied to calculate the cytokine concentrations in the supernatants. Statistical analysis was performed using the Student's *t*-test for matched pairs. Differences with a confidence level of 95% or higher were considered to be statistically significant ($P < .05$).

RESULTS

IL-12 and TNF-α Production Are More Sensitive for DEX Than IL-10 Production

Because GC favor Th2 type of immune responses, we were interested in their effects on IL-10 and IL-12 as cytokines that play a pivotal role in the development of Th1 and Th2 cells. TNF-α was studied as a positive control for inhibition by GC. We used whole-blood cultures because these are more representative for in vivo conditions than cultures of PBMC and because the induction of both IL-10 and IL-12 by LPS is much more efficient in whole blood cultures.[9] Figure 1 shows the results obtained after 24 hours of stimulation with LPS for IL-10 and IL-12(p40) production (mean of 13 different donors each) and TNF-α production (mean of 9 donors). DEX turned out to have differential suppressive effects on these cytokines. DEX dose-dependently inhibited the LPS-induced IL-12(p40) production to 26% of the initial response at a concentration of 10^{-6} mol/L DEX ($P < .01$). As shown in Table 1, on average 9.1×10^{-8} mol/L DEX was needed to achieve 50% inhibition of IL-12(p40). TNF-α was slightly more sensitive in that 50% inhibition was found with 2.6×10^{-8} mol/L DEX (Fig 1 and Table 1).

In contrast, IL-10 production was relatively insensitive to DEX: a significant ($P < .01$) inhibition of IL-10 production to 61% of the initial response was only found with 10^{-6} mol/L DEX. As shown in Fig 2A-C for three individual donors, DEX might not have an effect at all or even stimulate IL-10

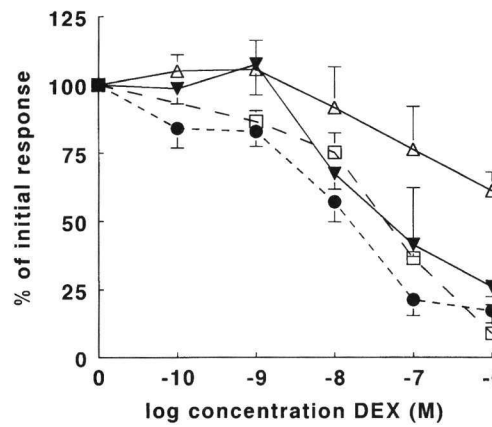


Fig 1. Dose-dependent effect of DEX on LPS-induced cytokine production in whole-blood cultures. IL-10, IL-12(p40), and TNF-α were induced in whole-blood cultures with 250 ng/mL LPS and in the presence of various concentrations DEX. For the induction of IL-12(p70), 1,000 U/mL IFN-γ was added to the cultures. Supernatants were obtained after 24 hours. The mean cytokine production is expressed as a percentage of the initial response in the absence of DEX. The results of IL-10 (△, n = 13), IL-12(p40) (▼, n = 13), IL-12(p70) (□, n = 4), and TNF-α (●, n = 9) are expressed as the mean of the percentage (±SEM) of the initial cytokine response. The mean absolute values (±SD) in the absence of DEX for IL-12(p70), IL-12(p40), IL-10, and TNF-α were 127 ± 35 pg/mL, 623 ± 419 pg/mL, 411 ± 611 pg/mL, and 2,318 ± 1,499 pg/mL, respectively.

Table 1. Relative Sensitivity of Cytokines to DEX

Donor	Sex	TNF-α		IL-12(p40)		IL-10	
		ng/mL	IC50*	pg/mL	IC50*	pg/mL	IC50*
4	M	1.53	0.00001†	360	0.011	101	1.70†
5	M	1.38	0.021	98	0.0042	172	>100†
6	F	2.76	0.0051	696	0.0071	274	>100†
7	F	2.95	0.0052	617	0.0061	2,498	0.0015
8	F	0.60	0.012	382	0.31	235	>100†
9	M	5.15	0.080	308	0.082	218	>100†
10	M	1.29	0.015	143	0.021	394	0.022
11	F	1.24	0.081	226	0.082	382	0.33
12	F	3.95	0.020	772	0.033	343	0.20
13	M	ND	ND	945	0.021	106	4.0†
14	M	ND	ND	1,321	0.12	101	1.0
15	M	ND	ND	1,498	0.063	155	>100†
16	F	ND	ND	734	0.43	370	>100†
Mean IC50 ± SEM			0.026 ± 0.010		0.091 ± 0.036		1.036 ± 0.546‡

Cytokine production in whole-blood cultures was induced with 250 ng/mL LPS. The supernatants were obtained after 24 hours of culture and the cytokines determined by ELISA.

Abbreviations: ND, not done; M, male; F, female.

*DEX concentration (μmol/L) required for 50% inhibition of cytokine production.

†Results obtained by extrapolation.

‡N = 7, excluding values >100.

production. In 7 of 13 donors it was possible to estimate (partly by extrapolation) that 1.04×10^{-6} mol/L DEX would be needed to achieve 50% inhibition of IL-10 production in these donors (Table 1). In 3 donors we found no inhibition at all, whereas in 3 additional donors the inhibition by 10^{-6} mol/L DEX did not exceed 25%, which made extrapolation impossible. For these donors an IC50 value >100 is given (Table 1). Similar differences in DEX sensitivity between IL-10 and the other cytokines were found after 48 hours of culture (data not shown). Since in several donors IL-10 production was insensitive to DEX, we performed additional experiments to assess if endogenous cortisol determined the sensitivity to DEX in vitro. High endogenous cortisol did not correlate with less suppression of IL-10 or of IL-12(p40) by DEX in vitro (N = 16, data not shown).

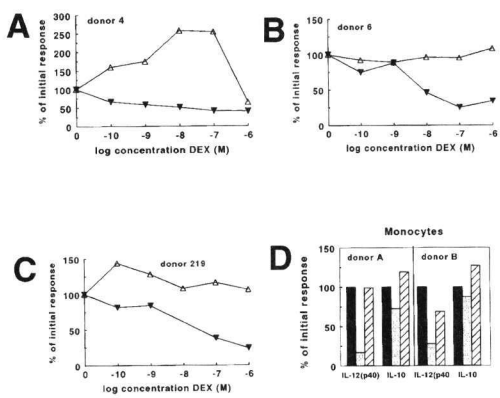
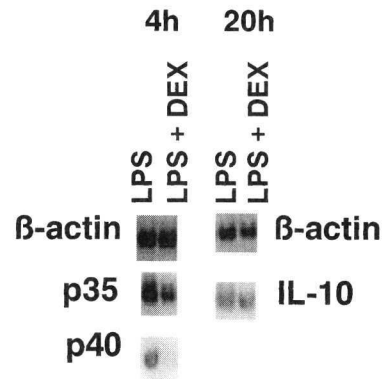


Fig 2. Differential regulation of IL-12(p40) and IL-10 in individual donors and monocytes. IL-12(p40) (▼) and IL-10 (△) were induced in whole-blood cultures with 250 ng/mL LPS and in the presence of various concentrations DEX. The results of three individual donors are shown (A, B, and C). Monocytes stained with PE-conjugated anti-CD14 antibodies were sorted with a flow cytometer to a purity of more than 98% and cultured as described in Materials and Methods (D). The results obtained with two individual donors are shown. Cells were stimulated with LPS in the absence (■) or presence (□) of 10^{-6} mol/L DEX or in the presence of 10^{-6} mol/L DEX and 10^{-6} mol/L RU486 (▨). The results are expressed as a percentage of the initial response with LPS.

In two subsequent experiments we studied B cells, T cells, and monocytes that were positively selected by flow cytometry using FITC-conjugated anti-CD19, PE-conjugated anti-CD14, or FITC-conjugated anti-CD3. The obtained cell populations were more than 98% pure. When stimulated with LPS for 24 hours, production of IL-10 and IL-12(p40) was only detectable in monocyte cultures. These cytokines were not detected in LPS-stimulated cultures of T cells or B cells (data not shown), which is in agreement with recent observations by Guery et al,[25] who showed that normal B cells are not capable of IL-12 production. As shown in Fig 2D, IL-12(p40) production by monocytes as opposed to IL-10 production was sensitive to DEX and this effect was antagonized by 1 μmol/L RU486. Effects of this glucocorticoid receptor antagonist will be discussed in more detail below. For the detection of the functional IL-12(p70) protein in our whole-blood culture system it was necessary to add exogenous IFN-γ, which upregulates IL-12(p35) mRNA.[26] The mean of results (obtained with whole blood from four different donors) of the effect of DEX on this protein are shown in Fig 1. On average, 50% inhibition was found with 6.4×10^{-8} mol/L DEX. As will be pointed out below it cannot be excluded that IFN-γ altered the sensitivity to DEX. However, additional studies performed in seven donors showed that in the presence of exogenous IFN-γ, 10^{-6} mol/L DEX caused on average 80% inhibition of IL-12(p70) and on average 25% inhibition of IL-10 (data not shown).

To obtain more insight into the effects of DEX in the absence

of IFN-γ, we studied the expression of the p40 and p35 subunits at the mRNA level in whole-blood cultures, using a semi-quantitative PCR. In unstimulated whole blood IL-12(p40) mRNA was below the detection limit whereas a more than 1,000-fold upregulation was found in response to LPS; in contrast, a constitutive expression of IL-12(p35) mRNA was observed which was enhanced threefold in response to LPS (data not shown). As illustrated in Fig 3 for six different donors, DEX consistently suppressed ($P < .05$) the expression of both IL-12(p40) and IL-12(p35) mRNA. As was already found at the protein level DEX had variable effects on IL-10 mRNA, ranging from inhibition to stimulation, but on average no inhibition was found.



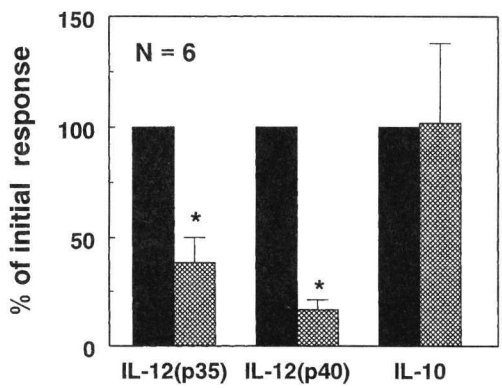


Fig 3. Sensitivity of IL-12(p40), IL-12(p35) mRNA, and IL-10 mRNA to DEX. mRNA was isolated from whole-blood cultures stimulated with 250 ng/mL LPS with (▨) or without (■) 10^{-6} mol/L DEX and used to perform semiquantitative RT-PCR assays for IL-12(p40), IL-12(p35), and IL-10 as described in Materials and Methods. mRNA for IL-12(p40) and IL-12(p35) were measured after 4 hours of culture, whereas IL-10 mRNA was measured after 20 hours of culture; these time points were previously established to be optimal for the expression of these particular mRNAs. Results shown are density scans of one typical donor (top) as well as the mean ± SEM of six different healthy donors, expressed as a percentage of the mRNA expression in the absence of DEX (bottom). *$P < .05$.

On the basis of the donors in which inhibition of IL-10 by DEX could be detected (Table 1), we conclude that—as far as DEX has suppressive effects—IL-12 is at least 12-fold more sensitive than IL-10. As will be discussed below, lack of inhibition in the other donors and the potential to even stimulate IL-10 production further support the hypothesis that GC may favor Th2 type of response by differential effects on IL-10 and IL-12.

Suppression of IL-12 by GC Is Mainly Mediated Via GC Receptors

The differences in DEX sensitivity of IL-12 and IL-10 suggested that different receptors may be involved in the regulation of these cytokines. It is known that DEX binds with high affinity to the GC receptor (GR) and with low affinity to the mineralocorticoid receptor (MR).[27-29] Because the reverse is true for the physiologic glucocorticoid HC,[29] we first compared the efficacy of DEX and HC. As shown in Fig 4 (lower panel) both HC and DEX were relatively ineffective in the inhibition of IL-10. As far as the suppression of IL-12(p40) and IL-12(p70) is concerned, HC was less effective than DEX. On average, a fivefold higher HC concentration was needed for 50% inhibition of IL-12(p40) (Fig 4, upper panel) whereas an eightfold higher concentration was needed for the inhibition of IL-12(p70) (Fig 4, middle panel). These results are in line with the fact that HC has an eightfold lower affinity for the GR as compared with DEX.

The role of the GR in the modulation of cytokines by DEX and HC was further addressed by using RU486 as an antagonist. Addition of 1 μmol/L of RU486 antagonized the suppressive actions of both DEX and HC on LPS-induced IL-12(p40), although this antagonizing effect was not complete (Fig 5, upper panel). A concentration of 50 μmol/L RU486, which increased the IL-12(p40) production more than twofold ($P < .05$), completely abrogated the inhibitory effect of DEX, but not of HC (Fig 5, upper panel). This appeared to be significant on the basis of results obtained in eight different donors ($P < .05$). Because HC has an eightfold lower affinity for the GR than DEX we had expected a complete antagonizing effect of RU486 on HC suppression. Because this appears not to be the case, HC may also mediate suppressive effects via the MR. Therefore, we studied the effect of the MR agonist aldosterone. The addition of 10^{-8} mol/L aldosterone caused 17% suppression of IL-12(p40) ($n = 15$, $P < .05$; data not shown). This shows that occupation of the MR may indeed contribute to inhibition of IL-12(p40). However, because the MR antagonist spironolactone did not antagonize suppression of IL-12(p40) by HC (data not shown), part of its effect might be mediated by a mechanism different from the GR and MR. RU486 enhanced IL-12(p70) production, probably by antagonizing the inhibitory effects of endogenous cortisol.

Compared with the effects on IL-12(p40), RU486 was less efficient in antagonizing the suppressive effect of DEX on the induction of IL-12(p70) (Fig 5, middle panel). This may have

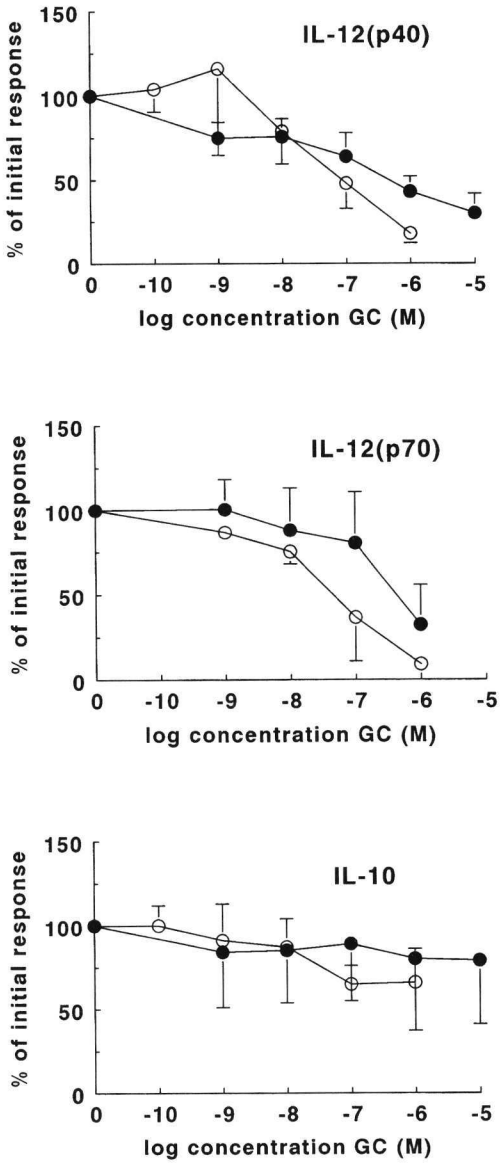


Fig 4. Sensitivity of cytokines to DEX and HC. Cytokines were induced in whole-blood cultures with 250 ng/mL LPS in the presence of DEX (○) or HC (●). For the induction of IL-12(p70), 1,000 U/mL IFN-γ was added to the cultures. The data are expressed as a percentage ± SEM of the cytokine production in the absence of GC. The results are the means of the production of IL-12(p40) and IL-10 in whole-blood cultures of 13 different healthy donors. IL-12(p70) production is the mean of the results obtained with four different healthy donors. Cytokines were determined by ELISA in supernatants harvested after 24 hours of culture.

been due to the presence of exogenous IFN-γ, which has previously been suggested to increase the sensitivity to GC.[30]

Stimulation of IL-10 by GC

Whereas RU486 significantly enhanced the IL-12 production and antagonized the suppression by GC, different effects were observed with regard to IL-10. Although the slight inhibitory effect of high GC concentrations was antagonized by 1 μmol/L RU486, 50 μmol/L RU486 surprisingly caused a significant inhibition ($P < .05$) of IL-10 production (Fig 5, lower panel), suggesting that complete inactivation of the GR was inhibitory for this cytokine. Because RU486 has been found to act as an agonist on the progesteron receptor when cyclic adenosine monophosphate (cAMP) levels are increased,[31] we first performed additional studies to establish whether the effects of RU486 could be mimicked by equimolar concentrations of progesterone. However, although we could show in whole-blood cultures of four different donors that RU486 stimulated IL-12(p40) and inhibited IL-10, we observed that 50 μmol/L progesterone did not have an effect in these cultures (data not shown). This suggested that the stimulatory effects of high concentrations of RU486 on IL-12, but also the inhibition of IL-10, are caused by inhibition of endogenous cortisol. Indeed, additional experiments with 11 individual donors showed a positive correlation between the stimulatory effects of RU486 on IL-12(p40) production and cortisol levels in serum, whereas for IL-10 such a correlation was not found (Fig 6).

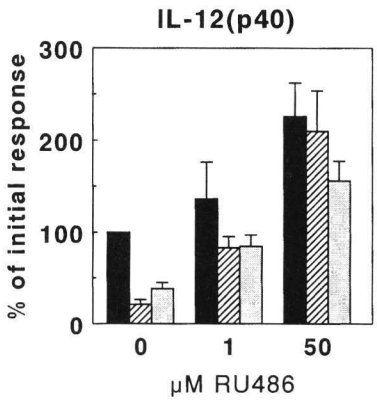
IL-10 Is Not an Intermediate in DEX-Mediated Suppression of IL-12 and TNF-α

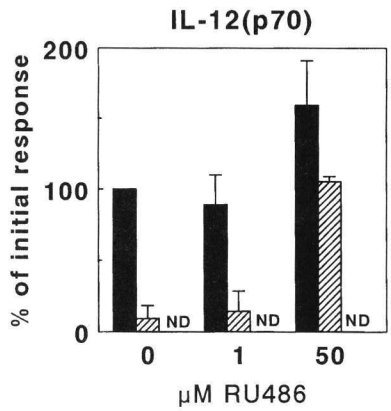
It is well-established that IL-10 can suppress IL-12(p40), IL-12(p70), and TNF-α production.[8,9,32] In view of the potential of GC to stimulate IL-10, we investigated whether IL-10 acted as an intermediate in the suppression of IL-12(p40), IL-12(p70), and TNF-α by DEX. As shown in Fig 7 (top), the addition of 5 μg/mL anti–IL-10 to the whole-blood cultures significantly enhanced the LPS-induced IL-12(p40), IL-12(p70), and TNF-α production ($P < .05$). Using the increased cytokine levels as reference values, 1 μmol/L DEX caused 80% inhibition of TNF-α, 86% inhibition of IL-12(p70), and 68% inhibition of IL-12(p40) in the presence of anti–IL-10. These values did not differ from the extent of inhibition by DEX found in the absence of anti–IL-10. Thus, in the presence of anti–IL-10, DEX was still able to suppress IL-12(p40), IL-12(p70), and TNF-α production. Likewise, we established whether RU486 would stimulate IL-12 under conditions where IL-10 was already neutralized. As shown in Fig 7 (bottom), RU486 enhanced IL-12(p70) production even in the presence of anti–IL-10. Moreover, because RU486 and anti–IL-10 synergistically enhanced the IL-12(p70) it is likely that IL-10 and cortisol suppress IL-12 by separate mechanisms.

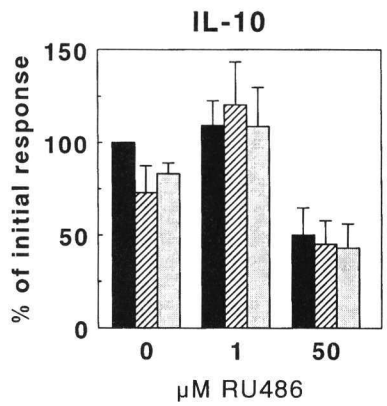
These results indicate that GC may suppress IL-12 by two complementary mechanisms: direct inhibition and possibly by upregulation of IL-10.

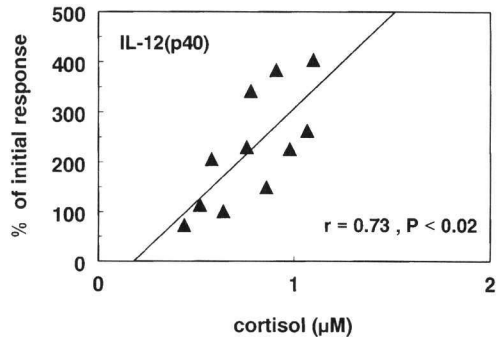
DISCUSSION

GC are generally regarded as immunosuppressive and are therefore widely used for clinical applications ranging from preventing rejection in transplantation to the treatment of









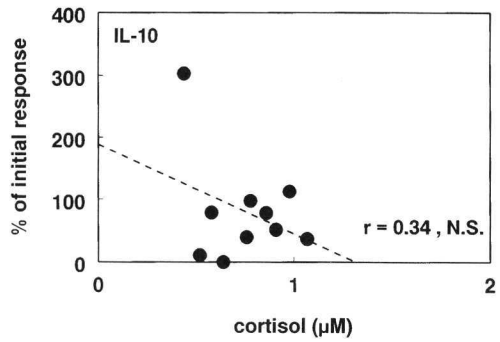


Fig 6. Correlation between antagonizing effects of RU486 and endogenous cortisol. The levels of cortisol were measured in the serum of 11 healthy donors. In these donors whole-blood cultures were performed as described in Fig 1. The effect of 50 μmol/L RU486 was studied on IL-10 and IL-12(p40) production. The level of cortisol in the serum of each donor is plotted against the effect of RU486 on the IL-10 (bottom) and IL-12(p40) (top) production. The effect of RU486 is expressed as the percentage of the initial response with LPS in each donor. Each symbol represents one individual donor. The blood was collected between 8 and 10 AM and put into culture immediately after collection.

Fig 5. The GR antagonist RU486 stimulates IL-12 and inhibits IL-10. Cytokines were induced in whole-blood cultures with 250 ng/mL LPS [for induction of IL-12(p70), 1,000 U/mL IFN-γ was added to the cultures] in the absence (■) or presence of 10^{-6} mol/L DEX (▨) or 10^{-6} mol/L HC (□). The influence of these GC on IL-12(p40) (upper panel), IL-12(p70) (middle panel), and IL-10 (lower panel) was studied in the absence or presence of 1 or 50 μmol/L RU486. The means of the results ± SEM obtained with eight different donors are shown. IL-12(p70) production is the mean of the results ± SEM obtained with four different healthy donors. The data are expressed as a percentage of the cytokine production in the absence of GC or antagonist. Cytokines were determined by ELISA in supernatants obtained after 24 hours of culture. ND, not done.

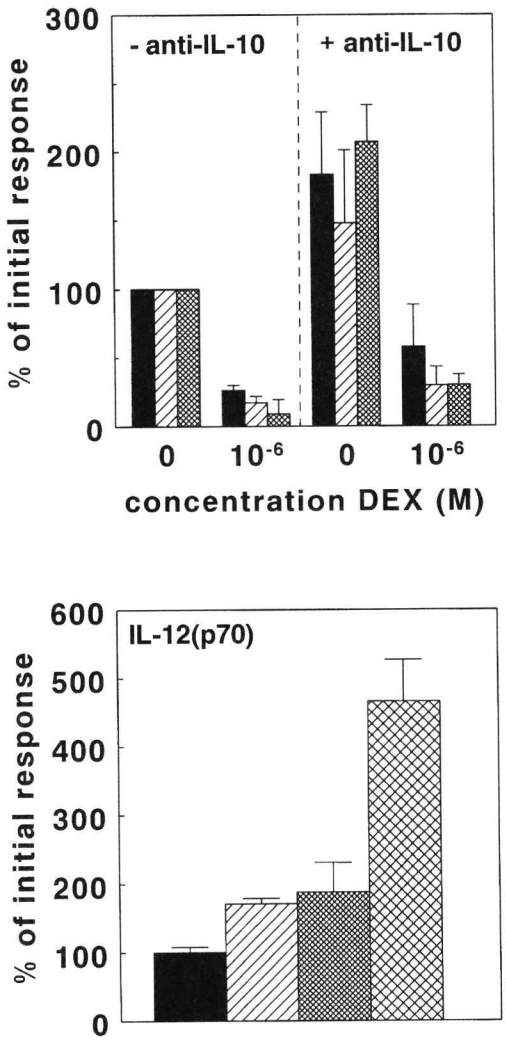


Fig 7. IL-10 is not an intermediate in the suppression of IL-12 by GC. Cytokines were induced in whole-blood cultures as described in the legend of Fig 1. Cultures were performed in the absence or presence of 5 μg/mL anti–IL-10 with or without 10^{-6} mol/L DEX (top panel). Results are expressed as a percentage ± SEM of the cytokine production found in the presence of LPS and in the absence of anti–IL-10. The results are the means of the IL-12(p40) (■) and TNF-α (▨) production obtained with nine different healthy donors; IL-12(p70) production (▩) is based on results obtained with four different healthy donors. In additional experiments, the effect of 50 μmol/L RU486 (▨), 5 μg/mL anti-IL-10 (▩), or both (▩) on LPS– and IFN-γ–induced IL-12(p70) was studied in nine separate donors (bottom panel). The results are expressed as a percentage ± SEM of the cytokine production in the presence of LPS and IFN-γ alone (■).

allergic asthma. Recent studies have pointed to the possibility that GC may have a selective effect in immune regulation by suppressing IFN-γ production and promoting IL-4 production by $CD4^+$ T cells.[12,15] Our data are in support of this hypothesis because they show—on the basis of whole-blood cultures stimulated with LPS—that IL-12 is 10 to 100 times more sensitive to suppression by GC than IL-10, dependent on the presence of IFN-γ.

A comparison between DEX and HC showed that the relative suppressive effects of these GC were consistent with the fact that DEX has a eightfold higher affinity for the GR than HC.[27-29] Antagonism of the suppressive effects by the GR antagonist RU486 and not by the MR antagonist spironolactone support the conclusion that suppression was largely mediated by the GR. However, because aldosterone showed a modest suppression of IL-12(p40), a minor contribution of MR to the effects of HC cannot be excluded completely. The addition of IFN-γ to the cultures—as a condition required for detectable IL-12(p70) induction in our system—may have affected the sensitivity of this cytokine to GC. As has been shown previously, IL-4 and IL-2 decrease the sensitivity to GC by decreasing the affinity of the GR[30]; in this system the effect of IL-4 and IL-2 was abolished by IFN-γ. Therefore, IFN-γ may have affected the sensitivity of IL-12 for GC by increasing the affinity of the GR. However, in the absence of exogenous IFN-γ—as illustrated with the use of a semi-quantitative reverse transcriptase-PCR—DEX also suppressed the LPS-induced expression of IL-12(p40) and IL-12(p35) mRNA but had on average no effect on the expression of IL-10 mRNA. T cells may express IL-12(p35) mRNA in the absence of IL-12(p40) mRNA, consistent with the inability of these cells to secrete bioactive IL-12.[23,26] Although our data show that expression of mRNA for both subunits can be suppressed by DEX, this does not prove that both mRNAs are equally suppressed in one and the same cell type. Additional studies using isolated monocytes are needed to establish whether suppression of the bioactive IL-12 by DEX is accounted for by an effect on one of the subunits in particular or on both.

With regard to IL-10 we observed in various donors stimulation by GC rather than an inhibitory effect. That this was not a consistent finding in all donors may be due to the fact that in most of the donors occupancy of the GR has already occurred by endogenous cortisol and, consequently, that positive regulation of IL-10 has already been achieved; such a condition will probably be present in the majority of the donors because the whole-blood cultures were performed in the presence of autologous plasma. Preliminary data indicate that stimulation of IL-10 by low concentrations of cortisol is found more frequently using cultures of isolated PBMC. A stimulatory role for GC was in particular evident from the fact that the GR antagonist RU486 inhibited IL-10. This appeared not to be a nonspecific effect because IL-12(p40) and IL-12(p70) were stimulated. Because RU486 may act as an agonist for the progesterone receptor,[31] we ruled out that progesterone had inhibitory effects on IL-10 or stimulatory effects on IL-12 (data not shown).

Additional experiments showed that the stimulatory effects of RU486 on IL-12(p40) production correlated with endogenous cortisol, suggesting that this effect of RU486 can be explained by blocking the effects of endogenous cortisol. The

fact that we made this observation despite the absence of a direct negative correlation between the IL-12(p40) production capacity and cortisol (data not shown) may be explained by assuming that only part of the hormone is not bound to cortisol-binding protein and available for suppression. In the case of IL-10 any correlation may be difficult to find if occupancy of the GR with low levels of cortisol would lead to stimulation and high concentrations of cortisol with slight suppression of this cytokine.

Our observations are in line with studies showing that hypercortisolemia results in increased plasma IL-10 concentrations in vivo.[33,34] However, the mechanism by which these effects may occur are so far unknown. The presence of a GRE in the IL-10 promoter[35] points to a potential mechanism of GC in the stimulation of IL-10, although it is unknown thus far whether this GRE is functional.

Apart from having stimulatory effects, high concentrations of GC were inhibitory for IL-10, which has also been shown for DEX on IL-10 production by PBMC and monocytes.[36] Mechanisms to be taken into account in these effects are interference at the level of transcription factors. Because IL-12(p40) production is regulated by NF-kB[37] this transcription factor may be one of the main targets of GC, for instance via the induction of IkB.[38,39] The relative resistance of IL-10 to GC would be in agreement with the absence of NF-kB binding sites in the promoter region of the IL-10 gene.[35] However, because binding sites for AP-1 and cAMP responsive element binding protein (CREB) are present in the promoter region of IL-10,[35] interference with these factors might be one of the mechanisms of IL-10 suppression at pharmacological concentrations of GC. That we did not always observe suppressive effects on IL-10 may be due to the lack of induction of such transcription factors in individual donors. Interestingly, suppression of IL-10 by high concentrations of GC appeared to correlate with the efficiency of LPS to induce IL-10 (data not shown).

Taken together our results indicate that physiological concentrations of GC inhibit IL-12, but do not affect or even stimulate IL-10. The overall outcome of increased levels of GC in vivo may thus be that antigen-presenting cells are modulated to display a functional phenotype that favors the development of a Th2 response. Indeed, recently GC have been found to modulate adherent cells in such a way that they promote Th2 development.[40] This bias toward Th2 may be amplified by the direct effects of GC on T cells.[11-13,15] The effects of GC on the level of antigen-presenting cells and the development of Th cells might explain why during pregnancy and diseases which are accompanied by excessive release of GC the cellular immunity is suppressed and the humoral immunity is enhanced.[41,42] Therefore, GR agonists and antagonists might be of use in selective modulation of Th activity.

ACKNOWLEDGMENT

We are grateful to Dr Kees Lucas for critically reviewing the manuscript and to Bep Blauw and Ellen Siemssen for technical assistance. The assistance of Guus Westra (Department of Hematology, Free University Academical Hospital, Amsterdam, The Netherlands) with the FACS-sorting is highly appreciated. Furthermore, we thank Dr Eef Lentjes (Department of Clinical Chemistry, University Hospital Leiden, Leiden, The Netherlands) for performing the cortisol measurements. We are also grateful to Prof G. Trinchieri (Wistar Institute, Philadelphia, PA) for providing us with the hybridomas C11.79 and C8.6.

REFERENCES

1. Mosmann TR, Coffman RL: Th1 and Th2 cells: Different patterns of lymphokine secretion lead to different functional properties. Annu Rev Immunol 7:145, 1989

2. Romagnani S: Lymphokine production by human T cells in disease states. Annu Rev Immunol 7:145, 1994

3. O'Garra A, Murphy K: T-cell subsets in autoimmunity. Curr Opin Immunol 5:880, 1993

4. Paul WE, Seder RA: Lymphocyte responses and cytokines. Cell 76:241, 1994

5. Hsieh CS, Macatonia SE, Tripp CS, Wolf SF, O'Garra A, Murphy KM: Development of Th1 $CD4^+$ T cells through IL-12 produced by *Listeria*-induced macrophages. Science 260:547, 1993

6. Trinchieri G: Interleukin-12: A proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen specific adaptive immunity. Annu Rev Immunol 13:251, 1995

7. Frankenberger M, Sternsdorf T, Pechumer H, Pforte A, Loms Ziegler-Heitbrock HW: Differential cytokine expression in human blood monocyte subpopulations. Blood 87:373, 1996

8. D'Andrea A, Aste-Amezaga M, Valiante NM, Ma X, Kubin M, Trinchieri G: Interleukin 10 inhibits human lymphocyte interferon-γ production by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells. J Exp Med 178:1041, 1993

9. van der Pouw Kraan T, Boeije L, Smeenk R, Wijdenes J, Aarden L: Prostaglandin-E2 is a potent inhibitor of human interleukin-12 production. J Exp Med 181:775, 1995

10. Hilkens CM, Snijders A, Vermeulen H, van der Meide PH, Wierenga EA, Kapsenberg ML: Accessory cell derived IL-12 and prostaglandin-E2 determine the IFN-γ level of activated human $CD4^+$ T cells. J Immunol 156:1722, 1996

11. Daynes RA, Araneo BA: Contrasting effects of glucocorticoids on the capacity of T cells to produce growth factors interleukin-2 and interleukin-4. Eur J Immunol 19:2319, 1989

12. Ramirez F, Fowell DJ, Puklavec M, Simmonds S, Mason D: Glucocorticoids promote a Th2 cytokine response by $CD4^+$ T cells in vitro. J Immunol 156:2406, 1996

13. Nijhuis EWP, Hinloopen B, van Lier RAW, Nagelkerken L: Differential sensitivity of human naive and memory $CD4^+$ T cells for dexamethasone. Int Immunol 7:591, 1995

14. Nijhuis EWP, Hinloopen B, Odding J, Nagelkerken L: Abrogation of the suppressive effects of dexamethasone by PKC activation or CD28. Cell Immunol 156:438, 1994

15. Brinkman V, Kristofic C: Regulation by corticosteroids of Th1 and Th2 cytokine production in human $CD4^+$ effector T cells generated from $CD45RO^-$ and $CD45RO^+$ subsets. J Immunol 155:3322, 1995

16. Bohle B, Willheim M, Baier K, Stadler B, Spitzauer S, Scheiner O, Ebner C: Hydrocortisone enhances total IgE levels—but not the synthesis of allergen specific IgE—in a monocyte-dependent manner. Clin Exp Immunol 101:474, 1994

17. Wu CY, Sarfati M, Heusser C, Fournier S, Rubio-Trujillo M, Peleman R, Delespesse G: Glucocorticoids increase the synthesis of immunoglobulin E by interleukin-4 stimulated human lymphocytes. J Clin Invest 87:870, 1991

18. Nüsslein HG, Weber G, Kalden JR: Synthetic glucocorticoids potentiate IgE synthesis. Allergy 49:365, 1994

19. MacPhee I, Antoni F, Mason D: Spontaneous recovery of rats from experimental allergic encephalomyelitis is dependent on regulation of the immune system by endogenous adrenal corticosteroids. J Exp Med 169:431, 1989

20. Mason D: Genetic variation in the stress response: Susceptibility to experimental allergic encephalomyelitis and implications for human inflammatory disease. Immunol Today 12:57, 1991

21. Mason D, MacPhee I, Antoni F: The role of the neuroendocrine system in determining genetic susceptibility to experimental allergic encephalomyelitis in the rat. Immunology 70:1, 1990

22. Chikanza IC, Petrou P, Kingsley G, Chrousos G, Panayi GS: Defective hypothalamic response to immune and inflammatory stimuli in patients with rheumatoid arthritis. Arthritis Rheum 35:1281, 1992

23. D'Andrea A, Rengaraju M, Valiante NM, Chehimi J, Kubin M, Aste M, Chan SH, Kobayashi M, Young D, Nickbarg E, Chizzonite R, Wolf SF, Trinchieri G: Production of natural killer cell stimulatory factor (interleukin-12) by peripheral blood mononuclear cells. J Exp Med 176:1387, 1992

24. Shire D: An invitation to an open exchange of reagents and information useful for the measurement of cytokine mRNA levels by PCR. Eur Cytokine Network 4:161, 1993

25. Guery JC, Ria F, Galbiati F, Adorini L: Normal B cells fail to secrete interleukin 12. Eur J Immunol 7:1632, 1997

26. Snijders A, Hilkens CM, van der Pauw Kraan TC, Engel M, Aarden LA, Kapsenberg ML: Regulation of bioactive IL-12 production in lipopolysaccharide-stimulated human monocytes is determined by the expression of the p35 subunit. J Immunol 156:1207, 1996

27. Evans RM, Arriza JL: A molecular framework for the actions of glucocorticoid hormones in the nervous system. Neuron 2:1105, 1989

28. de Kloet ER: Brain corticosteroid receptor balance and homeostatic control. Front Neuroendocrinol 12:95, 1991

29. Munck A, Guyre PM, Holbrook NJ: Physiological functions of glucocorticoids in stress and their relation to pharmacological actions. Endocrine Rev 5:25, 1984

30. Kam JC, Szefler SJ, Surs W, Sher ER, Leung DY: Combination IL-2 and IL-4 reduces glucocorticoid receptor-binding affinity and T cells response to glucocorticoids. J Immunol 151:3460, 1993

31. Beck CA, Weigel NL, Moyer ML, Nordeen SK, Edwards DP: The progesterone antagonist RU 486 acquires agonist activity upon stimulation of cAMP signaling pathways. Proc Natl Acad Sci USA 90:4441, 1993

32. de Waal Malefyt R, Abrams J, Bennet B, Figdor CG, de Vries JR: Interleukin-10 (IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. J Exp Med 174:1209, 1991

33. van der Poll T, Barber AE, Coyle SM, Lowry SF: Hypercortisolemia increases plasma interleukin-10 concentrations during human endotoxemia—a clinical research center study. J Clin Endocrinol Metab 81:3604, 1996

34. Tabardel Y, Duchateau J, Schmartz D, Marecaux G, Shahla M, Barvais L, Leclerc JL, Vincent JL: Corticosteroids increase blood interleukin 10 levels during cardiopulmonary bypass in men. Surgery 119:76, 1996

35. Platzer C, Meisel Ch, Vogt K, Platzer M, Volk HD: Upregulation of monocytic IL-10 by tumor necrosis factor-alpha and cAMP elevating drugs. Int Immunol 4:517, 1995

36. Fushimi T, Okayama H, Seki T, Shimura S, Shirato K: Dexamethasone suppressed gene expression and production of interleukin-10 by human peripheral mononuclear cells and monocytes. Int Arch Allergy Immunol 112:13, 1997

37. Murphy TL, Cleveland MC, Kulesza P, Magram J, Murphy KM: Regulation of Interleukin 12(p40) expression through an NF-kB half-site. Mol Cell Biol 10:5258, 1995

38. Scheinman RI, Cogswell PC, Lofquist AK, Baldwin AS Jr: Role of transcriptional activation of IkBα in mediation of immunosuppression by glucocorticoids. Science 270:283, 1995

39. Auphan N, Di Donato JA, Rosette C, Helmberg A, Karin M: Immunosuppression by glucocorticoids: Inhibition of NF-kB activity through induction of IkB synthesis. Science 270:286, 1995

40. Blotta MH, de Kruyff RH, Umetsu DT: Corticosteroids inhibit IL-12 production in human monocytes and enhance their capacity to induce IL-4 synthesis in $CD4^+$ lymphocytes. J Immunol 158:5589, 1997

41. Rook GA, Hernandez-Pando R, Lightman SL: Hormones, peripherally activated prohormones and regulation of the Th1/Th2 balance. Immunol Today 7:301, 1994

42. Wilder RL: Neuroendocrine-immune system interactions and autoimmunity. Annu Rev Immunol 13:307, 1995

Appendix

The kinetics of IL-10 and IL-12p40 mRNA expression is not affected by glucocorticoids

As was shown in chapter 3, the differential sensitivity of IL-10 and IL-12 for GC is demonstrated at both the protein and mRNA level. However, as was explained in the Methods section of chapter 3, the effects of DEX on mRNA expression was measured at the optimal time points of mRNA expression for IL-10 and IL-12. Addition of DEX to the whole blood cultures might delay the expression of cytokine mRNA, leading to an overestimation of the suppressive effects on IL-12.

To investigate this possibility we stimulated whole blood cultures with LPS in the absence or presence of 1 μM DEX and isolated mRNA at several time points. The mRNA levels of the cytokines IL-10 and IL-12p40 were measured with the use of a semi quantitative PCR as described in the Methods of chapter 3.

In figure 1, which is a representative for 2 separate experiments, it is demonstrated that the optimal time-point of IL-12p40 expression is reached after 4 to 8 hours of stimulation with LPS and that IL-10 mRNA expression reaches its optimum after 16 hours of stimulation with LPS. Addition of DEX to the cultures almost completely abolished the expression of IL-12p40 mRNA, whereas expression of IL-10 mRNA was even enhanced. The kinetics of mRNA expression of both cytokines was not significantly affected by DEX.

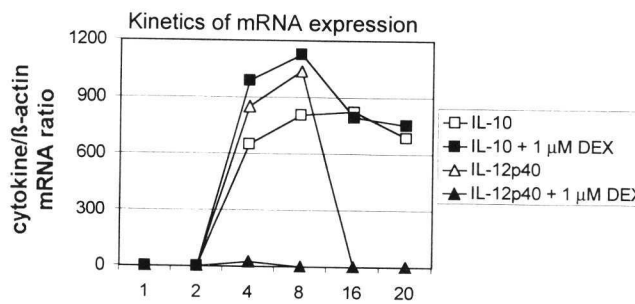


Fig 1. The kinetics of IL-10 and IL-12p40 mRNA expression is not affected by dexamethasone.

Taken together these results are strengthening the evidence as presented in chapter 3 for the selectivity in the effects of GC on IL-10 and IL-12.

CHAPTER FOUR

Disturbed regulation of cytokines by glucocorticoids in the Chronic Fatigue Syndrome

By Jeroen Visser [a, b], Willy Graffelman [c], Bep Blauw [a], Inge Haspels [a], Eef Lentjes [d], E. Ronald de Kloet [b] and Lex Nagelkerken [a].

a. Division of Immunological and Infectious Diseases, TNO Prevention and Health, Leiden, The Netherlands.
b. Division of Medical Pharmacology, Leiden Amsterdam Center for Drug Research, Leiden, The Netherlands.
c. Department of General Practice and Nursing Home Medicine, Leiden University Medical Center, Leiden, The Netherlands.
d. Department of Clinical Chemistry, Leiden University Medical Center, Leiden, The Netherlands.

Abstract

Previously $CD4^+$ T cells from Chronic Fatigue Syndrome (CFS)-patients were demonstrated to produce less IFN-γ and display an increased glucocorticoid sensitivity. Because IL-10 and IL-12 are important regulatory cytokines for IFN-γ production, the sensitivity of these cytokines for dexamethasone was studied in whole blood cultures from CFS-patients and controls.

CFS-patients showed an increased IL-10, a reduced IL-12p70 , but normal TNF-α production. The increased IL-10 was not due to viral IL-10 production.

IL-12 and TNF-α production by cells from patients and controls was equally sensitive to dexamethasone, but the patients showed increased dexamethasone sensitivity with regard to IL-10. Moreover, also IFN-γ induced IL-12p40 appeared more sensitive. Gender and duration of fatigue symptoms influenced the cytokine results, but not the sensitivity for dexamethasone. Interestingly, IL-10 and IL-12p40 production showed an inverse correlation with serum levels of cortisol in the controls, but not in the patients. Therefore, improper functioning of glucocorticoid-receptors may be involved in the regulation of these cytokines in CFS.

Introduction

The Chronic Fatigue Syndrome (CFS) is a disease of unknown origin characterized by severe disabling fatigue with a duration of more than six months and a reduction in normal daily activity of at least 50%. Several causes have been held responsible for the disease including viral infections, a disturbed hypothalamus-pituitary-adrenal-gland (HPA) axis and an altered immune function [1-4].

Although no infectious agent could thus far be related with the syndrome, several functional abnormalities of immune cells have been reported in CFS. These abnormalities include a decreased natural killer cell activity [5, 6], a reduced mitogenic response of lymphocytes [7, 8] and alterations in cytokine production [9-13]. These findings may in part be related to an altered composition of the peripheral blood mononuclear cells (PBMC) of the studied patients. In several reports CFS-patients showed increased numbers of $CD38^+$ $CD8^+$, $HLA\text{-}DR^+$ $CD8^+$, $CD5^+$ $CD19^+$ and $CD45RO^+$ $CD4^+$ cells and decreased numbers of $CD11b^+$ and $CD45RA^+$ $CD4^+$ cells [7-16]. However these findings are not consistent and show a lot of diversity. An explanation for this inconsistency could be the variation in the group of patients under investigation, since a study with a well-defined patient group and a well-matched control group showed no differences in the composition of the PBMC, although slight differences in cytokine production were observed [13].

Furthermore, when the patients were divided in subgroups according to disease onset or on the basis of how well they were feeling on the day of testing, the differences appeared to be more pronounced [13, 16]. Also when more homogenous subpopulations of cells are studied more pronounced differences can be detected. For example, we demonstrated that although no differences were found in PBMC cultures, purified $CD4^+$ T cells showed a decreased IFN-γ production [17]. Taken together the differences in immune parameters in CFS-patients demonstrated sofar, are subtle and this appears to be dependent on the parameter under investigation, the heterogeneity of the patient population and the quality of the control group.

As far as the functioning of the immune system is concerned, the integrity of the HPA-axis should also be taken into account. CFS-patients show lower levels of free cortisol in urine and reduced evening plasma cortisol levels, in conjunction with elevated plasma

levels of adrenocorticotropic hormone (ACTH) [18]. Moreover, a challenge with corticotropin-releasing hormone (CRH) or ACTH resulted in a reduced integrated ACTH or cortisol response, respectively, in CFS-patients compared to controls [18, 19]. These data are thus in support of an altered activity of the HPA-axis in relation to the disease. Various hormones belonging to the HPA-axis display immunomodulatory properties [20-22].

Whereas ACTH and CRH may activate monocytes and macrophages [20-23] , glucocorticoids (GC) in particular are known to display a wide variety of immunomodulatory properties. GC inhibit T cell proliferation and cytokine production [22, 24-29], cause apoptosis of lymphocytes [30] and reduce the capacity of dendritic cells to present antigen or activate T cells due to a downregulation of co-stimulatory molecules and cytokine production [31, 32].

In the past few years it has become clear that GC do not exclusively inhibit immune function, but dependent on the GC concentration, they may also have stimulatory effects. We have previously demonstrated that IL-10 synthesis is relatively resistant to GC as compared to IL-12, which is very sensitive to suppression by GC [28]. Because IL-12 is very important for a proper induction of IFN-γ production and the generation of Th1 responses [33] and because IL-10 can downregulate Th1 responses via the inhibition of IL-12 [34, 35], Th1 responses may be more easily suppressed by GC than Th2 responses.

Accordingly, low concentrations of GC have been found to stimulate the production of IL-4 and the proliferation of human PBMC and T cell clones *in vitro* [26] and to enhance ovalbumin-specific IL-4 production in the mouse both *in vivo* and *in vitro* [24]. Therefore, by altering the Th1/Th2 balance, GC may determine the outcome of an ongoing immune response. Our previous observation that $CD4^+$ T cells of CFS-patients produced less IFN-γ in association with an increased dexamethasone sensitivity is suggestive for a reduced Th1 activity in CFS-patients [17]. To establish whether such a decrease in cellular immunity would be due to an altered production of IL-10 and IL-12, we studied the production of these cytokines both on the level of protein and mRNA. Moreover, we established the sensitivity of both cytokines for endogenous and exogenous glucocorticoids in CFS-patients.

Materials and Methods

Subjects

SELECTION CRITERIA

We included patients between 18 and 50 years of age who met the CDC-criteria defined by Fukuda et al [2]. Patients suffering from somatic and psychiatric disorders and patients using beta-blockers, psychotropic drugs, immunosuppressive drugs or diuretics were excluded from the study. Furthermore patients with an alcohol intake of more than four units of alcohol per day and a body- mass index greater than 45 were excluded from the study.

RECRUITMENT OF PATIENTS AND CONTROLS

Patients were recruited by 38 general practitioners associated with the Department of General Practice and Nursing Home Medicine, Leiden University Medical Center (LUMC). We verified, by inspection of the available medical records, if the prospective patients met the above defined criteria. The general practitioners contacted a total of 66 patients to participate in the study. From these 66 patients, 59 patients gave their informed consent.

The patients were requested to recruit a healthy control matched for age, race and sex from their direct environment. From all the controls informed consent was obtained. The mean age of the CFS group was 38 ± 8 years; and the mean age of the control group was 38 ± 9 years. The female to male ratio in both groups was 2 to 1. Fasting blood samples of each patient and its matched control were obtained simultaneously at the patients home between 7.00 and 10.00 a.m.

This study was approved by the Medical Ethical Committee of the LUMC.

SUBGROUPING OF CASES

To reduce the heterogeneity of the patient population the patients were subgrouped by sex, duration of disease and a self reported energy score.

The duration of disease was expressed in months and the patients were divided in 2 groups with a duration of 6 to 60 months (N=24) or more than 60 months (N=32). The average duration of the illness was 83.7 months with a range from 11 to 274 months. From 3 patients the duration of the disease could not be determined. These 3 patients were all

fulfilling the Fukuda criteria being ill longer than six months, but it was not possible to determine ate the duration of the disease exactly and therefore assign them to one of the subgroup.

By a questionaire the patients and controls were asked to report their energy level. The self reported energy score was expressed on a scale from 1 to 100, being the lowest and highest energy level respectively. With the exception of one control person, all controls reported an energy score ≥ 50. The average energy score of the controls was 78.8 (45-100). Seven patients reported an energy score ≥ 50. The average energy score of the patients was 30 (5-90). These energy scores are very subjective, as they are based on the patients and controls own perception. Therefore we only subdivided our patient population in this report according to the objective criteria duration of the disease and gender.

Antibodies and reagents

Anti-IL-12 mAb C11.79, C8.6 and 20C2 were kindly provided by Dr. T van der Pouw Kraan (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands), and Dr. D.H. Presky (Hoffmann-La Roche, Nutley, NJ). These antibodies recognize both IL-12p40 and the bioactive heterodimer p70, consisting of p40 and p35 as previously described [35].

Anti- IL-10 mAb (JES3-9D7 and biotinylated JES3-12G8), biotinylated anti-viral IL-10 JES3-6B11, anti- human TNF-α mAb (MAb1 and biotinylated Mab11) were purchased from Pharmingen (San Diego, CA).

Recombinant human IL-12 was purchased from R&D Systems (Abington, UK), recombinant human IL-10 was kindly provided by Dr. S. Narula (Schering Plough Research Institute, Kenilworth, NJ), recombinant viral IL-10 and recombinant human TNF-α were obtained from Pharmingen.

The glucocorticoid-receptor (GR) antagonist RU486 (Roussel-UCLAF, Romaineville, France) was a kind gift of Dr. Win Sutanto (Division of Medical Pharmacology, LACDR, Leiden, The Netherlands). Dexamethasone (DEX) was purchased from Sigma (St. Louis, MO).

Phenotypic analysis

Forty µl of whole blood were incubated with a saturating amount of fluorescein-isothiocyanate (FITC), phycoeryhtrin (PE) or Peridinin chlorophyll protein (PercP)-conjugated mAbs.

The following mAbs were used: anti-CD3-FITC, anti-CD4-PercP, anti-CD8-PE, anti-CD14-FITC, anti-CD16-FITC, anti-CD19-FITC, (all from Becton Dickinson, Mountain View, CA) and anti-CD45RA-PE, anti-CD45RO-FITC, anti-CD56-PE (CLB, Amsterdam, The Netherlands).

Erythrocytes were lysed with FACS lysing solution (Becton Dickinson) according to the manufacturers description. After washing with PBS the cells were fixated with 0.5 % formaldehyde.

Cells were analyzed with the use of a FACScan (Becton Dickinson).

Whole blood cultures

Whole blood was collected by venapuncture in heparinized blood collecting tubes (Becton Dickinson). The blood was five-fold diluted in Iscoves Modified Dulbecco's Medium (IMDM) supplemented with glutamax (Gibco, Paisley, UK), 10% FCS (Sebak, Gmbh, Aidenbach, Germany), 50 µM ß-mercaptoethanol, 100 U/ml penicillin and 100 µg/ml streptomycin. Fifty µl of the diluted blood was cultured in 96-wells flat-bottomed microtiterplates (Costar, Cambridge, MA) in a final volume of 200 µl per well. Cells were stimulated with 250 ng/ml, 500 ng/ml or 1000 ng/ml LPS *(Escherichia coli* serotype 0127: B8, Sigma) to induce cytokine production; IL-12p70 was induced by stimulation with 250 ng/ml LPS in the presence of 1000 IU/ml IFN-γ (a kind gift of Peter van der Meide, BPRC, Rijswijk, The Netherlands).

A dose related response to DEX or HC (both from Sigma) was studied by the addition of these hormones to the culture wells in a final concentration ranging from 10^{-10} M to 10^{-5} M. Stock solutions of 10^{-2} M HC and $2x10^{-2}$ M DEX were prepared in dimethylsulfoxide (DMSO; Merck, Darmstadt, Germany) and stored at –20°C in 0.2 ml aliquots. To establish the specificity of the effects of these hormones, the GR antagonist RU486 was used at a concentration of 10 or 1 µM (diluted from a 0.1 M stock prepared in DMSO).

The whole blood was put into culture within 2 h after venapuncture. For assessment of cytokines the supernatants of the cultures were harvested after 24 h of culture and stored at –20°C.

Assays for cytokines

For the IL-12p40 ELISA, mAb C11.79 (2 μg/ml in 50 mM $NaHCO_3$, pH 9.5, 50 μl/well) was coated overnight at 4°C on round bottomed microtiterplates with high binding capacity (Greiner, Nürtingen, Germany). For the IL-12p70 ELISA plates were coated with mAb 20C2 (2 μg/ml in 50 mM $NaHCO_3$, pH 9.5, 50 μl/well). As for all subsequent washing steps, the plates were washed 6 times with PBS containing 0.05% Tween-20. Subsequently, the plates were blocked for 1.5 h with 200 μl PBS containing 0.2% gelatin and 0.05% Tween-20 (PTG).

After washing, 50 μl diluted biotinylated mAb C8.6 in PTG (final concentration 0.25 μg/ml) was added per well together with 50 μl of the undiluted samples and these were simultaneously incubated for 2 h. After washing, the plates were incubated for 1 h with 75 μl/well poly-streptavidin-horseradish peroxidase (CLB, Amsterdam, The Netherlands) 1:10,000 diluted in PTG. Finally, after washing, the plates were developed with 100 μl/well 0.1 M 3,5,3',5'-tetramethyl-benzidine (TMB; Merck) in 0.11 M sodium acetate pH 5.5 containing 0.003% H_2O_2. Colour development was stopped by the addition of 50 μl 2 M H_2SO_4 to each well. The absorbances were measured at 450 nm in a Biorad 3500 platereader (Biorad, Richmond, CA). Recombinant human IL-12 diluted in culture medium was used as a standard; the standard curves ranged from 4000 to 16 pg/ml.

The IL-10 ELISA was performed in an identical fashion. The plates were coated with JES3-9D7 mAb (0.5 μg/ml) and biotinylated JES3-12G8 mAb was used in a concentration of 2 μg/ml. Recombinant human IL-10 diluted in culture medium was used as a standard. The standard curves ranged from 2500 to 10 pg/ml.

For the detection of viral IL-10 ELISA, the plates were coated with JES3-9D7 mAb (1 μg/ml) and biotinylated JES3-6B11 mAb was used in a concentration of 2 μg/ml. Recombinant viral IL-10 diluted in culture medium was used as a standard. The standard curves ranged from 2500 pg/ml to 10 pg/ml.

For the TNF-α ELISA the plates were coated with 1 μg/ml MAb1; biotinylated MAb11 was used in a concentration of 1 μg/ml for detection. The supernatants were tested in

a five-fold dilution in culture medium. Recombinant TNF-α diluted in culture medium was used as a standard. The standard curves ranged from 10000 to 40 pg/ml.

Cortisol measurement

Blood from the patients and the controls was collected, immediately put on ice and allowed to coagulate. The tubes were spun down for 30 minutes (3000 rpm, 4°C); serum was collected and immediately stored at –20°C. Plasma total cortisol was measured using the fluorescent polarization immuno assay on the TDx from Abbott (Amstelveen, The Netherlands). Plasma free cortisol concentrations were measured in the filtrate obtained by temperature-controlled ultrafiltration using the amicon MPS-1 ultrafiltration device according to Lentjes et al. [36].

RNA quantitation using semi-quantitative PCR

mRNA was extracted from the PBMC and used to measure IL-10 , IL-12p35, IL-12p40 and IFN-γ mRNA in a semiquantitative manner as described previously [29, 37]. cDNA as a read-out of the mRNA was quantitated in a PCR using the PQB-3 and the PQA-1 vector [37] as an external standard, which contains primer sequences for human IL-10, β-actin and IFN-γ respectively. These vectors were kindly provided by Dr. D. Shire (Sanofi, Labège, France).

To enable quantitation of IL-12 p40 and IL-12p35 cDNA, 2 complementary 40-mer sequences each encompassing a 20-mer sequence of IL-12p40 and of IL-12p35 were cloned into the *HIND* III (for IL-12p40 and p35 sense primers) and *BAM*HI sites (for IL-12p40 and p35 antisense primers) of the PQA-1 vector. By means of a parallellism test, PQB-3 and PQA-1/IL-12 were verified to be amplified equally efficient as cDNA from mRNA encoding cytokines or β-actin, when amplified with IL-10, β-actin, IFN-γ, IL-12p40 or p35 specific primers. With regard to viral IL-10, a known amount of cDNA obtained from EBV transformed B cell lines both in the lytic and latent phase of infection was stepfold diluted and amplified with specific viral IL-10 primers.

In addition to measure viral IL-10 mRNA expression, mRNA was amplified using the following primers: Viral IL-10 sense: TGTGGAGGTACAGACCAATGT, Viral IL-10 antisense: CACCTGGCTTTAATTGTCATG.

PCR products were stained on 1% agarose gels with ethidium bromide or SYBR Green I (Biozym, Landgraaf, The Netherlands). Densities of the amplified vector (known amount in fg) and of the amplified cDNA (unknown amount) were analyzed using the

Bio-1D digital imaging system version 6 (Vilber Lourmat, Marne La Vallée, France). The comparison of these densities enabled the subsequent calculation of amplified β-actin or cytokine cDNA in fg. Results are expressed as a ratio of quantified cytokine product (in fg) over β-actin product (in fg).

Data processing and statistics

The curvefitting option in the Biorad microplatemanager software was applied to calculate the cytokine concentrations in the supernatants. Statistical analysis was performed using the Rank-Wilcoxon test for matched pairs and the Mann Whitney *U* test. Correlation's were established using the nonparametric Spearman correlation test. Differences with a confidence level of 95 % or higher were considered to be statistically significant ($p<0.05$).

Results

Increased IL-10 and a decreased IL-12 production in whole blood cultures of CFS-patients.

Previous experiments with purified T helper cells of CFS-patients revealed a decreased IFN-γ production [17]. Therefore we studied the production of IL-10 and IL-12, cytokines that inhibit and stimulate IFN-γ production, respectively [33-35]. As shown in Table 1, whole blood cultures of CFS-patients display an increased production of IL-10 ($p<0.05$) upon stimulation with LPS and a trend ($p=0.07$) towards a reduced production of IL-12p70 upon stimulation with LPS + IFN-γ. These findings are in line with the reduced IFN-γ production found in CFS-patients.

As was demonstrated previously subgrouping of CFS-patients can reveal differences in cytokine production in certain subgroups of CFS-patients [13, 14]. Likewise we subgrouped the CFS-patients according to gender and duration of the fatigue symptoms. Interestingly only the patients who were ill less than 5 years showed a significantly increased production of IL-10 as compared to their matched controls. Furthermore only the female CFS-patients demonstrated an increased IL-10 production as compared to the matched controls (data not shown). With regard to IL-12p70, a trend towards a reduced production was only observed in the total group of CFS-patients. The lack of any difference in the IL-12p40 and TNF-α production between patients and controls (Table 1) supports the specificity of our observations.

Table 1. Mean cytokine levels in whole blood cultures in subgroups of CFS-patients compared to matched controls

Cytokine [a]	CFS	Controls	N	p [b]
IL-10				
Total group	285 ± 27	229 ± 27	48	.047
≤ 5 yr	289 ± 21	203 ± 37	21	.022
> 5 yr	270 ± 48	214 ± 22	27	.453
IL-12p70				
Total group	113 ± 14	162 ± 19	29	.074
≤ 5 yr	101 ± 25	144 ± 28	11	.263
> 5 yr	123 ± 20	173 ± 29	18	.163
IL-12p40				
Total group	1087 ± 24	978 ± 100	43	.587
≤ 5 yr	628 ± 88	780 ± 77	19	.177
> 5 yr	1568 ± 93	1261 ± 80	24	.286
TNF-α				
Total group	2137 ± 528	1222 ± 85	35	.510
≤ 5 yr	1950 ± 1031	1080 ± 263	15	.534
> 5 yr	2521 ± 1011	1269 ± 288	20	.382

Note: Results are expressed in pg/ml ± SEM.

a. Results for the total group or patients with disease duration less or more than 5 years as compared to matched controls.

b. Wilcoxon rank sum test for matched pairs.

Since we did not observe differences between patients and controls with respect to plasma levels of these cytokines (Table 2), our data were suggestive for a different activation state of mononuclear cells in CFS-patients. To address this possibility, we employed a semi-quantitative RT-PCR analysis to investigate the mRNA expression of various cytokines in unstimulated PBMC of 20 randomly selected patients and matched controls. Both in patients and controls, the mRNA expression of IL-12p40 was below the detection limit, confirming previous data in the literature [38, 39]. Although mRNA for IL-10, IL-12p35 and IFN-γ was detectable, no differences in mRNA expression could be demonstrated for these cytokines (Table 3).

Table 2. Mean cytokine levels in plasma of CFS-patients and matched controls.

Cytokine	CFS	Controls	N	p[a]
IL-10	30 ± 15	29 ± 12	33	NS
TNF-α	352 ± 155	388 ± 142	33	NS
IL-12p40	216 ± 55	278 ± 86	33	NS

Note: Results are expressed in pg/ml ± SEM. NS: not significant.
a. Wilcoxon rank sum test for matched pairs.

Because the ELISA for IL-10 potentially detects both human and viral IL-10, the latter being encoded by the Epstein Barr virus, we also verified the production and mRNA expression of viral IL-10. We were unable to detect significant levels of viral IL-10 protein by a specific viral IL-10 ELISA in the supernatants of whole blood cultures of all tested patients and controls. Likewise, mRNA specific for viral IL-10 was undetectable in unstimulated PBMC from patients and controls (Table 3).

EBV transformed B-cell lines that were used as a positive control showed high levels of viral IL-10 mRNA expression both in the latent and lytic phase of infection.

Table 3. Mean cytokine mRNA expression in unstimulated PBMC of CFS-patients compared to matched controls.

Cytokine	CFS	Controls	N	P[a]
viral IL-10	nd	nd	20	-
IL-12p35	60 ± 17	150 ± 98	20	NS
IL-12p40	nd	nd	20	-
IFN-γ	105 ± 64	29 ± 6	20	NS

Note: Results are expressed as a ratio (x 10^{-4}) of quantified cytokine product (in femtograms) over β-actin product (in femtograms) ± SEM. NS: not significant. nd: not detectable.
a. Wilcoxon rank sum test for matched pairs.

CFS-patients show increased levels of CD45RA positive T helper cells

Several reports have shown changes in the distribution of lymphocyte subsets in the whole blood of CFS-patients [7-16]. Upon stimulation with LPS especially the monocytes can become potent producers of IL-10 and IL-12. Therefore a difference in the number of monocytes or other cell types in whole blood of CFS-patients might be responsible for the observed differences in IL-10 and IL-12 production upon stimulation with LPS. However as demonstrated in Table 4, extensive flowcytometric analysis of the whole blood and peripheral blood mononuclear cells, revealed that cell subsets that are capable of IL-10 and IL-12 production upon stimulation with LPS like monocytes, B cells and granulocytes, were not more or less prevalent in CFS-patients. However, a slight increase in the fraction of

$CD45RA^+$ $CD4^+$ T cells in CFS patients ($p<0.05$) was observed. This increase was detected in all the subgroups of CFS-patients and no differences were found with regard to the composition of the other lymphocyte subsets.

Table 4. Mean percentage of lymphocyte subsets in whole blood of CFS-patients and matched controls.

	CFS	Controls	N
Leukocytes (10^6/ml)	7.0 ± 2.3	6.4 ± 2.2	49
PMNL	55.8 ± 2	56.2 ± 2	49
CD3+	27,3 ± 6.4	25.2 ± 7.3	49
CD4+	16.1 ± 5.0	14.7 ± 5.0	49
CD8+	6.9 ± 2.5	7.2 ± 2.9	46
CD14+	5.3 ± 1.6	5.6 ± 1.5	48
CD19+	4.1 ± 1.9	4.0 ± 2.1	49
CD16+/CD56+	5.1 ± 5	4.2 ± 2.1	49
CD45RA+ within CD4	39.9 ± 12.4	32.2 ± 11.0[a]	49
CD45RO+ within CD4	35.2 ± 10.2	38.9 ± 12.0	49

Note: Results are expressed as percentages ± SD. PMNL: Polymorphonuclear leukocytes.
a. $P < 0.05$ Mann-Whitney U test.

Cytokine production in whole blood cultures of CFS-patients is more sensitive to glucocorticoids

As shown above, whole blood cultures of the CFS-patients have an increased production of IL-10. In previous experiments we have demonstrated that the cytokine IL-10 is relatively resistant to GC or can even be stimulated by this hormone, whereas IL-12 and TNF-α are very sensitive to inhibition by GC [29]. Because purified $CD4^+$ T cells of CFS-patients displayed an increased sensitivity to DEX [17], the increased production of IL-10 in whole blood of CFS-patients might be the result of an altered regulation of cytokine production by

GC in CFS-patients. To investigate this possibility we studied the regulation of IL-10, IL-12 and TNF-α by exogenous GC in whole blood cultures of CFS-patients.

For each donor we calculated the amount of DEX in nmol/l that was required to achieve 50% inhibition of cytokine production (IC50) and calculated the mean IC50 of the tested donors for each cytokine. As shown in Table 5 there was no difference in sensitivity to DEX between CFS-patients and controls with regard to LPS induced IL-12p40 and TNF-α production. Furthermore, the DEX sensitivity of these cytokines was not related to duration of disease or gender.

IL-12p70 induced with LPS +IFN-γ did not show differences in sensitivity to DEX when CFS patients and controls were compared (Table 5). However the LPS + IFN-γ induced IL-12p40 production was more suppressed by DEX in the CFS-patients.

IL-10 was relatively resistant for suppression by DEX and an IC50 concentration for DEX could only be calculated in 28 out of 48 controls and in 34 out of 48 CFS-patients. When the sensitive donors were compared, it appeared that patients needed less DEX to achieve 50% inhibition of IL-10 production as compared to the sensitive donors in the control group (P=0.001, Mann Whitney *U* test). This increased sensitivity was not related to disease duration or gender.

The suppression of cytokine production by DEX was mediated via the GR, because RU486 antagonized the suppression completely (data not shown).

Table 5. Mean relative sensitivity (IC50) of cytokines to dexamethasone in LPS stimulated whole blood cultures in subgroups of CFS-patients compared to matched controls.

Cytokine [a]	CFS	N	Controls	N	P [b]
IL-10					
Total group (insensitive)	>>10000	14	>>10000	20	-
Total group (sensitive)	342 ± 84	34	1983 ± 519	28	.001[c]
≤ 5 yr (insensitive)	>>10000	6	>>10000	6	-
≤ 5 yr (sensitive)	353 ± 127	15	2683 ± 879	15	.004[c]
> 5 yr (insensitive)	>>10000	8	>>10000	14	-
> 5 yr (sensitive)	376 ± 136	19	1340 ± 667	13	.091[c]
TNF-α					
Total group	42 ± 12	35	38 ± 7	35	.727
≤ 5 yr	39 ± 13	12	24 ± 9	15	.197
> 5 yr	53 ± 22	19	48 ± 10	20	.407
IL-12p40					
Total group	141 ± 43	43	145 ± 44	43	.868
≤ 5 yr	80 ± 33	18	163 ± 71	19	.518
> 5 yr	180 ± 76	22	126 ± 64	24	.671
LPS + IFN-γ induced [d]					
IL-12p70	59 ± 20	15	29 ± 8	15	.523
IL-12p40	30 ± 21	15	952 ± 729	15	.040

Note: Results are expressed in nmol/L ± SEM.
IC50: GC concentration in nmol/L required for 50% inhibition of cytokine production.
a. Results of the total group or patients with a disease duration less or more than 5 years.
b. Wilcoxon rank sum test for matched pairs.
c. Mann-Whitney U test.
d. Whole blood stimulated with 0.25 μg/ml LPS + 1000 IU/ml IFN-γ

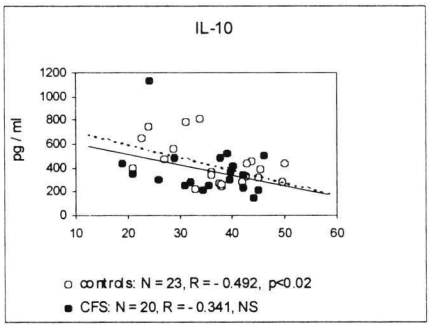
Disturbed relation between endogenous cortisol and cytokine production in whole blood cultures of CFS-patients.

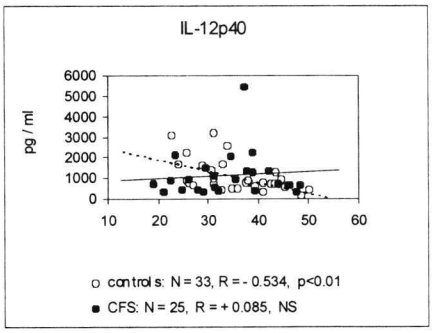
We next investigated whether a relation existed between absolute cytokine levels and endogenous cortisol.There was no difference in cortisol levels in serum between CFS-patients and the controls. Serum of the patients showed 0.68 ±0.26 μmol/liter total cortisol and 41.4 ± 10 nmol/liter free cortisol, whereas the serum concentrations in the controls were 0.73 ± 0.2 μmol/liter and 38.2 ± 16.6 nmol/liter respectively.

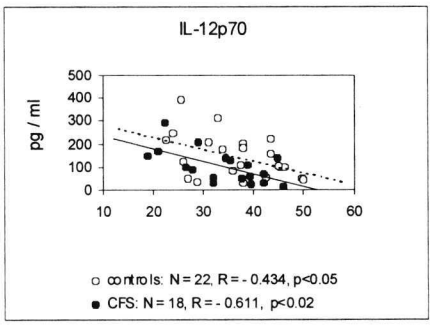
As shown in Figure 1, cytokine production in whole blood cultures of healthy controls was inversely related to the endogenous levels of free cortisol. This was found for LPS induced IL-10, IL-12p40 and LPS + IFN-γ induced IL-12p70. In contrast, CFS-patients showed such a correlation only with IL-12 p70, whereas IL-10 and IL-12p40 production appeared independent of endogenous cortisol.

In neither the patients or controls a relation between endogenous cortisol and the absolute number of leucocytes was found (data not shown). In the controls, however, the relative and absolute number of T cells and naive T helper cells was positively correlated with endogenous cortisol, while in the patients, which showed an increased number of naive cells (see Table 4), such a relation was not found (data not shown). With regard to the other lymphocyte subpopulations no such correlation was found both in patients and controls.

Figure 1 IL-10 and IL-12p40 production in whole blood cultures of CFS-patients is not related to endogenous free cortisol.







Whole blood was stimulated with 0.25µg/ml LPS for IL-10 (top left) and IL-12p40 (top right) production. IL-12p70 (bottom) was induced by 0.25 ug/ml LPS + 1000 IU/ml IFN-γ Cytokines were determined in the supernatants by ELISA after 24 h of culture.
The correlation coefficient and statistical significance was calculated according to the method of Spearman. Closed symbols represent the patients, open symbols the controls.

Discussion

In this study we observed subtle changes in cytokine production in whole blood cultures of carefully selected CFS-patients, and a disturbed regulation of cytokine production by endogenous and exogenous GC.

In whole blood cultures of CFS-patients we detected an increased production of IL-10 and a trend to a lower production of IL-12p70. When the cytokine production was corrected for the number of leukocytes, both the increase in IL-10 production and the decrease in IL-12p70 production appeared to be significant ($p<0.05$). Although human eosinophils and dendritic cells have been shown to produce IL-12 [31, 32, 40], the most significant contribution to IL-12p70 production in whole blood cultures is most likely from monocytes.

Also on the basis of monocyte numbers, CFS-patients showed an increased IL-10 production and a reduced IL-12p70 production. Because the balance between IL-10 and IL-12 is a major determinant in the regulation of IFN-γ production [33-35], the results presented in this study can explain our previous observation that IFN-γ production by PHA stimulated $CD4^+$ T cells of CFS-patients is decreased [17]. The cytokine production in the whole blood cultures of the CFS-patients was influenced by the heterogeneity of the patient population. When the patients were subdivided according to duration of fatigue symptoms and gender, the increased IL-10 production appeared to be prominent in the female CFS-patients and the patients with a disease duration less than 5 years. The trend to a reduced IL-12p70 production was only detected in the total patient group.

Flowcytometric analysis revealed that CFS-patients displayed a higher percentage of $CD45RA^+$ cells within the $CD4^+$ T cell subset and this was also detected in the subgroups of the patients compared to their matched controls. These results are conflicting with data reported in the literature [7-16] which are suggestive for a decrease or no change in naive T helper cells of CFS-patients. Possibly the composition of the patient and control groups could be responsible for these observed differences.

It has been shown that the naive T helper cells produce approximately tenfold less IL-10 than memory T helper cells [41], which makes it unlikely that the increase in IL-10 production in CFS patients can be attributed to this subset.

In unstimulated PBMC of CFS-patients and controls it was impossible to detect IL-12p40 mRNA, confirming previous data in the literature [38]. It also indicates that the

cells of CFS-patients are not activated *in vivo*, in contrast to for instance PBMC of MS patients that do not require stimulation for the detection of IL-12p40 mRNA expression [39]. This is further supported by our observation that mRNA expression of IL-10, IL-12p35 and IFN-γ was not different between patients and controls.

We were unable to detect viral IL-10 in the supernatants of the whole blood cultures or on the level of mRNA. Our data suggest that these CFS-patients do not experience an active EBV infection, or the reactivation of EBV, because viral IL-10 can be detected in this class of patients [42]. Accordingly an active EBV infection or reactivation of EBV could not be established by serology [9].

The results presented in this article indicate that cytokine production in CFS-patients is differentially regulated by GC. One of the findings in favor of this idea, is the increased sensitivity of IL-10 and IL-12p40 to suppression by DEX in whole blood cultures. Categorizing the patients according to gender or duration had no influence on cytokine sensitivity for DEX as is shown in Table 5. These data are in line with our previous observation of an increased DEX-sensitivity of proliferation and IL-4 production using purified $CD4^+$ T cells from a different population of CFS-patients [17].

Further evidence for an altered regulation of cytokines by GC in CFS-patients comes from the lack of correlation between endogenous cortisol and IL-10 and IL-12p40 production in whole blood cultures. This uncoupling between endogenous cortisol and immune parameters in CFS-patients was also demonstrated by Cannon et al [43], who showed no relation between endogenous cortisol and stress induced neutrophil mobilization.

The addition of saturating amounts of IFN-γ to the whole blood cultures of the 15 tested patient-control combinations (see Table 5), increased the IC-50 for DEX with regard to IL-12p40 production in the controls from 101 ± 60 nmol/liter to 952 ± 729 nmol/liter (data not shown; $p=0.09$). As opposed to the controls, IFN-γ did not change the sensitivity for DEX with regard to IL-12p40 in the patients. These results suggest that an impaired response to IFN-γ may be responsible for the reduced IL-12p70 production in the patients. However, such a difference in response to IFN-γ was only observed with regard to the DEX sensitivity, as IFN-γ caused a similar increase of IL-12p40 production and a suppression of IL-10 production in both patients and controls (data not shown).

It has been demonstrated that IFN-γ can interfere with the affinity of the GR [44]; therefore differences in effects of IFN-γ on DEX sensitivity between patients and controls might be due to differences in the effects of IFN-γ on the GR rather than a dysfunctioning of the IFN-γ receptor.

The anti-inflammatory cytokines IL-4 and IL-10 show an increased sensitivity for GC in CFS-patients, while the pro-inflammatory cytokines IL-12 and TNF-α were equally sensitive for GC as compared to healthy controls. This is of interest because normally the proinflammatory cytokines are much more sensitive for GC suppression as compared to the anti-inflammatory cytokines [22, 28, 29].

A mechanism explaining these results could be a disturbed interaction between the GC-GR complex and transcription factors like AP-1 and NFkB, which are intermediates in the suppressive effects of GC, or via upregulation of inhibitory proteins like IkB [45, 46]. An impaired interaction between GC-GR complexes and such transcription factors might result in an altered response to GC. Barnes et al. [47] have substantiated this hypothesis by demonstrating that such an impaired interaction between AP-1 and the GC-GR complex causes steroid resistance in a subset of asthma patients.

Whereas AP-1 is of importance for the regulation of the production of the cytokines IL-2, IL-4 and IL-10 [25, 48-50], NFkB is important in the regulation of IL-12 and TNF-α [45, 46, 51]. An impaired interaction between AP-1 and the GC-GR complex in CFS-patients might thus be responsible for the observed increase in sensitivity for GC with regard to proliferation (which is IL-2 dependent), IL-4 and IL-10 production as demonstrated previously [17] and in this paper. The fact that RU486 is equally effective in antagonizing the suppression by GC in both patients and controls (data not shown) is in support of this possibility, since this GR antagonist inhibits the binding of GC to the GR without having an effect on the interaction between the GR and transcription factors.

In our study neither of the groups showed a relation between endogenous cortisol and the absolute number of leucocytes (data not shown). In the controls the relative and absolute numbers of T cells and naive T helper cells were positively correlated with endogenous cortisol, while in the patients such a relation was not found (data not shown). With regard to the other lymphocyte subpopulations such a correlation was not found in patients and controls.

Taken together the cytokine profiles in whole blood cultures of CFS-patients are much more affected by the heterogeneity of the patient population as compared to the alterations in GC sensitivity. This means that studying the mechanisms involved in GC sensitivity could be promising for the development of an objective diagnostic marker and therapy for CFS.

In conclusion our results demonstrate an altered cytokine profile in whole blood cultures of CFS-patients that may be the result of an impaired regulation by glucocorticoids. This impaired regulation is evidenced by the increased sensitivity for GC and the disturbed relation between endogenous GC and cytokine production in whole blood cultures. The possibility that an altered functionality of the GR or an impaired interaction between the GC-GR complexes and transcription factors is responsible for this impaired regulation, is currently under investigation.

Acknowledgements

We thank Arrianne Plomp for providing us with the EBV transformed B cell lines. Furthermore we would like to thank Dr. C. Lucas for critically reading the manuscript.

References

1. Holmes GP, Kaplan JE, Gantz NM, et al. Chronic fatigue syndrome: a working case definition. Ann Intern Med 1988; 108: 387-9.

2. Fukuda K, Strauss SE, Hickie I, et al. The chronic fatigue syndrome: a comprehensive approach to its definition and study. Ann Intern Med 1994; 121: 953-9.

3. Bearn J and Wessely S. Neurobiological aspects of the chronic fatigue syndrome. Eur J Clin Invest 1994; 24: 79-90.

4. Strauss SE, Komaroff AL, Wedner HJ. Chronic Fatigue syndrome: Point and counterpoint. J Infect Dis 1994; 170: 1-6.

5. Aoki T, Miyakoshi H, Usuda Y, Herberman RB. Low NK syndrome and its relationship to chronic fatigue syndrome. Clin Immunol Pathology 1993; 69 (no 3): 253-65.

6. Caliguiri M, Murray C, Buchwald D, et al. Phenotypic and functional deficiency of natural killer cells in patients with chronic fatigue syndrome. J Immunol 1987; 139: 3306-13.

7. Barker E, Fujimura SF, Fadem MB, Landay AL, Levy JA, Immunologic abnormalities associated with chronic fatigue syndrome. Clin Infect Dis 1994; 18 (suppl): s157-9.

8. Strauss SE, , Fritz S, Dale JK, Gould B, Strober W. Lymfocyte phenotype and function in the chronic fatigue syndrome. J Clin Immunol 1993; 13: 30-40 .

9. Swanink CMA, Vercoulen JHMM, Bleijenberg G, Fennis JFM, Galema JMD, van der Meer. Chronic fatigue syndrome: a clinical and laboratory study with a well matched control group. J Intern Med 1995; 237: 499-506.

10. Klimas NG, Salvato FR, Morgan R, Fletcher MA. Immunologic abnormalities in chronic fatigue syndrome. J Clin Microbiol 1990; 28: 1404-10.

11. Chao CC, Janoff EN, Hu S, et al. Altered cytokine release in peripheral blood mononuclear cell cultures from patients with the chronic fatigue syndrome. Cytokine 1991; 3: 292-8.

12. Swanink CMA, Vercoulen JHMM, Galama JMD, et al. Lymphocyte subsets, apoptosis, and cytokines in patients with chronic fatigue syndrome. J Infect Dis 1996; 173: 460-3.

13. Mawle AC, Nisenbaum R, Dobbins JG, et al. Immune responses associated with chronic fatigue syndrome: A case-control study. J Infect Dis 1997; 175: 136-41.

14. Hassan IS, Bannister BA, Akbar A, Weir W, Bofill M. A study of the immunology of the chronic fatigue syndrome: correlation of immunologic parameters to health dysfunction. Clin Immunol Immunopathol 1998; 87 (1): 60-7.

15. Tirelli U, Marotta G, Improta S, Pinto A. Immunological abnormalities in patients with chronic fatigue syndrome. Scand J Immunol 1994; 40: 601-08.

16. Landay AL, Jessop C, Lennette ET, Levy JA. Chronic fatigue syndrome: clinical condition associated with immune activation. Lancet 1991; 338: 707-12.

17. Visser J, Blauw B, Hinloopen B, Brommer E, de Kloet ER, Kluft C, Nagelkerken L. CD4 T lymphocytes from patients with chronic fatigue syndrome have decreased interferon-γ production and increased sensitivity to dexamethasone. J Infect Dis 1998; 177: 451-4.

18. Demitrack MA, Dale JK, Strauss SE, et al. Evidence for impaired activation of the hypothalamic-pituitery-adrenal axis in patients with chronic fatigue syndrome. J Clin Endocrinol Metab 1991; 73: 1224-34.

19. Scott LV, Medbak S, Dinan TG. The low dose ACTH test in chronic fatigue syndrome and health. Clin Endocrinol 1998; 48: 733-37.

20. Blalock JE. The syntax of immune-neuroendocrine communication. Immunol Today 1994; 15: (No 11): 504-11.

21. Wilder, RL Neuroendocrine-immune system interactions and autoimmunity. Annu Rev Immunol 1995; 13: 307-38.

22. Rook GAW, Hernandez-Pando R, Lightman S. Hormones, peripherally activated prohormones and regulation of the Th1/Th2 balance. Immunol Today 1994; 15: 301-3

23. Mastorakos G, Bouzas EA, Silver PB, et al. Immune corticotropine releasing hormone is present in the eyes of and promotes experimental autoimmune uveoretinitis in rodents. Endocrinology 1995; 136: 4650-58.

24. Daynes RA, Araneo BA. Contrasting effects of glucocorticoids on the capacity of T cells to produce growth factors Interleukin-2 and Interleukin-4. Eur J Immunol 1989; 19: 2319-25.

25. Vacca A, Felli MP, Farina AR, et al. Glucocorticoid receptor-mediated suppression of the Interleukin-2 gene expression through impairment of the cooperativity between nuclear factor activated t cells and AP-1 enhancer elements. J Exp Med 1992; 175: 637-46.

26. Snijdewint FGM, Kapsenberg ML, Wauben-Penris PJJ, Bos JD. Corticosteroids class-dependently inhibit Th1- and Th2-type cytokine production. ImmunoPharmacol 1995; 29: 93-101.

27. Brinkman V, Kristofic C. Regulation by corticosteroids of Th1 and Th2 cytokine production in human $CD4^+$ effector T cells generated from $CD45RO^-$ and $CD45RO^+$ subsets. J Immunol 1995; 155: 3322-28.

28. Wilckens T, de Rijk R. Glucocorticoids and immune function: unknown dimensions and new frontiers. Immunol Today 1997; 18(9): 418-24.

29. Visser J, van Boxel-Dezaire A, Methorst D, Brunt T, de Kloet ER, Nagelkerken L. Differential regulation of Interleukin-10 (IL-10) and IL-12 by glucocorticoids in vitro. Blood 1998; 91: (No 11): 4255-64.

30. Brunetti M, Martelli N, Colasante A, Piantelli M, Musiani P, Aiello FB. Spontaneous and glucocorticoid-induced apoptosis in human mature T lymphocytes. Blood 1995; 86: (No 11): 4199-4205.

31. Moser M, de Smedt T, Sornasse T, et al. Glucocorticoids down-regulate dendritic cell function in vitro and in vivo. Eur J Immunol 1995; 25: 2818-24.

32. Vieira PL, Kalinski P, Wierenga EA, Kapsenberg ML, de Jong EC. Glucocorticoids inhibit bioactive IL-12p70 production by in vitro generated human dendritic cells without affecting their T cell stimulatory potential. J. Immunol. 1998; 161: 5245-51.

33. Trinchieri G. Interleukin-12: A proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen specific adaptive immunity. Annu. Rev. Immunol 1995; 13: 251-76.

34. Moore KW, O'Garra A, de Waal Malefyt R, Vieira P, Mosmann TR. Interleukin-10. Annu Rev Immunol 1993; 11: 165-90.

35. D'Andrea A, Aste-Amezaga M, Valiante NM, Ma X, Kubin M, Trinchieri G. Interleukin 10 inhibits human lymphocyte interferon production by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells. J Exp Med 1995; 178: 1041-48.

36. Lentjes EGWM, Griep EN, Boersma JW, Romijn FPTHM, de Kloet ER. Glucocorticoid receptors, fibromyalgia and low back pain. Psychoneuroendocrinol 1997; 22: (no 8): 603-14.

37. Shire D. An invitation to an open exchange of reagents and information useful for measurement of cytokine mRNA levels by PCR. Eur Cytokine Network 1993; 4: 161.

38. Snijder A, Hilkens CM, van der Pauw Kraan TC, Engel M, Aarden LA, Kapsenberg ML. Regulation of bioactive IL-12 production in lipopolysacharide-stimulated human monocytes is determined by the expression of the p35 subunit. J Immunol 1996; 156: 1207.

39. van Boxel-Dezaire AHH, Hoff SCJ, van Oosten BW et al. Decreased IL-10 and increased IL-12p40 mRNA are associated with disease activity and characterize different disease stages in multiple sclerosis. Ann Neurol 1999, June; 45 (6): 695-703.

40. Grewe M, Czech W, Morita A, et al. Human eosinophils produce biological active IL-12: Implications for control of T cell responses. J Immunol 1998; 161: 415-20.

41. Yssel H, de Waal Malefyt R, Roncarolo MG, Abrams JS, Lahesmaa R, Spits H, de Vries JE. IL-10 is produced by subsets of human CD4 positive T cell clones and peripheral blood T cells. J Immunol 1992; 149: 2378-84.

42. Kanegane H, Wakiguchi H, Kanegane C, Kurashige T, Tosato G. Viral interleukin-10 in chronic active Epstein-Barr virus infection. J Infect Dis 1997; 176: 254-7.

43. Cannon JG, Angel JB, Abad LW, O'Grady J, Lundgren N, Fagioli L, Komaroff AL. Hormonal influences on stress-induced neutrophil mobilization in health and chronic fatigue syndrome. J Clin Immunol 1998; 18 (4): 291-98.

44. Kam JC, Szefler SJ, Surs W, Sher ER, Leung DY. Combination of IL-2 and IL-4 reduces glucocorticoid receptor-binding affinity and T cells response to glucocorticoids. J Immunol 1993; 151: 3460.

45. Scheinman RI, Cogswell PC, Lofquist AK, Baldwin AS jr. Role of transcriptional activation of IkBα in mediation of immunosuppression by glucocorticoids. Science 1995; 270: 283-86.

46. Auphan N, Di Donato JA, Rosette C, Helmberg A, Karin M. Immunosuppression by glucocorticoids: Inhibition of NF-kB activity through induction of IkB synthesis. Science 1995; 270: 286.

47. Barnes PJ, Greening AP, Crompton GK. Glucocorticoid resistance in asthma. Am J Respir Crit Care Med 1995; 152: s125-s142.

48. Nijhuis EWP, Hinloopen B, Odding J, Nagelkerken L. Abrogation of the suppressive effects of dexamethasone by PKC activation or CD28. Cell Immunol 1994; 156: 438-47.

49. Platzer C, Meisel Ch, Vogt K, Platzer M, Volk HD. Upregulation of monocytic IL-10 by tumor necrosis factor-alpha and cAMP elevating drugs. Int Immunol 1995; 4: 517.

50. Li-Weber M, Salgame P, Hu C, Krammer PH. Characterization of constitutive and inducible transcription factors binding to the P2 NF-AT site in the human interleukin-4 promotor. Gene 1997; 188 (2): 253-60.

51. Murphy TL, Cleveland MC, Kulesza P, Magram J, Murphy KM. Regulation of IL-12p40 expression through an NFkB half-site. Mol Cell Biol 1995; 10: 5258.

CHAPTER FIVE

Glucocorticoid receptors and glucocorticoid responsiveness in Chronic Fatigue Syndrome (CFS)

Jeroen Visser [a,b], Eef Lentjes [c], Inge Haspels [a], Willy Graffelman [d], Bep Blauw [a], E. Ronald de Kloet [b] and Lex Nagelkerken [a]

a. Division of Immunological and Infectious Diseases, TNO Prevention and Health, Leiden, The Netherlands.
b. Division of Medical Pharmacology, Leiden Amsterdam Center for Drug Research, Leiden, The Netherlands.
c. Department of Clinical Chemistry, Leiden University Medical Center, Leiden the Netherlands.
d. Department of General Practice and Nursing Home Medicine, Leiden University Medical Center, Leiden, The Netherlands.

Abstract

In this study the hypothesis was tested whether the increased sensitivity for glucocorticoids in CFS-patients, could be attributed to an altered functioning of their glucocorticoid receptors (GR). For this purpose, affinity and distribution of the GR were studied in purified peripheral blood mononuclear cells (PBMC) of 10 CFS-patients and 14 controls, simultaneously with the responsiveness of these cells to glucocorticoids *in vitro*.

Affinity (Kd) and number (Bmax) of GR was not different in PBMC of CFS-patients as compared to the controls; Kd: 12.9 ± 8.9 nM versus 18.8 ± 16.2 nM and GR number: 4839 ± 2824 per cell versus 4906 ± 1646 per cell. Moreover, RT-PCR revealed no differences in GR mRNA expression.

Nevertheless, PBMC from CFS-patients showed an increased sensitivity to glucocorticoids *in vitro*. In CFS-patients 0.01 μM dexamethasone suppressed PBMC proliferation for 37%, while the controls were only suppressed for 17% ($p<0.01$). Addition of PMA to the cultures rendered the cells resistant to dexamethasone with regard to proliferation, IL-10 and IFN-γ production, but not IL-2 and TNF-α production in both patients and controls. No difference between patients and controls was observed in this respect.

In conclusion, PBMC of CFS-patients display an increased sensitivity to glucocorticoids, which cannot be explained by number or affinity of the GR, but should rather be attributed to molecular processes beyond the actual binding of the ligand to the GR.

Introduction

The Chronic Fatigue Syndrome (CFS) is a disease of unknown etiology with severe disabling fatigue lasting longer than six months [1, 2]. Several causes have been held responsible for this syndrome including an impaired functioning of the immune response, chronic viral infections and endocrinological disturbances [3, 4]. Several reports have shown that CFS-patients display an altered functioning of the immune response, i.e. an altered cytokine production, low NK-cell function and differences in the expression of activation markers on lymphocytes [5-18]. Others have suggested that an anti viral pathway in CFS-patients is upregulated as the consequence of an increased viral load or viral reactivation in CFS-patients [19]. However, until now no infectious agent could be linked to the syndrome.

Furthermore the observed immunological disturbances show a great diversity in results. Most of this diversity can be explained by the heterogeneity of the patient population and the employed read out systems [13]. Endocrinological studies performed by several groups demonstrated an impaired functioning of the Hypothalamus Pituitary Adrenal gland (HPA)-axis in CFS-patients [20-22]. CFS-patients showed significantly reduced levels of urinary free cortisol and a reduced production of Adreno Corticotropin Hormone (ACTH) upon stimulation with Corticotropin Releasing Hormone (CRH). Furthermore, a blunted response in cortisol production to ACTH was shown [20-22].

These alterations are quite different as compared to depression, which is rather characterized by a hyperfunctioning of the HPA-axis resulting in high levels of cortisol [23, 24]. Also, most patients with depression show reduced dexamethasone (DEX) suppression, probably because of a reduced functioning of the GR [23, 24]. In patients with fibromyalgia, a slight impairment of the GR has been demonstrated. Although these patients showed a reduced affinity of the GR, they have normal responses to GC in vitro [25].

In CFS-patients we have previously demonstrated an altered reactivity to GC evidenced by the increased suppression by DEX of proliferation and IL-4 production of CD4 positive T cells [17]. Moreover, immunological responses in CFS are not related with endogenous cortisol [26, Visser et al. submitted for publication]. Furthermore HC replacement therapy in CFS gave some improvement, but this also lead to adrenal insufficiency [27]. Taken together evidence is emerging that the GR function is altered in CFS-patients.

To investigate this possibility, we studied the affinity and distribution of the GR in PBMC of 10 CFS-patients and matched controls. In addition, RT-PCR was used to investigate the mRNA expression of the GR and HSP90. Simultaneously, PBMC were studied with regard to their sensitivity to DEX and HC *in vitro*, during mitogen induced proliferation and cytokine production.

Materials and Methods

Subjects

SELECTION CRITERIA

We included patients between 18 and 50 years of age who met the CDC-criteria defined by Fukuda et al [2]. Patients suffering from somatic and psychiatric disorders and patients using beta-blockers, psychotropic drugs, immunosuppressive drugs or diuretics were excluded from the study. Furthermore patients with an alcohol intake of more than four units of alcohol per day and a body-mass index greater than 45 were excluded from the study.

RECRUITMENT OF PATIENTS AND CONTROLS

Patients were recruited by 38 general practitioners associated with the Department of General Practice and Nursing Home Medicine, Leiden University Medical Center (LUMC). We verified, by inspection of the available medical records, if the prospective patients met the above-defined criteria. The general practitioners contacted a total of 66 patients to participate in the study. Of these 66 patients, 59 patients gave their informed consent.

The patients were requested to recruit a healthy control matched for age, race and sex from their direct environment. Three patients failed in recruiting their own control. Of all the 56 controls informed consent was obtained.

The mean age of the CFS group was 38 ± 8 years; the mean age of the control group was 38 ± 9 years. The female to male ratio in both groups was 2 to 1.

Fasting blood samples of each patient and its matched control were obtained simultaneously at the patients home between 7.00 and 10.00 a.m.

The Medical Ethical Committee of the LUMC approved this study.

Isolation of cells

PBMC were isolated from EDTA-blood by Ficoll (Sigma, St.Louis, MO) density centrifugation. The cells were cryopreserved and thawed according to standard procedures, to enable batchwise analysis of patients and controls at a later time point.

Cell cultures

Cultures of PBMC were performed in Iscoves Modified Dulbecco's Medium (Gibco, Paisley, UK) supplemented with 10% fetal calf serum (Sebak Gmbh, Aidenbach, Germany), 100 U/ml penicillin, 100 µg/ml streptomycin and 50 µM ß-mercaptoethanol. Cells were cultured at a density of 40,000 cells/well in a volume of 200 µl in flat-bottomed 96 wells microtiter plates (Costar, Cambridge, MA) and stimulated with 0.5 or 1 µg/ml phytohaemagglutinin (PHA; Murex, Dartford, UK). Dexamethasone (DEX; Sigma) or Hydrocortisone (HC; Sigma) was added to obtain a final concentration of 10^{-6}, 10^{-7} or 10^{-8} M. Finally, phorbol 12-myristate 13-acetate (PMA; Sigma) was added to the cultures in a concentration ranging from 0.1 to 0.3 ng/ml. Supernatants of PBMC cultures were harvested on day 4 and proliferation was measured on day 5 by adding 18.5 KBq (=0.5 µCi) methyl-^{3}H-thymidine (specific activity: 74 GBq/mmol; Radiochemical Centre, Amersham, UK) during the last 6 hrs of culture. The culture conditions described above and time points to measure cytokines and proliferation were previously established to represent suboptimal conditions for cryopreserved cells to allow the detection of stimulatory and inhibitory effects of PMA, DEX and HC. Supernatants were stored at –20°C until assay. Labeled cells were harvested onto glass fiber filters (Canberra Packard, Meriden, CT) and the filters were counted using a Matrix 96 ß-counter (Canberra Packard).

Cytokine assays

Cytokines were determined by ELISA, as described elsewhere [17]. For the IFN-γ and IL-2 detection, commercially available detection kits were used (BioSource, Fleurus, Belgium). For the measurement of IL-4 , mouse-anti-human-IL-4 (clone 8D4-8, 1 µg/ml) was used as a capture antibody, biotinylated rat-anti-human IL-4 (clone MP4-25D2, 2 µg/ml) as detecting antibody, whereas human recombinant IL-4 (standard curve ranged from 5000 to 2.5 pg/ml) served as a standard (these reagents were purchased from Pharmingen, San Diego, CA).

For the detection of IL-10 the plates were coated with JES3-9D7 mAb (0.5 μg/ml) and biotinylated JES3-12G8 mAb was used in a concentration of 2 μg/ml (both antibodies were purchased from Pharmingen). Recombinant human IL-10 (kindly provided by Dr. S. Narula, Schering-Plough Research Institute, Kenilworth, NJ) diluted in culture medium was used as a standard. The standard curves ranged from 2500 pg/ml to 10 pg/ml.

For the TNF-α ELISA, the plates were coated with 1 μg/ml Mab1; biotynilated Mab11 was used in a concentration of 1 μg/ml for detection. Recombinant TNF-α diluted in culture medium was used as a standard. The standard curves ranged from 10000 to 40 pg/ml. All these reagents were purchased from Pharmingen.

Hormone detection

Blood from the patients and the controls was collected, immediately put on ice and allowed to coagulate. The tubes were centrifuged for 30 minutes (3000 rpm, 4°C); serum was collected and immediately stored at –20°C. Serum total cortisol was measured using the fluorescent polarization immunoassay on the TDx from Abbott (Amstelveen, The Netherlands). Serum levels of free cortisol were determined in the filtrate obtained by temperature-controlled ultrafiltration using the Amicon MPS-1 ultrafiltration device. Cortisol binding globulin (CBG) concentrations were determined by radioimmunoassay (Medgenix diagnostics, Fleurus, Belgium) according to Lentjes et al. [25]. ACTH was measured in EDTA-plasma as described previously [28]. Of 4 patients and controls no ACTH was measured, of 4 patients no total- and free-cortisol was measured and from 24 patients and 20 controls no CBG was measured because of shortage of serum and plasma.

Glucocorticoid receptor assay

The number and affinity of the GR in PBMC of the patients and matched controls was determined using the technique as described by Lentjes et al. [25].

PBMC were washed twice with phosphate buffered saline (NPBI, Emmercompascum, the Netherlands) and resuspended in RPMI 1640 (Gibco) medium, supplemented with 2 mM L-glutamin, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% FCS (Sebak). This medium is further indicated as RPMI/FCS. The cells were pre-incubated for 1 h at 37° C, washed three times and then the cells were incubated for 1 h at 37° C in a shaking waterbath

with step-fold diluted (range 2.5-60 nmol/l) [^{3}H]-DEX (Amersham). Non-specific binding was measured in the presence of a thousand-fold excess of unlabeled DEX (Sigma). The incubations were terminated by adding 2 ml ice-cold RPMI/FCS. Cell-bound DEX was separated from free DEX by washing the cells twice with ice-cold RPMI/FCS followed by centrifugation (2.5 min at 12000 x g) of the cells through a Ficoll/PBS layer (density 1.030 g/ml). The tube contents were frozen in CO_2-acetone and the bottoms of the tubes, containing the cell pellet, were cut off and transferred into 10-ml liquid scintillation cocktail.

The amount of [^{3}H]-DEX bound was determined with a β-counter (Canberra Packard). The number of binding sites per cell and the Kd were calculated from a Woolf plot according to Keightley et al. [29].

RNA quantitation of HSP90 and GRα using semi-quantitative PCR

mRNA was extracted from the PBMC and used to measure HSP90 and GRα mRNA in a semiquantitative manner as described previously [30, 31]. PCR products were separated on 1% agarose gels and stained with ethidium bromide or SYBR Green I (Biozym, Landgraaf, The Netherlands). Densities of the the amplified cDNA were analyzed using the Bio-1D digital imaging system version 6 (Vilber Lourmat, Marne La Vallée, France). Results are expressed as a ratio of quantified HSP90 and GRα product over β-actin product.
Primers for the GRα: Sense: 5'CAA AAG AGC AGT GCA AGG ACA3', anti-sense: 5'GAG GTT TCT TGT GAG ACT CCT GT3'.
Primers for HSP90: Sense: 5'GTC TGG GTA TCG GAA AGC AAG 3', anti-sense: 5'CTG AGG GTT GGG GAT GAT GTC 3'.
Primers for β-actin: Sense: 5'GGG TCA GAA GGA TTC CTA TG 3', anti-sense: 5'GGT CTC AAA CAT GAT CTG GG 3'.

Statistical analysis

Statistical analysis was performed using the Rank-Wilcoxon test for matched pairs. Differences with a confidence level of 95% or higher were considered to be statistically significant ($P<0.05$). Significance of correlation was calculated using the method of Spearman.

Results

ACTH and cortisol levels in CFS-patients and matched controls

In the group of 59 carefully selected CFS-patients and 56 healthy controls the levels of total- and free-cortisol, ACTH and CBG were measured. In both groups the users of contraceptives (N=20) were excluded from the analysis.

As shown in Table 1 no differences were found in the levels of the measured hormones or CBG between patients and controls.

Correlation plots between total- and free-cortisol and between ACTH and free/total cortisol were made to study the relation between the several hormones involved in the HPA-axis. As shown in Fig. 1A, a positive correlation was observed between ACTH and total cortisol both in patients ($R=0.597$, $p<0.001$) and controls ($R=0.596$, $p<0.001$). Figure 1B demonstrates also a positive correlation between ACTH and free cortisol in patients ($R=0.741$, $p<0.001$) and controls ($R=0.429$, $p<0.02$).

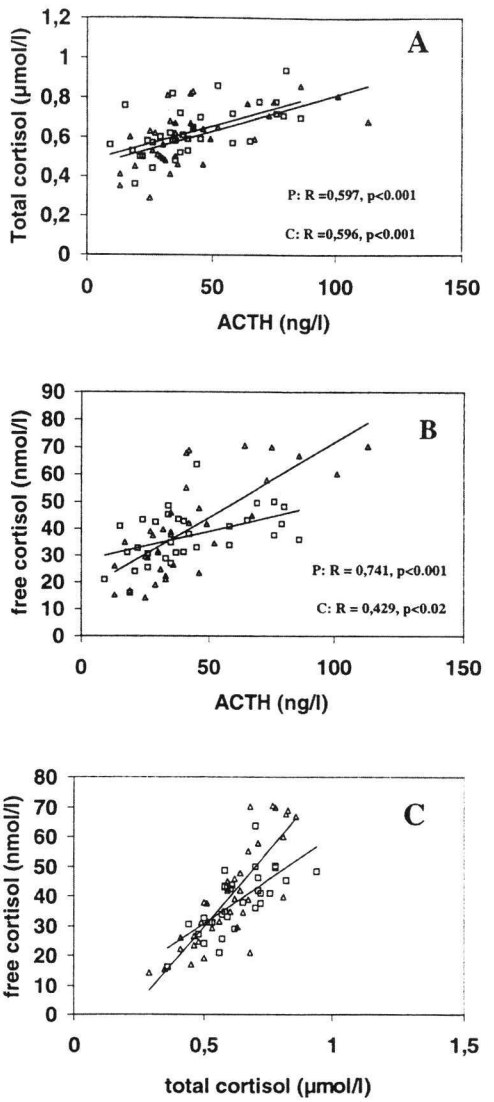
As expected, in both groups a positive correlation was observed between total and free cortisol (Fig. 1C, $p<0.001$).

Table 1. Cortisol, ACTH and CBG levels in CFS-patients and healthy matched controls.

	ACTH (ng/l)	total cortisol (μM)	free cortisol (nM)	CBG (μM)
CFS	43.7 ± 24.0 (n=38)	0.60 ± 0.14 (n=35)	40.0 ± 17.2 (n=35)	0.85 ± 0.10 (n=27)
Controls	42.5 ± 20.7 (n=33)	0.64 ± 0.12 (n=36)	38.0 ± 9.8 (n=36)	0.89 ± 0.12 (n=28)

Results are expressed as means ± SD.
Hormones and CBG were measured as described in the Methods section

Figure 1. Relation between ACTH and cortisol in CFS-patients and matched controls.



The figures represent correlation plots between ACTH vs. total cortisol (A), ACTH vs free cortisol (B) and total cortisol vs free cortisol (C). Squares represent the controls and triangles the patients.
Correlations are calculated according to the method of Spearman.

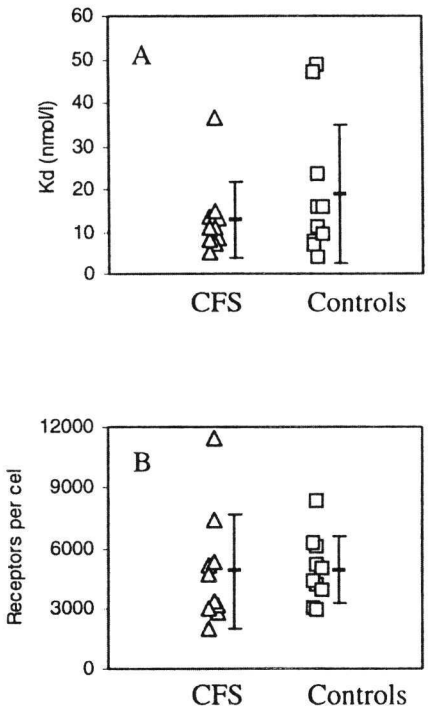
No alterations in affinity and number of GR in PBMC of CFS-patients

Because lymphocytes from CFS-patients appeared to have an increased sensitivity to GC *in* vitro [17, Visser et al, submitted for publication], we randomly selected 10 CFS-patients and their matched controls in order to investigate the affinity and number of the GR in the PBMC. From these patients and controls as well as 10 additional patients and matched controls RNA was isolated, to investigate the expression of the GR and the HSP90 by RT-PCR.

As shown in Fig. 2 no differences were detected in the affinity or the number of receptors per cell between CFS-patients and matched controls. In addition, 4 internal lab controls did not differ in GR affinity and density from the analyzed patients and matched controls (data not shown).

Likewise RT-PCR revealed no differences in the expression of GR and HSP90 mRNA between PBMC of CFS-patients and the matched controls (data not shown).

Figure 2. No changes in GR density or affinity in PBMC of CFS-patients.

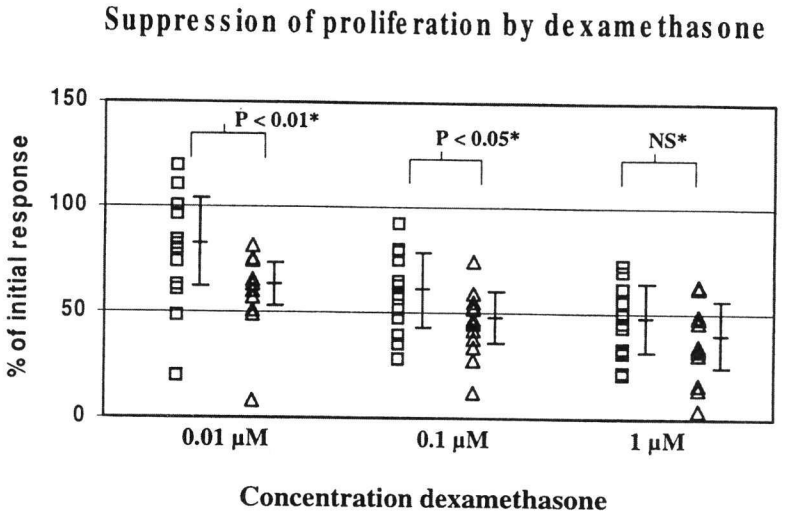


*Affinity (**A**) and number (**B**) of receptors were determined as described in the Methods section. The mean Kd ± SD was 12.9 ± 8.9 nmol/l in CFS (n =10) and 18.8 ± 16.2 nmol/l in the matched controls (n =10). The mean number of receptors per cell ± SD was 4839 ± 2834 in the patients and 4906 ± 1646 in the matched controls.*

Increased sensitivity of PBMC to glucocorticoids in CFS-patients

Simultaneously, the cells from the 10 patients used for the receptor study, as well as 2 additional patients, were stimulated with PHA in the presence of various concentrations of DEX or HC to establish their sensitivity to GC *in vitro*. The *in vitro* proliferation of the PBMC was comparable between patients (8785 ± 2732 cpm) and controls (8073 ± 2755 cpm). Nevertheless, as shown in Fig. 3, the *in vitro* proliferation of the PBMC from CFS-patients was more sensitive to DEX as compared to the cells from the controls. Moreover, the patients also showed an increased sensitivity to HC. Addition of 0.01 μM HC to the cultures suppressed the proliferation of the PBMC of the CFS-patients with 24% as compared to 10% in the controls ($p<0.05$, data not shown).

Figure 3. Increased sensitivity of PBMC from CFS-patients to DEX as compared to matched controls.



The figure represents the proliferation by PBMC from 12 CFS-patients (triangles) and matched controls (squares) after stimulation with 1 μg/ml PHA in the presence of various concentrations DEX. The results are expressed as percentage of the initial response to PHA only. The mean proliferation ± SD of PBMC in the absence of DEX was 8785 ± 2732 cpm in CFS-patients and 8073 ± 2755 cpm in the matched controls.
**: Rank Wilcoxon test for matched pairs.*

As depicted in Table 2, CFS-patients showed no differences in the production of IL-2, IFN-γ, TNF-α, IL-4 and IL-10. The increased sensitivity for GC in CFS-patients with regard to proliferation was not demonstrated at the level of cytokine production. However, in agreement with the literature [30, 32, 33], IL-4 and IL-10 were less sensitive to DEX than IL-2, IFN-γ and TNF-α (see Table 3). With regard to IL-2 it was not possible to calculate an IC50 concentration because addition of low concentrations of DEX already suppressed IL-2 to levels below the detection limit of the ELISA.

Taken together these results demonstrate that although no differences were detected in affinity and the number of glucocorticoid receptors per cell, PBMC of CFS-patients display an increased sensitivity to GC *in vitro*.

Table 2. Cytokine production in PBMC of CFS-patients and matched controls.

	IL-2 (n=15)	IFN-γ (n=10)	TNF-α (n=15)	IL-10 (n=15)	IL-4 (n=15)
CFS	159 ± 32	1556 ± 533	1775 ± 177	108 ± 21	17 ± 2
Controls	342 ± 134	1468 ± 400	1972 ± 220	91 ± 14	17 ± 3

Results are expressed in pg/ml as means ± SEM.
PBMC were stimulated with 0.5 µg/ml PHA and the supernatants were harvested after 4 days of culture.
The supernatants were stored at –20°C and the cytokines levels were measured by ELISA as described in the Methods section.

Table 3. Relative sensitivity of cytokines to DEX expressed as IC50 in CFS-patients and matched controls.

	IFN-γ (n = 10)	TNF-α (n = 15)	IL-10 (n = 15)	IL-4 (n= 15)
CFS	22 ± 8.5	51 ± 16.8	180 ± 57.9	427 ± 246.7
Controls	27 ± 7.6	64 ± 38.8	546 ± 210.3	315 ± 123.5

IC50 is expressed as the mean ± SEM DEX concentration (nmol/l) required for 50% inhibition of cytokine production. PBMC were stimulated with 0.5 µg/ml PHA in the absence or presence of various concentrations DEX and the supernatants were harvested after 4 days of culture. The supernatants were stored at –20°C and the cytokines levels were measured by ELISA as described in the Methods section.

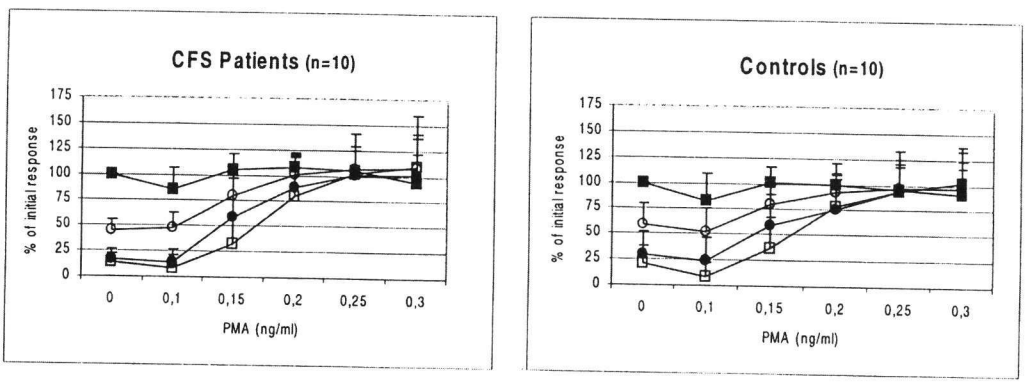
Addition of PMA renders cells resistant for dexamethasone.

The increased sensitivity of CFS-patients for GC, in the absence of an increased affinity or density of the GR, suggests an increased suppression of transcription factors by activated GR in PBMC of CFS-patients. Several authors [34, 35] demonstrated that an impaired interaction between the GR and the transcription factor AP-1 or overexpression of this transcription factor rendered cells unresponsive for GC. As shown in Fig. 4, addition of PMA (a strong inducer of AP-1) to the cultures rendered the cells resistant for DEX with regard to proliferation which is in agreement with the literature [34, 35]. Although the patients displayed a increased sensitivity to DEX, the amount of PMA needed to abrogate inhibition of proliferation by DEX did not differ between patients and controls.

Interestingly, as is shown in Fig. 5A-D, addition of PMA to the cultures potently enhanced IL-2, suppressed IFN-γ and had not much effect on TNF-α and IL-10 production. Interestingly, more PMA was needed to achieve comparable levels of IL-2 production in the patients as compared to the controls.

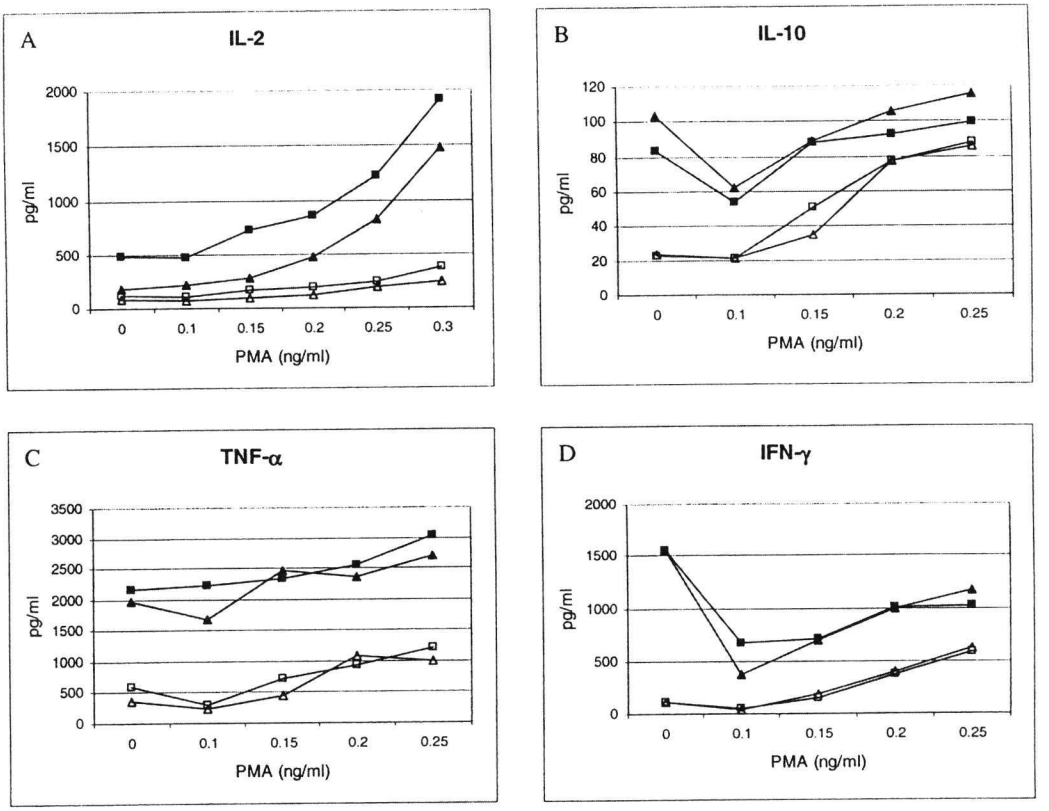
PMA rendered the cells resistant for DEX with regard to the suppression of IL-10 (Fig. 5D) and to a lesser extent IFN-γ (Fig. 5B), while IL-2 (Fig. 5A) and TNF-α (Fig. 5C) still remained sensitive. No differences between patients and controls could be observed in this respect.

Figure 4. Addition of PMA renders lymphocyte proliferation resistant to DEX both in CFS-patients and matched controls.



PBMC were stimulated with 0.5 μg/ml PHA in the presence of various concentrations of dexamethasone and/or PMA. The proliferation was measured after 5 days of culture as described in the methods. In this figure the proliferation is expressed as the mean percentage ± SD of the initial response of 10 patients and controls in the absence of DEX and PMA. PHA induced proliferation by PBMC of the controls was 7805 ± 2555 cpm and by PBMC of the patients 7955 ± 2455 cpm. The cells were stimulated with 0.5 μg/ml PHA(■), PHA + 0.01 μM DEX (○), PHA + 0.1 μM DEX(●) and PHA + 1 μM DEX(□). PMA was added to the cultures as indicated.

Figure 5. Addition of PMA renders IL-10 and IFN-γ resistant to DEX, while IL-2 and TNF-α remain sensitive.



PBMC of 10 CFS-patients and matched controls, were stimulated with 0.5 μg/ml PHA in the presence of various concentrations of dexamethasone and/or PMA. The supernatants were harvested after 4 days of culture and the cytokines were measured by ELISA as described in the Methods section. In this figure the cytokine production is expressed as the mean pg/ml in the absence of DEX and PMA.

The triangle marked lines represent the CFS-patients and the square marked lines the matched controls. The closed symbols represent the cultures stimulated with 0.5 μg/ml PHA and the open symbols represent the cultures stimulated with 0.5 μg/ml PHA in the presence of 1 μM DEX. PMA was added to the cultures as indicated.

Discussion

The results presented in this report demonstrate an increased sensitivity for glucocorticoids by PBMC of CFS-patients, without evidence for an altered density or affinity of the GR. The sensitivity to GC is dependent on several factors like affinity and density of the GR [34, 36, 37] , the interaction of the GR with transcription factors and the control of the gene under investigation by a glucocorticoid responsive element (GRE) [37-40]. Barnes et al. have elegantly demonstrated that all these mechanisms are involved in steroid resistant asthma [34]. In particular an impaired interaction between the transcription factor AP-1 and the GR appeared to be explanatory for steroid resistance in a subset of these patients [34]. Furthermore upregulation of AP-1 levels *in vitro* rendered lymphocytes insensitive for GC [35].

The fact that PBMC of CFS-patients tend to produce less IL-2 as compared to controls suggests a reduced induction of AP-1 by PHA in CFS-patients, because AP-1 is a dominant transcription factor in the regulation of IL-2 expression [32]. The reduced AP-1 levels might lead to a reduced buffer capacity in the PBMC of CFS-patients for the activated GR and therefore subsequently be responsible for the increased GC sensitivity. In addition, because the increased sensitivity for GC *in vitro* in CFS-patients is not accompanied by alterations at the level of the GR, suggests disturbances in the interaction between the GR and transcription factors in CFS-patients. To investigate these possibilities, we added PMA to the PBMC cultures. PMA is a potent stimulator of protein kinase C (PKC) causing a strong induction of AP-1 and to a lesser extent of NFkB, but it is detrimental for the expression of CREB [35, 40].

Addition of PMA to the cultures resulted in a strong induction of IL-2 in both patients and controls, but it had no stimulatory effect on the other cytokines. PMA rendered the cells resistant to DEX with regard to proliferation, IL-10 production, to a lesser extent IFN-γ production, while TNF-α and IL-2 remained sensitive. These results demonstrate that multiple mechanisms are involved in the suppressive effects of glucocorticoids on cytokine production and proliferation of lymphocytes.

More PMA was needed to achieve comparable IL-2 levels in the PBMC of CFS-patients as compared to the controls, which is supportive for reduced AP-1 levels in CFS-patients. To what extent reduced AP-1 levels are involved in the difference in GC sensitivity is still unclear, because no differences were found between patients and controls with regard to the resistance inducing capacity of PMA. Moreover, if an increased suppressive effect of the

activated GR on AP-1 expression was present in the lymphocytes of the CFS-patients, PMA should be less capable to induce steroid resistance in the patients. Probably, addition of PMA is not specific enough to identify subtle changes in GR and transcription factor interactions, because PMA can induce phosphorylation of the GR via activation of protein kinases.

Phosphorylation of the GR has been shown to interfere with the transcriptional activity of the GR [37, 41]. Therefore more specific molecular biological methods like electro-mobility shift assays may be required for a proper investigation of the interactions between transcription factors and the GR in CFS-patients.

Also, a polymorphism in the GR gene of CFS-patients explaining the altered responsiveness to GC, cannot be excluded. Huizenga et al have shown that a polymorphism at nucleotide position 1220, is associated with an altered sensitivity to GC [42]. Carriers of this mutation gave a higher suppression of cortisol by DEX *in vivo*. The affinity and number of the GR in lymphocytes of these mutation carriers were not affected, but they showed a trend towards a higher suppression of proliferation by DEX [41]. Again this shows that a normal affinity and number of the GR can be associated with an increased sensitivity for GC *in vivo* or *in vitro*. It would be therefore of interest to investigate whether CFS-patients display polymorphisms of the GR gene in association with the observed increase in GC sensitivity.

If we assume that the responsiveness to GC is comparable in all tissues, the increased sensitivity for GC as observed in lymphocytes from CFS-patients [17, this report, Visser et al submitted for publication], might be responsible for the moderate dysfunctioning of the HPA-axis in CFS-patients. CFS-patients display a slight hypocortisolism and an altered response to a challenge with ACTH or CRH *in vivo* [20-22]. Demitrack et al. demonstrated that low doses of ACTH give a higher increase of cortisol in CFS-patients, despite the fact that the maximal cortisol response is blunted [20]. Such an increased sensitivity was not reproduced by Scott et al [22], but again a blunted cortisol response was found.
We did not observe reduced levels of cortisol in serum of CFS-patients as opposed to the study by Demitrack et al. [20]. This might be explained by the fact that we measured the hormone levels at only one time-point. Therefore, twenty-four hours urinary cortisol levels are probably more informative in this regard. However, it is remarkable that 7 CFS-patients displayed high free cortisol levels without increased ACTH levels. These 7 CFS-patients are responsible for a stronger correlation between ACTH and free cortisol in CFS-patients as compared to the controls. The relatively high free cortisol levels in these 7 CFS-patients

could not be explained by reduced CBG levels, because these patients showed no reduction in CBG.

Patients with depression show a dysfunctioning of the HPA-axis which is quite the opposite to that found in CFS-patients [23, 24]. Whereas CFS-patients tend to show a hypofunctioning of the HPA-axis [3, 20-22], depressed patients display a hyperfunctioning of the HPA-axis [23]. These patients display elevated levels of cortisol, that escape DEX suppression, have elevated levels of CRH in their cerebrospinal fluid, increased mRNA expression of CRH and a blunted ACTH response to CRH [23, 24, 43-45]. In depressed patients a reduced number of GR has been demonstrated so it can therefore be hypothesized that a failure in the negative feedback by GC is one of the major reasons for the dysfunctioning of the HPA-axis in depression [24, 46, 47].

Post Traumatic Stress Disorder (PTSD) patients and fibromyalgia-patients, disorders with which CFS is often compared, show some resemblance in HPA-axis activity with CFS-patients. Like CFS-patients, PTSD and fibromyalgia-patients show low 24-h urinary cortisol levels [25, 48, 49]. Furthermore, PTSD-patients show exaggerated cortisol responses to a DEX challenge and have increased numbers of GR in their PBMC [50]. In fibromyalgia-patients a modest impairment of the GR has been demonstrated. These patients show a reduced affinity of the GR, but have normal responses to GC *in vitro* [25].

Taken together impaired GR function can lead to HPA-axis abnormalities which in turn may increase the vulnerability to affective disorders, fatigue syndromes and immune dysfunction [23, 51-54].

As suggested above, the increased sensitivity of PBMC from CFS-patients for GC may be the result of a decreased buffer capacity of transcription factors like AP-1, CREB or NFkB. However, it should be taken into account that we measured affinity and the number of receptors during steady state conditions of unstimulated PBMC. It is known that GC by itself as well as other soluble factors like the cytokines IL-2, IL-4 and IFN-γ can influence the affinity and the number of the GR in lymphocytes [36, 37]. So besides an altered interaction with transcription factors also the plasticity of the GR could be different in CFS-patients. Therefore, it will be of interest to study GR function in PBMC of CFS-patients under influence of such cytokines and after a challenge with GC. These hypotheses are currently under investigation and may give more insight into the HPA-axis disturbances in CFS-patients and potential therapy.

Acknowledgements

The technical assistance of Fred Romijn from the Central Clinical Chemistry Laboratory of the LUMC is highly appreciated. We would like to thank Dr. Kees Lucas for critically reading this manuscript.

References

1. Holmes GP, Kaplan JE, Gantz NM, et al. Chronic fatigue syndrome: a working case definition. Ann Intern Med 1988; 108: 387-9.

2. Fukuda K, Strauss SE, Hickie I, et al. The chronic fatigue syndrome: a comprehensive approach to its definition and study. Ann Intern Med 1994; 121: 953-59.

3. Bearn J and Wessely S. Neurobiological aspects of the chronic fatigue syndrome. Eur J Clin Invest 1994; 24: 79-90.

4. Strauss SE, Komaroff AL, Wedner HJ. Chronic Fatigue syndrome: Point and counterpoint. J Infect Dis 1994; 170: 1-6.

5. Aoki T, Miyakoshi H, Usuda Y, Herberman RB. Low NK syndrome and its relationship to chronic fatigue syndrome. Clin Immunol Pathology 1993; 69 (no 3): 253-65.

6. Caliguiri M, Murray C, Buchwald D, et al. Phenotypic and functional deficiency of natural killer cells in patients with chronic fatigue syndrome. J Immunol 1987; 139: 3306-13.

7. Barker E, Fujimura SF, Fadem MB, Landay AL, Levy JA, Immunologic abnormalities associated with chronic fatigue syndrome. Clin Infect Dis 1994; 18 (suppl): s157-9.

8. Strauss SE, , Fritz S, Dale JK, Gould B, Strober W. Lymfocyte phenotype and function in the chronic fatigue syndrome. J Clin Immunol 1993; 13: 30-40.

9. Swanink CMA, Vercoulen JHMM, Bleijenberg G, Fennis JFM, Galema JMD, van der Meer J. Chronic fatigue syndrome: a clinical and laboratory study with a well matched control group. J Intern Med 1995; 237: 499-506.

10. Klimas NG, Salvato FR, Morgan R, Fletcher MA. Immunologic abnormalities in chronic fatigue syndrome. J Clin Microbiol 1990; 28: 1404-10.

11. Chao CC, Janoff EN, Hu S, et al. Altered cytokine release in peripheral blood mononuclear cell cultures from patients with the chronic fatigue syndrome. Cytokine 1991; 3: 292-8.

12. Swanink CMA, Vercoulen JHMM, Galama JMD, et al. Lymphocyte subsets, apoptosis, and cytokines in patients with chronic fatigue syndrome. J Infect Dis 1996; 173: 460-3.

13. Mawle AC, Nisenbaum R, Dobbins JG, et al. Immune responses associated with chronic fatigue syndrome: A case-control study. J Infect Dis 1997; 175: 136-41.

14. Hassan IS, Bannister BA, Akbar A, Weir W, Bofill M. A study of the immunology of the chronic fatigue syndrome: correlation of immunologic parameters to health dysfunction. Clin Immunol Immunopathol 1998; 87 (1): 60-7.

15. Tirelli U, Marotta G, Improta S, Pinto A. Immunological abnormalities in patients with chronic fatigue syndrome. Scand J Immunol 1994; 40: 601-08.

16. Landay AL, Jessop C, Lennette ET, Levy JA. Chronic fatigue syndrome: clinical condition associated with immune activation. Lancet 1991; 338: 707-12.

17. Visser J, Blauw B, Hinloopen B, Brommer E, de Kloet ER, Kluft C, Nagelkerken L. CD4 T lymphocytes from patients with chronic fatigue syndrome have decreased interferon-γ production and increased sensitivity to dexamethasone. J Infect Dis 1998; 177: 451-4.

18. Borish L, Schmaling K, DiClementi JD, Streib J, Negri J, Jones JF. Chronic fatigue syndrome: Identification of distinct subgroups on the basis of allergy and psychologic variables. J Allergy Clin Immunol 1998; 102: 222-230.

19. Suhadolnik RJ, Reichenbach NL, Hitzges P. et al. Upregulation of the 2-5A synthetase / Rnase L antiviral pathway associated with chronic fatigue syndrome. Clin Infect Dis 1994; 18: S96-S104.

20. Demitrack MA, Dale JK, Strauss SE, et al. Evidence for impaired activation of the hypothalamic-pituitery-adrenal axis in patients with chronic fatigue syndrome. J Clin Endocrinol Metab 1991; 73: 1224-34.

21. Dinan TG, Majeed T, Lavelle E, Scott LV, Berti C, Behan P. Serotonin mediated activation of the hypothalamic-pituitary-adrenal-axis in chronic fatigue syndrome. Psychoneuroendocrinol 1997; 22: 261-68.

22. Scott LV, Medbak S, Dinan TG. The low dose ACTH test in chronic fatigue syndrome and health. Clin Endocrinol 1998; 48: 733-37.

23. Holsboer F, Barden N. Antidepressants and hypothalamic-pituitary adrenocortical regulation. Endocr Rev 1996; 17 (2): 187-205.

24. Modell S, Yassouridis A, Huber J, Holsboer F. Corticosteroid receptor function is decreased in depressed patients. J Neuroendocrinol 1997; 65 (3): 216-22.

25. Lentjes EGWM, Griep EN, Boersma JW, Romijn FPTHM, de Kloet ER. Glucocorticoid receptors, fibromyalgia and low back pain. Psychoneuroendocrinol 1997; 22: (no 8): 603-14.

26. Cannon JG, Angel JB, Abad LW, O'Grady J, Lundgren N, Fagioli L, Komaroff AL. Hormonal influeces on stress-induced neutrophil mobilization in health and chronic fatigue syndrome. J Clin Immunol 1998; 18 (4): 291-98.

27. McKenzie R, O'Fallon A, Dale J, Demitrack M, Sharma G, Deloria M, Garcia-Borreguero D, Blackwelder W, Straus SE. Low-Dose hydrocortisone for treatment of chronic fatigue syndrome: a randomized controlled trial. *JAMA* 1998; 280: 1061-66.

28. Griep EN, Boersma JW, Lentjes EG, Prins AP, van der Korst JK, de Kloet ER. Function of the hypothalamic-pituitary-adrenal axis in patients with fibromyalgia and low back pain. J Rheumatol. 1998 Jul; 25 (7): 1374-81.

29. Keightley DD, Fisher RJ, Cressie NAC. Properties and interpretation of the woolf and scatchard plots in analyzing data from steroid receptor assays. J Ster Biochem 1983; 19: 1407-12.

30. Visser J, van Boxel-Dezaire A, Methorst D, Brunt T, de Kloet ER, Nagelkerken L. Differential regulation of Interleukin-10 (IL-10) and IL-12 by glucocorticoids in vitro. Blood 1998; 91: (No 11): 4255-64.

31. Vachier I, Chiappara G, Vignola AM, Gagliardo R, Altieri E, Terouanne B, Vic P, Bousquet J, Godard P, Chanez P. Glucocorticoid receptors in bronchial epithelial cells in asthma. Am J. Respir Crit Care Med 1998; 158: 963-70.

32. Vacca A, Felli MP, Farina AR, et al. Glucocorticoid receptor-mediated suppression of the Interleukin-2 gene expression through impairment of the cooperativity between nuclear factor activated T cells and AP-1 enhancer elements. J Exp Med 1992; 175: 637-46.

33. Daynes RA, Araneo BA. Contrasting effects of glucocorticoids on the capacity of T cells to produce growth factors Interleukin-2 and Interleukin-4. Eur J Immunol 1989; 19: 2319-25.

34. Barnes PJ, Greening AP, Crompton GK. Glucocorticoid resistance in asthma. Am J Respir Crit Care Med 1995; 152: s125-s142.

35. Nijhuis EWP, Hinloopen B, Odding J, Nagelkerken L. Abrogation of the suppressive effects of dexamethasone by PKC activation or CD28. Cell Immunol 1994; 156: 438-47.

36. Kam JC, Szefler SJ, Surs W, Sher ER, Leung DY. Combination of IL-2 and IL-4 reduces glucocorticoid receptor-binding affinity and T cells response to glucocorticoids. J Immunol 1993; 151: 3460.

37. Bamberger CM, Schulte HM and Chrousos GP. Molecular determinants of glucocorticoid receptor function and tissue sensitivity to glucocorticoids. Endocrine Reviews 1996; 17 (3): 245-61.

38. Scheinman RI, Cogswell PC, Lofquist AK, Baldwin AS jr. Role of transcriptional activation of IkBα in mediation of immunosuppression by glucocorticoids. Science 1995; 270: 283-86.

39. Auphan N, Di Donato JA, Rosette C, Helmberg A, Karin M. Immunosuppression by glucocorticoids: Inhibition of NF-kB activity through induction of IkB synthesis. Science 1995; 270: 286.

40. Adcock IM, Brown CR, Gelder CM, Shirasaki H, Peters MJ, Barnes PJ. Effects of glucocorticoids on transcription factor activation in human peripheral blood mononuclear cells. Am J Physiol 1995; 268 (2 Pt 1): C331-8.

41. Rogatsky I, Logan SK and Garabedian MJ. Antagonism of glucocorticoid receptor transcriptional activation by the c-jun N-terminal kinase. Proc Natl Acad Sci USA 1998; 95: 2050-55.

42. Huizenga NATM, Koper JW, de Lange P et al. A polymorphism in the glucocorticoid receptor gene may be associated with and increased sensitivity to glucocorticoids in vivo. J Clin Endocrinol Metab 1998; 83: 144-51.

43. Banki CM, Bisette G, Arato M, O'Conner L, Nemeroff CB. Cerobrospinal fluid corticotropin-releasing factor-like immunoreactivity in depression and schizophrenia. Am J Psych 1987; 144: 873-77.

44. Nemeroff CB, Widerlov E, Bisette G, Walleus H, Karlsson I, Eklund K, Kilts CD, Loosen PT, Valk W. Elevated concentration of CSF corticotropin-releasing factor-like immunoreactivity in depressed patients. *Science* 1984; 226: 1342-44.

45. Holsboer F, Von Bardeleben U, Gerken A, Stalla GK, Muller O. Blunted corticotropin and normal cortisol response to human corticotropin-releasing factor in depression. New England J Med 1984; 311: 1127.

46. Gormley GJ, Lowy MT, Reder AT, Hospelhorn VD, Antel JP, Meltzer HY. Glucocorticoid receptors in depression: relationship to the dexamethasone suppression test. Am J Psych 1985; 132: 1278-84.

47. Whalley LJ, Borthwick N, Copolov D, Dick H, Christie JE, Fink G. Glucocorticoid receptors and depression. Br Med J 1986; 292: 859-61.

48. Yehuda R, Southwick SM, Nussbaum G, Giller EL, Mason JW. Low urinary cortisol excretion in patients with post-traumatic stress disorder. J Nerv Mental Dis 1990; 178: 366-69.

49. Griep EN, Boersma JW, de Kloet ER. Altered reactivity of the hypothalamic-pituitary-adrenal axis in the primary fibromyalgia syndrome. J Rheumatol 1993; 20: 469-74.

50. Yehuda R, Boisoneau D, Lowy MT, Giller EL. Dose-response changes in plasma cortisol and lymphocyte glucocorticoid receptors following dexamethasone administration in combat veterans with and without posttraumatic stress disorder. Arch Gen Psych 1995; 52: 583-93.

51. Wilckens T, de Rijk R. Glucocorticoids and immune function: unknown dimensions and new frontiers. Immunol Today 1997; 18 (9): 418-24.

52. Wilder, RL Neuroendocrine-immune system interactions and autoimmunity. Annu Rev Immunol 1995; 13: 307-38.

53. Michelson D, Stone L, Galliven E, Magiakou MA, Chrousos GP, Sternberg EM, Gold PW. Multiple sclerosis is associated with alterations in hypothalamic-pituitary-adrenal axis function. J Clin Endocrinol Metab 1994; 79 (3): 848-53.

54. Rook GAW, Hernandez-Pando R, Lightman S. Hormones, peripherally activated prohormones and regulation of the Th1/Th2 balance. Immunol Today 1994; 15: 301-3

CHAPTER SIX

Summary and General Discussion

Hormones belonging to the HPA-axis are strongly involved in immunoregulation [1-4]. In particular GC are generally accepted to play a major role in this regard [1-4]. Changes in immunological parameters might be due to an altered regulation by these hormones, for instance as a consequence of GR dysfunction. Such changes may occur in conjunction with impaired functioning of the HPA-axis, which is also dependent on proper GR function. For instance, in depressive disorders the increased cortisol levels are presumed to result from a deficit in negative feedback because of a reduced affinity or density of the GR [5-8]. Although the etiology of CFS is unknown, altered GR function might disturb both the immune function and the integrity of the HPA-axis in these patients. Accordingly, the reduced levels of cortisol in CFS-patients may be due to an enhanced negative feedback because of an increased efficacy of GR-mediated effects [9-12].

In view of the importance of GC in immunoregulation, the main subject of this thesis are the immunomodulatory effects of GC on cytokine production in CFS-patients and healthy controls.

Furthermore, I will discuss whether the alterations in immune function, as found in the CFS-patients, can be attributed to differences in their responsiveness to GC. Especially the molecular mechanisms involved in the regulation of immune responses by GC will be discussed for a better understanding of the differential effects of GC on the nature of the immune response in general. Moreover, the mechanisms involved in regulating the functional properties of the GR will be thoroughly discussed in order to explain the increased sensitivity of lymphocytes for GC in CFS-patients.

Regulation of Th1 and Th2 responses by glucocorticoids in CFS

The supposed increased negative feedback action of GC in CFS-patients might be a generalized phenomenon, and therefore also affect their immune response. As was mentioned in the introduction of this thesis, Th1 responses are much more sensitive for suppression by GC than Th2 responses [2, 13-16]. Therefore it was hypothesized that CFS-patients would display reduced Th1 responses, increased Th2 responses and an increased responsiveness to GC *in vitro*.

Indeed, the studies on CFS described in the literature are suggestive for a reduced Th1 response and an increased Th2 response, as is apparent from a reduced NK activity [17, 18].

Also a reduced DTH response [19], increased allergic reactions [20-22] and reactivation of viral infections have been reported [23-27].

In order to investigate whether CFS-patients display differences in Th1 or Th2 responses we report in **chapter 2** the characteristics of purified CD4 positive T cells from the peripheral blood of CFS-patients compared to age and sex matched controls. Since the balance between Th1 and Th2 cells, is proposed to be regulated by GC [2], it was also studied to what extent immunological alterations in CFS are associated with an altered sensitivity of T helper cells to GC.

Stimulation of these CD4 positive T cells of CFS-patients with PHA resulted in a reduced IFN-γ production, but normal IL-4 production and proliferation. These results therefore suggest a normal Th2 activity accompanied by a reduced Th1 activity in CFS-patients. However, because CFS-patients have been reported to show reduced levels of cortisol [9-12] we rather had expected increased production of IFN-γ *in vitro*.

The reduced IFN-γ production could not be attributed to an altered balance between naive and memory T helper cells, because flow cytometric analysis showed no differences in these cell-types between patients and controls. Interestingly, we demonstrated in CFS-patients an increased sensitivity of the T helper cells for dexamethasone *in vitro* as judged from the enhanced suppression of their proliferation and IL-4 production. The increased sensitivity was not found with regard to IFN-γ, probably due to the fact that the IFN-γ production is already suppressed in CFS-patients. In as much as IFN-γ is more suppressed by GC than IL-4 [2, 13-16], increased *in vivo* suppression by endogenous GC, might have caused elimination of IFN-γ production in a sensitive subset of cells leading to a lower IFN-γ production *in vitro*.

It is however of importance to realize that besides a direct suppression of the IFN-γ production by T cells, GC might also regulate IFN-γ production indirectly via modulation of IL-12 and IL-10 production, which were demonstrated to be crucial regulatory cytokines for IFN-γ production [28, 29]. In the experiments described in chapter 2, we used monocyte-dependent stimulation of the T helper cells.

Therefore the reduced IFN-γ production by T helper cells of CFS-patients could be due to an altered IL-10 and IL-12 production by monocytes. Furthermore, the increased sensitivity for GC may have also affected the balance between IL-10 and IL-12 in vivo in CFS-patients.

The possibility that the Th2 skewing effect of glucocorticoids in general is due to specific downregulation of IL-12 and an upregulation of IL-10, was addressed in **chapter 3**.

Differential regulation of IL-10 and IL-12 by glucocorticoids in vitro

In **chapter 3** the effects of GC on IL-10 and IL-12 production were described. The goal of these experiments was whether the Th2 skewing effects of GC in general are exerted via selective effects on IL-10 or IL-12 production.

In this chapter our studies demonstrate in whole blood cultures and in purified monocytes, that IL-12 and TNF-α are very sensitive for suppression by GC, while IL-10 is relatively resistant. Significant suppression of IL-10 occurred only by pharmacological (1μM) concentrations of DEX. In some donors, the production of IL-10 was even enhanced by DEX, which is in line with the results shown in the literature [30, 31]. It is well established that one of the modes of action of IL-10 in suppressing cellular immunity is the inhibition of IL-12 [29]. However, because DEX could still suppress IL-12 and TNF-α in the presence of neutralizing antibodies for IL-10, IL-10 was not an intermediate in the suppression of these cytokines. GC mediated the suppressive effects of DEX and HC via the GR, because the GR antagonist RU486 could abolish the suppression. Interestingly, high concentrations of RU486 completely antagonized the suppressive effects of DEX on IL-12p40 production, whereas the abrogation of suppression by HC was not complete. This suggests that also the mineralocorticoid receptor is involved in the suppression of IL-12p40 by hydrocortisone. However we could not substantiate this hypothesis, because aldosterone, a specific mineralocorticoid receptor agonist, and spironolactone a specific mineralocorticoid receptor antagonist did not affect the suppression of IL-12p40 by HC.

An important observation reported in chapter 3, was the suppression of IL-10 and the enhancement of IL-12 production in whole blood cultures by high concentrations of RU486. Because of the presence of endogenous cortisol in the whole blood cultures it is allowed

to speculate that a basal occupation of the GR is a prerequisite for an efficient induction of IL-10 and simultaneously causes suppression of IL-12. Indeed with regard to IL-12p40 production a positive correlation between endogenous cortisol and the enhancing effect of RU486 was observed. With regard to IL-10 such a correlation could not be found possibly because of a saturating effect of the endogenous cortisol. This enhancing effect of RU486 on IL-12 was not affected by neutralizing antibodies for IL-10. The observation that neutralizing antibodies to IL-10 and RU486 synergistically enhanced the IL-12 production, suggests that different mechanisms are involved in the regulation of IL-12 by IL-10 and GC.

Transcription factors involved in the regulation of cytokine production

The differential effects of GC on TNF-α, IL-10 and IL-12 production as presented in chapter 3 can be largely explained by the involvement of different transcription factors in the regulation of these cytokines (see table 1).

Table 1. Transcription factors involved in cytokine regulation and the sensitivity of cytokines to glucocorticoids.

Cytokine	Transcription factors	Suppression by GC
IL-2	AP-1, NFkB, NFAT, OCT1	↓↓↓↓
IL-4	AP-1, NFAT,	↓↓
TNF-α	CREB, NFkB, AP-1	↓↓↓↓
IFN-γ	STAT-4, AP-1	↓↓↓↓
IL-12p40	NFkB	↓↓↓
IL-12p35	???	↓↓↓
IL-10	AP-1, CREB, GRE	↓↑

The promoter region of the TNF-α gene contains binding sites for the transcription factors AP-1, CREB and NFkB [32], transcription factors that can all be suppressed by GC [33-35]. With the use of reporter assays and the study of the effect of selective mutations in the binding sites for the different transcription factors it was found that NFkB and CREB are dominantly involved in the transcription of TNF-α after stimulation with LPS [32]. The involvement of the transcription factors CREB and NFkB in TNF-α production and the important role of NFkB in the regulation of IL-12p40 gene expression [32, 36] may therefore explain the strong suppression of these cytokines by GC. It is unknown which transcription factors are involved in the expression of IL-12p35. However in view of the fact that both IL-10 and GC can suppress IL-12p35 [this thesis, 37], it is allowed to conclude that to some extent NFkB is involved in the regulation of IL-12p35 expression.

The reason for this assumption is the fact that IL-10 has been shown to suppress the activity of NFkB, but not the activity of AP-1, CREB, OCT1 and NF-IL-6 [38].

IL-10 was observed to be relatively resistant to suppression by GC and we could only detect significant suppression by high concentrations of dexamethasone. Furthermore, high concentrations of RU486 prevented optimal induction of IL-10 by LPS. This observation is in line with the findings by van der Pol et al. [30], who showed an increase in IL-10 plasma levels in response to HC.

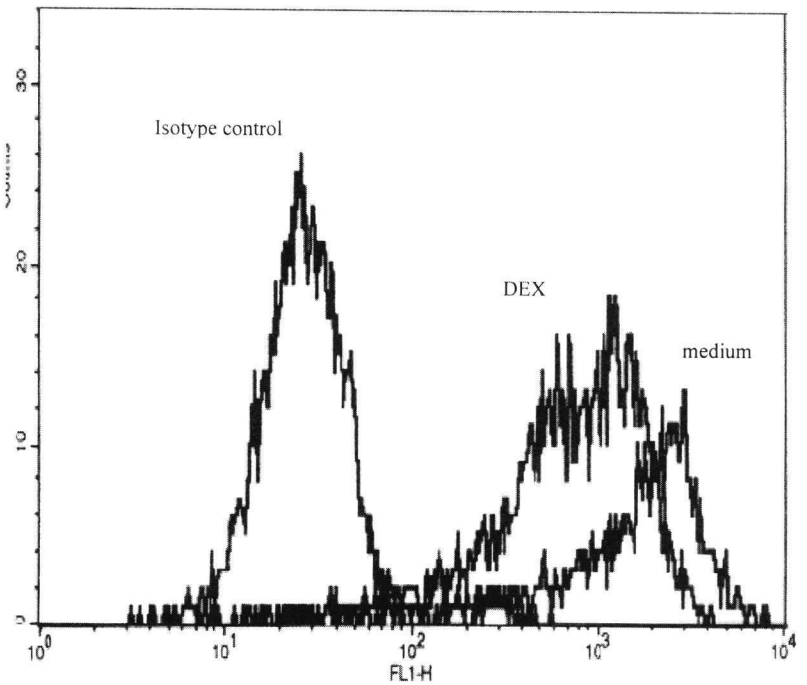
In addition to responsive elements for AP-1 and CREB, the promoter region of IL-10 also contains a glucocorticoid responsive element (GRE) [39].

Therefore, it can be stated that GC might act as an enhancer for IL-10 expression via the occupation of the GRE by the activated GR. Since AP-1 and CREB are rather inhibited by GC, the balance between the positive action via the GRE and the negative actions via downregulation of CREB and AP-1 will determine the net effect of GC on the production of IL-10.

The differential effects of GC on IL-10 and IL-12 production appear to occur in parallel to a differential effect on the receptors for these cytokines. Wu and colleagues [40] have shown that DEX suppresses the expression of the IL-12 receptor on T cells and that the T cells loose their responsiveness for IL-12 for at least 24 hours. On the other hand, Michel et al. [41] have demonstrated that DEX enhances the expression of the IL-10 receptor on keratinocytes of psoriasis patients.

Experiments by our group employing PBMC of healthy donors, demonstrated that the expression of the IL-10 receptor on monocytes was only slightly influenced by DEX (see Fig. 1). The same results were obtained with T cells. These results indicate that GC and IL-10 can work in concert to suppress inflammatory responses. Accordingly it has been demonstrated by Herfarth et al [42], that suboptimal concentrations of DEX potentiate the ability of IL-10 to prevent chronic intestinal and systemic inflammation in rats.

Fig 1. Expression of the IL-10 receptor on monocytes is relatively resistant to dexamethasone suppression.



In this figure it is demonstrated that the IL-10 receptor on Monocytes is relatively resistant to dexamethasone. The cells were incubated with or without 1 μM dexamethasone for 24 hours. The expression of the IL-10 receptor was studied using biotynilated IL-10 applying a commercially available detection kit (R&D systems, Abingdon, UK) according the manufacturers instructions. The figure is a representative of 2 separate experiments.

In conclusion it can be stated, that as a consequence of the differential effects on IL-10 and IL-12, GC are able to modulate the function of antigen presenting cells in such a way that they become Th2 inducing APC. Several authors have substantiated this hypothesis by demonstrating that monocytes and dendritic cells treated with GC are strong inducers of Th2 responses [43-47]. These effects are most likely due to inhibition of IL-12 in addition to the downregulation of co-stimulatory molecules [43-47].

Altered regulation of IL-10 and IL-12 by glucocorticoids in CFS

As discussed above IL-12 is a key cytokine in the induction of IFN-γ [28]. It is important to realize that only IL-12p70 (the heterodimer of IL-12p40 and IL-12p35) is biologically active in this respect [28]. IL-10 is a potent suppressor of IL-12p70 and as a consequence of IFN-γ production [29]. However there are reports that demonstrate a direct suppressive effect of IL-10 on IFN-γ production [48, 49]. The observed reduced production of IFN-γ by T helper cells of CFS-patients might therefore be caused by an altered balance between IL-10 and IL-12 production. To investigate this possibility we studied in **chapter 4** the production of IL-10 and IL-12 in whole blood cultures of CFS-patients. We used whole blood cultures, because it is possible to induce both IL-10 and IL-12 in this system.

The experiments presented in chapter 4 demonstrate an increased production of IL-10 and a trend to a reduced production of IL-12p70 in CFS-patients. When the production was corrected for monocyte counts, both IL-10 and IL-12p70 were significantly different in CFS-patients as compared to the controls. Monocytes which express low CD14 levels are not very capable in producing IL-10 [50]. However, the density of CD14 on the monocytes did not differ between patients and controls.

Therefore the reduced production of IFN-γ by $CD4^+$ T cells of CFS-patients in a monocyte dependent system as described in chapter 2, may be explained by increased IL-10 and reduced IL-12 levels.

In chapter 4 cytokines induced by LPS in whole blood cultures were studied with regard to their sensitivity to GC. Both in CFS-patients and controls IL-10 production was relatively resistant to GC as compared to TNF-α and IL-12 production. However, IL-10 production was more suppressed in whole blood cultures of CFS-patients as compared to the controls. With regard to TNF-α and IL-12p40 production no differences in the sensitivity for DEX was observed between patients and controls.

For an optimal induction of IL-12p70 we stimulated the whole blood cultures with LPS and IFN-γ. IL-12p70 production was equally sensitive to GC in CFS-patients and controls, however, in this system, IL-12p40 appeared more sensitive to DEX in CFS-patients than in controls. This was probably due to the fact that IFN-γ rendered the IL-12p40 production in controls less sensitive to DEX, while having no effect on the sensitivity for DEX in the patients.

Therefore, our observations also suggested a diminished response to IFN-γ in the CFS-patients as compared to the controls. Such a difference would than be explanatory for a reduced IL-12p70 production in CFS. However, the fact that IFN-γ gave a comparable inhibition of IL-10 and stimulation of IL-12p40 in CFS-patients and controls, would therefore suggest a reduced inducability of IL-12p35 in CFS. Secondly, with regard to IL-12p40 suppression by GC, IFN-γ may have affected a GR mediated mechanism, but only in the controls.

As discussed in chapter 3 endogenous cortisol present in whole blood influenced the induction of cytokine production. Indeed in controls an inverse correlation was observed between endogenous cortisol and the quantity of cytokines that could be induced by LPS in whole blood cultures. In contrast, CFS-patients only displayed a significant correlation between endogenous cortisol and IL-12p70, while no relation was observed between endogenous cortisol and IL-12p40 or IL-10. An uncoupling between endogenous cortisol and immune parameters was also demonstrated by Cannon et al [51] with regard to the distribution of neutrophils in the peripheral blood. Taken together these results indicate that GC differentially regulate cytokine production in CFS-patients.

For a long time infectious agents were being regarded responsible for the etiology of CFS. A lot of attention has been paid to the involvement of the Epstein Barr virus, which was hypothesized by Strauss and Komaroff to be a likely cause of CFS [23, 26].

However, intensive research by several groups [27, 52-55] did not provide evidence for an involvement of EBV or other infectious agents in the etiology of CFS. In these studies a serological approach was used to establish an involvement of EBV, with the risk to overlook subclinical infections. Since we observed an increased IL-10 production in CFS, we investigated by PCR analysis and ELISA whether this could be attributed to viral IL-10, the EBV homologue of human IL-10. In none of the donors PBMC, we could detect significant expression of viral IL-10. Also the culture supernatants appeared negative for viral IL-10. Because expression and production of viral IL-10 can be detected after EBV infection [56], active EBV infection or reactivation of EBV is unlikely to play a role in CFS.

Nevertheless, a subset of CFS-patients may originate from people who have been subject to infections, diagnosed with symptoms of post viral fatigue and after several years of persistent fatigue complaints considered to suffer from CFS. To identify such a subset of CFS-patients, longitudinal studies are recommended. Lloyd and colleagues have started such a longitudinal study in Australia [AACFS conference, Boston, August 1998]. They included recently infected patients and are documenting their immune status, persistence of the infectious agent in conjunction with physical examinations and fatigue complaints. The outcome of such a study will clarify the involvement of infectious agents in the onset of CFS with more certainty.

As was suggested by Mawle et al. [57], the diversity in alterations of immune parameters found in CFS is probably caused by the heterogeneity of the patient population. Also the different approaches to test these immune parameters, contribute to the variety in the results. As shown in chapter 4, the heterogeneity of the patient population also influenced the results in our study. Subdividing the patients according to objective criteria such as gender and duration of the disease, already revealed differences between these subgroups with regard to cytokine profile and sensitivity for DEX. The cytokine production appeared more affected by the heterogeneity of the patients population than their sensitivity to GC. This implies that immune parameters like cytokine profiles or expression of lymphocyte subsets are not very useful as a diagnostic marker for the severity of the disease. However, the sensitivity to GC might still be helpful in the development of a diagnostic test.

In conclusion these results demonstrate an altered cytokine profile in whole blood cultures of CFS-patients that may be the consequence of an impaired regulation by GC. This impaired regulation is evidenced by the increased sensitivity of IL-10 and IL-12p40 to GC in whole blood cultures of CFS-patients as well as the absence of a relation between these cytokines and endogenous cortisol.

In **chapter 5** studies are reported addressing the question whether an altered GR function or an impaired interaction of the GR with transcription factors may explain this impaired regulation in CFS-patients.

Mechanisms involved in glucocorticoid sensitivity

GC diffuse across the cell membrane and exert their effects after binding to the GR in the cytosol [58]. This GR-GC complex can then exert transcriptional activities. After formation of GR-GC homodimers, these complexes can act as transcription factors and regulate genes with a GRE in their promoter region, a phenomenon termed transactivation [58]. Monomers of the activated GR influence the transcription of several genes by interfering with the expression of several transcription factors in the cell, a phenomenon known as transrepression [58]. These monomers inhibit the expression of transcription factors like AP-1 and NFkB by protein-protein interactions resulting in repression of expression of genes regulated by these transcription factors. Most of the anti-inflammatory actions of GC are thought to be mediated by this mechanism [58-60].

The increased sensitivity to GC as demonstrated in chapters 2 and 4 suggests altered properties of the GR in CFS-patients. These altered properties may be caused by several factors including:

1. Availability of the hormone

In order to mediate its effects, the hormone has to be available for binding to its receptor. Especially in the kidney the bio-availability of the hormone is decreased by the enzyme 11β-HSD that converts cortisol into its inactive form cortisone [58]. This enzyme has been shown to be present in other tissues than the kidney and is hypothesized to be involved in regulating the local effects of GC in tissues, such as the lungs [Rook, personal communication].

2. Density of the GR

The response of cells to GC depends on the properties of the GR, of which affinity and density are important features for the responsiveness of cells to GC. It has been demonstrated that steroid resistance and hypersensitivity are associated with low and high expression of the GR respectively [58, 61].

Mutations in the GR gene have been identified which are responsible for diminished expression of the GR and which subsequently cause steroid resistance [62].

3. Affinity of the GR

Cytokines have been shown to influence the expression and affinity of the GR. Kam et al. [63] and Sher et al. [61] demonstrated that combinations of IL-2 and IL-4 cause a marked reduction of the affinity of the GR and render lymphocytes unresponsive to GC. How cytokines influence the affinity of the GR is unclear, but phosphorylation of these receptors might be involved.

4. Binding of the GR with a GRE

The GR can be phosphorylated at four major sites (Thr-171, Ser-224, Ser-232 and Ser-246) in its N-terminal transcriptional regulatory region [64-66]. Recent studies have shown that these sites can be differentially phosphorylated by the protein kinases MAPK, JNK, ERK and CdK as opposed to P38. This phosphorylation process may enhance or suppress the transcriptional activity of the GR , leading to an altered GRE/DNA binding of the GR homodimers [64-66]. Whether phosphorylation of the GR results also in altered transrepression or affinity is unclear.

The observation that IL-2 and IL-4 are activators of protein kinases [67] might explain their ability to reduce the transcriptional activity of the GR and as a consequence the responsiveness of lymphocytes to GC.

5. Stability of the GR

In the cytosol the GR forms a complex with the heat shock proteins 90 and 56. These heat shock proteins maintain the GR in a stable form allowing the formation of a complex with its ligand [58]. Several studies have demonstrated that mutations in the heat shock protein complex or diminished expression of the heat shock proteins cause destabilization of the GR, reduction of its affinity and steroid resistance [67-69]. Likewise polymorpisms of the ligand binding subunit of the GR were shown to cause reduced affinity and steroid resistance [70].

6. Interaction of the GR with transcription factors

As mentioned earlier in this thesis GC control immune responses by downregulating transcription factors like AP-1, NFkB and CREB via protein-protein interactions, via upregulation of inhibitor proteins or the induction of anti inflammatory cytokines.
Altered responses to GC may be due to a disturbed interaction of the GR with transcription factors. Barnes and coworkers [59]have demonstrated that steroid resistance in a subgroup of asthma patients can be attributed to a reduced protein-protein interaction with AP-1.
The mechanisms by which GC upregulate for instance IkB or IL-10 are not fully understood, although with regard to IL-10 transactivation via a GRE might be involved [39, this thesis]. In the promoter region of IkB no GRE has been demonstrated, so it is unclear whether transactivation is involved in IkB upregulation [34, 35].

7. The expression of endogenous inhibitors like GR-β.

Recently an alternative splice variant of the GR termed GR-β, has been identified [71]. This variant lacks the hormone binding domain and can therefore not be activated by its ligand. Although it has been demonstrated that GR-β can interfere with transactivation and block the activity of a GRE [71], this endogenous antagonizing effect of GR-β is still controversial.

Affinity and density of the GR in CFS in relation to glucocorticoid sensitivity in vitro

As explained above, disturbed responses to GC are associated with several factors of which in particular the affinity or density of the GR have been studied. In **chapter 5** a study was reported, which addresses the question whether the increased sensitivity of CFS-patients for GC was due to an increased sensitivity or density of the GR in their lymphocytes.

We were unable to demonstrate a difference in affinity and density of the GR in PBMC of 10 randomly selected CFS-patients as compared to their matched controls. With the exception of 2 patients and 1 matched control, the affinity and receptor density were shown to be in the normal range. Accordingly, no differences were found in the expression level of GR mRNA. Nevertheless PBMC from the same CFS-patients were more sensitive to GC *in vitro*. This suggests that other mechanisms are involved. As explained above, disturbed interactions of the GR with transcription factors or other coactivators of transcription can lead to altered sensitivity for GC without changes in affinity and density of the receptors.

Also Huizenga et al. [72] have demonstrated steroid hypersensitivity in a subset of healthy people without a change in affinity or density of the GR. A point mutation in the GR resulting in a substitution of an asparagine by a serine residue was associated with this increased sensitivity. Because serine residues of the GR can be phosphorylated, such a process may alter the transcriptional activity of the GR.

In chapter 5 the PBMC were stimulated with increasing dosages of the phorbol ester PMA, which is a strong activator of protein kinase C and causes strong upregulation of AP-1 and to a lesser extent of NFkB [33]. In both patients and controls, the production of IL-2 was strongly enhanced by adding PMA to the cultures, but more PMA was required to achieve comparable IL-2 levels in the patients as compared to the controls. The trend towards reduced production of IL-2 was not completely restored by addition of PMA. In view of the importance of AP-1 in the regulation of IL-2 expression [73], these results suggests that PBMC of CFS-patients are less capable in the upregulation of AP-1 after mitogen stimulation. A reduced buffer capacity of AP-1 in the cells of CFS-patients might therefore be responsible for the increased sensitivity for GC in CFS-patients.

In agreement with studies by Barnes et al. [59] and Nijhuis et al. [74], PMA induced overexpression of AP-1 rendered the PBMC unresponsive to DEX with regard to the

suppression of lymphocyte proliferation. Furthermore PMA rendered the cells resistant to DEX in the suppression of IFN-γ and IL-10, while IL-2 and TNF-α remained sensitive.

No difference was found between patients and controls with regard to the resistance inducing capacity of PMA. We had expected that more PMA would have been required to induce steroid resistance in CFS-patients than the controls, because the results presented in chapter 5 suggested reduced AP-1 levels and/or an increased interaction of the GR with transcription factors such as AP-1. Possibly, costimulation with PMA is not subtle enough to discriminate between patients and controls, because besides its AP-1 inducing capacity, PMA might via activation of protein kinases [75] phosphorylate the GR, and thereby cause an altered transcriptional activity of these GR.

In conclusion, we postulate that PBMC of CFS-patients display an increased sensitivity to GC in vitro, which cannot be explained by alterations in number or affinity of the GR, but should rather be attributed to molecular processes beyond the actual binding of the ligand to the GR. Therefore more specific molecular biological methods like electromobility shift assays may be useful to investigate GR-transcription factor interactions in lymphocytes of CFS-patients.

Glucocorticoid sensitivity in relation to affective disorders

If it is assumed that GR are present in nearly almost al the tissues and cell-types of the human body, GR dysfunction is not tissue specific and will affect all the functions under control of GC. Importantly, the feedback of GC on the pituitary and the hypothalamus will be altered, resulting in altered regulation of the HPA-axis, with subsequent changes in regulation of the stress system, metabolic processes, brain development, behavior and immune function. Experiments performed by the groups of Sternberg and Mason [76-79] have demonstrated that a reduced capacity to generate high levels of GC, or an inappropriate response to GC, render rat and mice strains sensitive for experimental autoimmune diseases. Likewise in humans steroid resistance or reduced functioning of the HPA-axis are supposed to play a role in autoimmunity [2, 3, 4, 80]. Interestingly, affective disorders like depression and post traumatic stress disorder (PTSD) are associated with HPA-axis dysfunction [7, 81]. In depression a reduced responsiveness to GC was observed, probably as a result of a reduced affinity and expression of the GR [5, 8]. Moreover an altered cross-talk between GR and transcription factors cannot be excluded. The reduced GR function in depression is probably responsible for the fact that the majority of the depressed patients escape dexamethasone-suppression *in vivo* [6, 8]. With regard to PTSD and Fybromyalgia a hypoactivity of the HPA-axis is observed [82, 83]. In PTSD-patients the hypoactivity of the HPA-axis might result from an increased negative feedback, because increased GR levels were observed in their PBMC [82]. With regard to Fibromyalgia a reduced affinity of the GR has been described, despite normal responses to GC *in vitro* [84].

Several groups have demonstrated an impaired functioning of the HPA-axis in CFS [9-12]. CFS-patients showed reduced levels of cortisol and an impaired response to ACTH. The cause of this HPA-axis dysfunction is unclear, but the results presented in this thesis suggest that an increased responsiveness to GC might be involved. However, although it was demonstrated in this thesis that PBMC of CFS-patients display an increased sensitivity to GC *in vitro*, this does not prove an increased sensitivity at the level of the HPA-axis. However, hydrocortisone replacement therapy gave no improvement of the syndrome but rather lead to adrenal insufficiency [85]. To establish whether CFS-patients react stronger on GC *in vivo*, a dexamethasone-suppression test needs to be performed on CFS-patients.

In view of the data presented in this thesis, I expect CFS-patients to be more sensitive in this regard. Therefore, such studies may provide additional evidence that the symptomatology of CFS is quite distinct from depression, because in depression a reduced dexamethasone suppression of HPA-axis activity is observed.

Concluding remarks

1. *It is known that glucocorticoids are beneficial for Th2 responses and detrimental for Th1 development. The present study has demonstrated that this Th2 skewing effect by glucocorticoids involves enhancement of IL-10 and suppression of IL-12.*

2. *CFS-patients display an altered cytokine profile. However, potential confounding factors are heterogeneity of the patient population (such as gender, duration of disease, etc) and the various lymphocyte subsets under investigation. Therefore, immune parameters are expected to be only useful for diagnostic purposes under well defined disease conditions.*

3. *Lymphocyte function of CFS-patients displays an increased sensitivity to glucocorticoids. Therefore the study of glucocorticoid responsiveness in CFS may be promising for a more objective diagnosis of the syndrome.*

4. *The mechanisms underlying the increased glucocorticoid sensitivity in CFS are not due to changes in the affinity or density of the glucocorticoid receptor. Therefore, future studies should focus on other aspects of the life cycle of this receptor (i.e. interactions with transcription factors, co-activators and heat shock proteins), in order to explain its role in the pathogenesis of CFS.*

References

1. Blalock JE. The syntax of immune-neuroendocrine communication. Immunol Today 1994; 15: (No 11): 504-11.

2. Rook GAW, Hernandez-Pando R, Lightman S. Hormones, peripherally activated prohormones and regulation of the Th1/Th2 balance. Immunol Today 1994; 15: 301-3.

3. Wilder RL: Neuroendocrine-immune system interactions and autoimmunity. Annu Rev Immunol 1995; 13: 307.

4. Wilckens T, de Rijk R. Glucocorticoids and immune function: unknown dimensions and new frontiers. Immunol Today 1997; 18 (9): 418-24.

5. Holsboer F, Von Bardeleben U, Gerken A, Stalla GK, Muller O. Blunted corticotropin and normal cortisol response to human corticotropin-releasing factor in depression. New England J Med 1984; 311: 1127.

6. Gormley GJ, Lowy MT, Reder AT, Hospelhorn VD, Antel JP, Meltzer HY. Glucocorticoid receptors in depression: relationship to the dexamethasone suppression test. Am J Psych 1985; 132: 1278-84.

7. Holsboer F, Barden N. Antidepressants and hypothalamic-pituitary adrenocortical regulation. Endocr Rev 1996; 17 (2): 187-205.

8. Modell S, Yassouridis A, Huber J, Holsboer F. Corticosteroid receptor function is decreased in depressed patients. J Neuroendocrinol 1997; 65 (3): 216-22.

9. Demitrack MA, Dale JK, Strauss SE, et al. Evidence for impaired activation of the hypothalamic-pituitery-adrenal axis in patients with chronic fatigue syndrome. J Clin Endocrinol Metab 1991; 73: 1224-34

10. Bearn J and Wessely S. Neurobiological aspects of the chronic fatigue syndrome. Eur J Clin Invest 1994; 24: 79-90.

11. Dinan TG, Majeed T, Lavelle E, Scott LV, Berti C, Behan P. Serotonin mediated activation of the hypothalamic-pituitary-adrenal-axis in chronic fatigue syndrome. Psychoneuroendocrinol 1997; 22: 261-68.

12. Scott LV, Medbak S, Dinan TG. The low dose ACTH test in chronic fatigue syndrome and health. Clin Endocrinol 1998; 48: 733-37.

13. Daynes RA, Araneo BA. Contrasting effects of glucocorticoids on the capacity of T cells to produce growth factors Interleukin-2 and Interleukin-4. Eur J Immunol 1989; 19: 2319-25.

14. Brinkman V, Kristofic C. Regulation by corticosteroids of Th1 and Th2 cytokine production in human $CD4^+$ effector T cells generated from $CD45RO^-$ and $CD45RO^+$ subsets. J Immunol 1995; 155: 3322-28.

15. Snijdewint FGM, Kapsenberg ML, Wauben-Penris PJJ, Bos JD. Corticosteroids class-dependently inhibit Th1- and Th2-type cytokine production. ImmunoPharmacol 1995; 29: 93-101.

16. Ramirez F, Fowell DJ, Puklavec M, Simmonds S, Mason D: Glucocorticoids promote a Th2 cytokine response by $CD4^+$ T cells in vitro. J Immunol 1986; 156: 2406.

17. Caliguiri M, Murray C, Buchwald D, et al. Phenotypic and functional deficiency of natural killer cells in patients with chronic fatigue syndrome. J Immunol 1987; 139: 3306-13.

18. Aoki T, Miyakoshi H, Usuda Y, Herberman RB. Low NK syndrome and its relationship to chronic fatigue syndrome. Clin Immunol Pathology 1993; 69 (no 3): 253-65.

19. Lloyd A, Hickie I, Hickie C, Dwyer J, Wakefield D. Cell mediated immunity in patients with chronic fatigue syndrome, healthy control subjects and patients with major depression. Clin Exp Immunol 1992; 87: 76-9.

20. Straus SE, Dale JK, Whright R, Metcalfe DD. Allergy and the chronic fatigue syndrome. J allergy Clin Immunol 1988; 81 (5): 791-5.

21. Komaroff AL. Buchwald DS. Chronic Fatigue syndrome: an update. Ann Rev Med 1998; 49: 1-13.

22. Borish L, Schmaling K, DiClementi JD, Streib J, Negri J, Jones JF. Chronic fatigue syndrome: Identification of distinct subgroups on the basis of allergy and psychologic variables. J Allergy Clin Immunol 1998; 102: 222-230.

23. Komaroff AL. Chronic Fatigue Syndromes: relationship to chronic viral infections. J Virol Methods 1988; (1-4): 3-10.

24. Josephs SF, Henry B, Balachandran N, Strayer D, Peterson D, Komaroff AL, Ablashi DV. HHV-6 reactivation in Chronic Fatigue Syndrome. Lancet 1991; 337 (8753): 1346-7.

25. Manian FA. Simultaneous measurement of antibodies to Epstein-Barr virus, human herpesvirus-6, herpes simplex virus types 1 and 2, and 14 enteroviruses in chronic fatigue syndrome: is there evidence of activation of a nonspecific polyclonal immune response? Clin Infect Dis 1994; 19 (3): 448-53.

26. Strauss SE, Komaroff AL, Wedner HJ. Chronic Fatigue syndrome: Point and counterpoint. J Infect Dis 1994; 170:1-6.

27. Mawle AC, Reyes M, Scmid DS. Is chronic Fatigue Syndrome an infectious disease? Infect Agents Dis 1994; 2: 333-41.

28. Trinchieri G: Interleukin-12: A proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen specific adaptive immunity. Annu Rev Immunol 1995; 13: 251.

29. D'Andrea A, Aste-Amezaga M, Valiante NM, Ma X, Kubin M, Trinchieri G: Interleukin 10 inhibits human lymphocyte interferon-γ production by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells. J Exp Med 1993; 178: 1041,

30. van der Poll T, Barber AE, Coyle SM, Lowry SF. Hypercortisolemia increases plasma interleukin-10 concentrations during human endotoxemia-a clinical research center study. J Clin Endocrinol Metab 1996; 81: 3604.

31. Tabardel Y, Duchateau J, Schmartz D, Marecaux G, Shahla M, Barvais L, Leclerc JL, Vincent JL. Corticosteroids increase blood interleukin 10 level during cardiopulmonary bypass in men. Surgery 1996; 119: 76.

32. Yao J, Mackman N, Edgington TS, Fan ST. Lipopolysacharide induction of the Tumor Necrosis Factor-α promoter in human monocytic cells. J Biol Chem 1997; 272 (28): 17795-801.

33. Adcock IM, Brown CR, Gelder CM, Shirasaki H, Peters MJ, Barnes PJ. Effects of glucocorticoids on transcription factor activation in human peripheral blood mononuclear cells. Am J Physiol. 1995; 268 (2 Pt 1): C331-8.

34. Scheinman RI, Cogswell PC, Lofquist AK, Baldwin AS jr. Role of transcriptional activation of IkBα in mediation of immunosuppression by glucocorticoids. Science 1995; 270: 283-86.

35. Auphan N, Di Donato JA, Rosette C, Helmberg A, Karin M. Immunosuppression by glucocorticoids: Inhibition of NF-kB activity through induction of IkB synthesis. Science 1995; 270: 286.

36. Murphy TL, Cleveland MC, Kulesza P, Magram J, Murphy KM. Regulation of interleukin-12p40 expression through a NFkB half-site. Mol Cell Biol 1995; 10: 5258.

37. Aste-Amezaga M, Ma X, Sartori A, Trichieri G. Molecular mechanisms of the induction of IL-12 and its inhibition by IL-10. J Immunol 1998; 160: 5936.

38. Wang P, Wu P, Siegel MI, Egan RW, Billah MM. Interleukin-10 inhibits nuclear factor κB activation in human monocytes. J Biol Chem 1995; 270 (16): 9558.

39. Platzer C, Meisel Ch, Vogt K, Platzer M, Volk HD. Upregulation of monocytic IL-10 by tumor necrosis factor alpha and cAMP elevating drugs. Int Immunol 1995; 4: 517.

40. Wu CY, Wanf K, McDyer JF, Seder RA. Prostaglandin E2 and dexamethasone inhibit IL-12 receptor expression and IL-12 responsiveness. J Immunol 1998; 161 (6): 2723.

41. Michel G, Mirmohammadsadegh A, Olasz E, Jarzebska-Deussen B, Muschen A, Kemeny L, Abts HF, Ruzicka T. Demonstration and functional analysis of IL-10 receptors in human epidermal cells: Decreased expression in psoriatic skin, down-modulation by IL-8, and up-regulation by an anti-psoriatic glucocorticosteroid in normal cultured keratinocytes. J Immunol 1997; 159: 6291.

42. Herfarth HH, Bocker U, Janardhanam R, Balfour Sartor R. Subtherapeutic corticosteroids potentiate the ability of Interleukin-10 to prevent chronic inflammation in rats. Gastroenterology 1998; 115: 856.

43. Moser M, de Smedt T, Sornasse T, et al. Glucocorticoids down-regulate dendritic cell function in vitro and in vivo. Eur J Immunol 1995; 25: 2818-24.

44. Blotta MH, de Kruyff RH, Umetsu DT: Corticosteroids inhibit IL-12 production in human monocytes and enhance their capacity to induce IL-4 synthesis in $CD4^+$ lymphocytes. J Immunol 158: 5589, 1997.

45. DeKruyff R, Fang HY, Umetsu D. Corticosteroids enhance the capacity of macrophages to induce Th2 cytokine synthesis in $CD4^+$ lymphocytes by inhibiting IL-12 production. J Immunol 1998; 160: 2231.

46. Girndt M, Sester U, Kaul H, Hunger F, Kohler H. Glucocorticoids inhibit activation dependent expression of costimulatory molecule B7-1 in human monocytes. Transplantation 1998; 66 (3): 370-5.

47. Vieira PL, Kalinski P, Wierenga EA, Kapsenberg ML, de Jong EC. Glucocorticoids inhibit bioactive IL-12p70 production by in vitro generated human dendritic cells without affecting their T cell stimulatory potential. J. Immunol. 1998; 161: 5245-51.

48. Moore KW, O'Garra A, de Waal Malefyt R, Viera P, Mosman TR. Interleukin-10. Annu Rev Immunol 1993; 11: 165.

49. Wang F, Sengupta TK, Zhong Z, Ivashkiv LB. Regulation of the balance of cytokine production and the signal transducer and activator of transcription (STAT) transcription factor activity by cytokines and inflammatory synovial fluids. J Exp Med 1995; 182: 1825.

50. Frankenberger M, Sternsdorf T, Pechumer H, Pforte A, Loms Ziegler-Heitbrock HW: Differential cytokine expression in human blood monocyte subpopulations. Blood 1996; 87: 373.

51. Cannon JG, Angel JB, Abad LW, O'Grady J, Lundgren N, Fagioli L, Komaroff AL. Hormonal influences on stress-induced neutrophil mobilization in health and chronic fatigue syndrome. J Clin Immunol 1998; 18 (4): 291.

52. Straus SE, Dale JK, Tobi M, et al. Acyclovir treatment of the chronic fatigue syndrome. Lack of fficacy in a placebo-controlled trial. N Engl J Med 1988; 319 (26): 1692-8.

53. Swanink CMA, Vercoulen JHMM, Bleijenberg G, Fennis JFM, Galema JMD, van der Meer. Chronic fatigue syndrome: a clinical and laboratory study with a well matched control group. J Intern Med 1995; 237: 499-506.

54. Viral serologies in patients with chronic fatigue and chronic fatigue syndrome. Buchwald D, Ashley RL, Pearlman T, Kith P, Komaroff AL. J Med Virol 1996; 50 (1): 25-30.

55. Wallace HL 2nd, Natelson B, Gause W, Hay J. Human Herpes Viruses in chronic Fatigue syndrome. Clin Diagn Lab Immunol 1999; 6 (2): 216-23.

56. Kanegane H, Wakiguchi H, Kanegane C, Kurashige T, Tosato G. Viral interleukin-10 in chronic active Epstein-Barr virus infection. J Infect Dis 1997; 176: 254.

57. Mawle AC, Nisenbaum R, Dobbins JG, et al. Immune responses associated with chronic fatigue syndrome: A case-control study. J Infect Dis 1997; 175: 136-41.

58. Bamberger CM, Schulte HM, Chrousos GP. Molecular determinants of glucocorticoid receptor function and tissue sensitivity to glucocorticoids. Endocrine Reviews 1996; 17 (3): 245-61.

59. Barnes PJ, Greening AP, Crompton GK. Glucocorticoid resistance in asthma. Am J Respir Crit Care Med 1995; 152: s125-s142.

60. Scheinman RI, Gualberto A, Jewell CM, Cidlowski JA, Baldwin AS. Characterization of mechanisms involved in transrepression of NFkB by activated glucocorticoid receptors. Mol Cell Biol 1995; 15: 943.

61. Sher ER, Leung DYM, Surs W, Kam JC, Zieg G, Kamada AK, Szefler SJ. Steroi-resistant Asthma. Cellular mechanisms contributing to inadequate response to glucocorticoid therapy. J Clin Invest 1994; 93: 33-9.

62. Karl M, Lamberts SW, Detera-Wadleigh SD, Encio Ij, Stratakis CA, Hurley DM, Accili D, Chroussos GP. Familial glucocorticoid resistance caused by a splice site deletion in the human glucocorticoid receptor gene. J Clin Endocrinol Metab 1993; 76: 683.

63. Kam JC, Szefler SJ, Surs W, Sher ER, Leung DY. Combination of IL-2 and IL-4 reduces glucocorticoid receptor-binding affinity and T cells response to glucocorticoids. J Immunol 1993; 151: 3460.

64. Krstic MD, Rogatsky I, Yamamoto KR, Garabedian MJ. Mitogen activated and cyclin dependent protein kinases selectively and differentially modulate transcriptional enhancement by the glucocorticoid receptor. Mol Cell Biol 1997; 17: 3947.

65. Rogatsky I, Logan SK, Garabedian MJ. Antagonism of glucocorticoid receptor transcriptional activation by the c-jun N-terminal kinase. Proc Natl Acad Sci USA 1998; 95: 2050.

66. Webster JC, Jewell CM, Bodwell JE, Munck A, Sar M, Cidlowski JA. Mouse glucocorticoid receptor phosphorylation status influences multiple functions of the receptor protein. J Biol Chem 1997; 272: 9287.

67. Oakes SA, Candotti F, Johnston JA, Chen YQ, Ryan JJ, Taylor N, Liu X, Hennighousen L, Notarangelo LD, Paul WE, Blaese RM, O'Shea JJ. Signaling via IL-2 and IL-4 in JAK3-deficient severe combined immunodefiency lymphocytes: JAK3-dependent and independent pathways. Immunity 1996; 5: 605.

68. Picard D. Reduced levels of hsp90 compromise steroid receptor action *in vivo*. Nature 1990; 348: 166.

69. Cadepont F, Binart N, Chambraud B, Jibard N, Schweizer-Groyer G, Segard-Maurel I, Baulieu EE. Interaction of glucocorticoid receptor and wild-type or mutated 90-kDa heat shock protein coexpressed in baculovirus-infected Sf9 cells. Proc Natl Acad Sci USA 1993; 90: 10434.

70. Hurley DM, Accili D, Stratakis CA, Karl M, Vamvakopoulos N, Rorer E, Constantine K, Taylor SI, Chrousos GP. Point mutation causing a single amino acid substitution in the hormone binding domain of the glucocorticoid receptor in familial glucorticoid resistance. J Clin Invest 1991; 87: 680.

71. Bamberger CM, Bamberger AM, DeCastro M, Chroussos GP. Glucocorticoid receptor beta, a potential endogenous inhibitor of glucorticoid action in humans. J Clin Invest 1995; 95: 2435.

72. Huizenga NATM, Koper JW, de Lange P, Pols HAP, Stolk RP, Burger H, Grobbee DE, Brinkmann AO, de Jong, Lamberts SWJ. J Clin Endocrinol Metab 1998; 83: 144-51.

73. Vacca A, Felli MP, Farina AR, et al. Glucocorticoid receptor-mediated suppression of the Interleukin-2 gene expression through impairment of the cooperativity between nuclear factor activated t cells and AP-1 enhancer elements. J Exp Med 1992; 175: 637-46.

74. Nijhuis E, Hinloopen B, Odding J, Nagelkerken L. Abrogation of the suppressive effects of dexamethasone by PKC activation or CD28. Cell Immunol 1994; 156: 438.

75. Liu WS, Heckman CA. The sevenfold way of PKC regulation. Cell Signal 1998; 10: 529.

76. Sternberg EM, Young WS, Bernardini R, Calogero AE, Chrousos GP, Gold PW, Wilder RL. A central nervous system defect in biosynthesis of corticotropin-releasing hormone is associated with susceptibility to streptococcal cell wall-induced arthritis in Lewis rats. Proc Natl Acad Sci USA 1989; 86 (12): 4771-5.

77. MacPhee I, Antoni F, Mason D: Spontaneous recovery of rats from experimental allergic encephalomyelitis is dependent on regulation of the immune system by endogenous adrenal corticosteroids. J Exp Med 1989; 169: 431.

78. Mason D: Genetic variation in the stress response: susceptibility to experimental allergic encephalomyelitis and implications for human inflammatory disease. Immunol Today 1991; 12: 57.

79. Mason D, MacPhee I, Antoni F: The role of the neuroendocrine system in determining genetic susceptibility to experimental allergic encephalomyelitis in the rat. Immunology 1990; 70: 1.

80. Chikanza IC, Petrou P, Kingsley G, Chrousos G, Panayi GS: Defective hypothalamic response to immune and inflammatory stimuli in patients with rheumatoid arthritis. Arthritis Rheum 1992; 35: 1281.

81. Yehuda R, Southwick SM, Nussbaum G, Giller EL, Mason JW. Low urinary cortisol excretion in patients with post-traumatic stress disorder. J Nerv Mental Dis 1990; 178: 366-69.

82. Yehuda R, Boisoneau D, Lowy MT, Giller EL. Dose-response changes in plasma cortisol and lymphocyte glucocorticoid receptors following dexamethasone administration in combat veterans with and without posttraumatic stress disorder. Arch Gen Psych 1995; 52: 583-93.

83. Griep EN, Boersma JW, de Kloet ER. Altered reactivity of the hypothalamic-pituitary-adrenal axis in the primary fibromyalgia syndrome. J Rheumatol 1993; 20: 469-74.

84. Lentjes EG, Griep EN, Boersma JW, Romijn F, de Kloet ER. Glucocorticoid receptors, fibromyalgia and low back pain. Psychoneuroendocrinol 1997; 22: 603.

85. McKenzie R, O'Fallon A, Dale J, Demitrack M, Sharma G, Deloria M, Garcia-Borreguero D, Blackwelder W, Strauss SE. Low dose hydrocortisone for treatment of chronic fatigue syndrome. A randomized Controlled Trial. JAMA 1998; 280: 1061.

Nederlandse samenvatting

Het chronisch vermoeidheidsyndroom (CVS) dat in Nederland beter bekend is als ME (Myalgische Encaphalo Myelitis), is een nog grotendeels onbegrepen ziekte met als belangrijkste kenmerk een langdurige invaliderende vermoeidheid. In de wetenschappelijke wereld wordt echter liever gesproken over het Chronisch Vermoeidheid Syndroom (CVS), omdat dit een correctere term is. ME suggereert dat er sprake is van een hersenontsteking, maar tot op heden is hiervoor geen overtuigend bewijs geleverd.

Zoals hierboven vermeldt, is de oorzaak van deze ziekte grotendeels onbekend. De diagnose van de ziekte kan niet gesteld worden op basis van een objectief criterium, maar geschiedt eigenlijk op basis van exclusie.

Echter na jaren van debat in de internationale wetenschap, zijn er door het Centre of Disease Control in de Verenigde Staten eind jaren tachtig internationale criteria opgesteld waaraan iemand moet voldoen om gekarakteriseerd te worden als CVS-patiënt. Deze criteria, in 1994 nog een keer gereviseerd, zijn in principe niet geschikt voor diagnostische doeleinden, maar wel geschikt voor het karakteriseren van CVS-patiënten voor wetenschappelijk onderzoek, zodat internationaal wetenschappelijke resultaten met elkaar te vergelijken zijn. Aan al het onderzoek beschreven in dit proefschrift is geheel vrijwillig en goed geïnformeerd deelgenomen door via deze criteria geselecteerde CVS-patiënten en gezonde controles.

Ondanks het feit dat de ontstaanswijze van CVS nog steeds grotendeels onbegrepen is, zijn er in de loop der jaren vele hypotheses gesteld en verworpen over de oorzaak van de ziekte. In eerste instantie dacht men dat een virusinfectie een belangrijke oorzaak kon zijn voor CVS. Hierbij werd dan voornamelijk gedacht aan het Epstein barr virus dat verantwoordelijk is voor de ziekte van Pfeiffer, omdat de symptomen van deze ziekte grote gelijkenis vertonen met CVS. Echter, uitgebreid onderzoek door onder andere de groepen van Dr. Strauss in de Verenigde Staten en Prof. Dr. Van der Meer in Nederland, heeft laten zien dat dit virus en andere virale, bacteriële en andere infecties niet de oorzaak kunnen zijn voor het syndroom.

Vanwege het feit dat CVS-patiënten reactivaties laten zien van virale infecties, ook vatbaarder lijken te zijn voor infecties en er regelmatig gerapporteerd wordt over een

toename van allergieën, werd er gedacht aan verstoringen in het afweersysteem van CVS-patiënten. Verder werd men gesterkt in deze veronderstelling, omdat mensen die behandeld werden met stoffen die gemaakt worden door je eigen afweersysteem verschijnselen laten zien zoals vermoeidheid en concentratie problemen die sterk lijken op CVS.

Uitgebreid onderzoek over de gehele wereld heeft laten zien dat er inderdaad verstoringen zijn in het afweersysteem van CVS-patiënten. Samenvattend wekken al deze onderzoeken de indruk dat het onderdeel van het afweersysteem van CVS-patiënten dat belangrijk is voor een goede afweer tegen virusinfecties wat minder goed functioneert. Dit gedeelte van het afweersysteem wordt ook wel de cellulaire immuniteit of het cellulaire immuunsysteem genoemd. De andere tak van het afweersysteem, het humorale immuunsysteem, dat belangrijk is voor verdediging tegen bacteriën lijkt daarentegen niet aangetast te zijn. Al deze bevindingen zijn echter subtiel van aard en worden sterk beïnvloed door de diversiteit van de patiëntengroep. De gevonden verschillen berusten op groepseffecten en zijn daarom niet geschikt voor een diagnostische test.

Lange tijd werd gedacht dat het CVS een soort depressie was en dat de klachten van CVS-patiënten puur psychosomatisch waren. Dit misverstand is door verschillende onderzoeken opgelost en er is overtuigend bewijs geleverd dat CVS een totaal andere aandoening is dan een depressie. Zo heeft onderzoek door Dr. Demitrack en andere groepen laten zien dat de neuro-endocrinologische verschijnselen die optreden bij CVS-patiënten bijna tegenovergesteld zijn aan die bij depressie.

CVS-patiënten laten een verstoring zien in het functioneren van hun hersenstam hypofyse bijnier-as (HPA-as). Dit systeem is een belangrijk regelsysteem in het menselijk lichaam voor de regulatie van stress gerelateerde responsen. Dit regelsysteem kan geactiveerd worden door stress wat leidt tot afgifte van het stresshormoon cortisol (een corticosteroïd) door de bijnier.

Bij CVS-patiënten is er sprake van een verminderd functioneren van dit regelsysteem, wat leidt tot chronisch verlaagde niveaus van cortisol. Bij depressieve patiënten daarentegen is er sprake van een versterkt functioneren van dit systeem wat zich uit in verhoogde niveaus van cortisol.

Verder heeft onderzoek door Dr. Vercoulen uit de groep van Prof. Dr. van der Meer in Nijmegen laten zien dat CVS-patiënten niet te behandelen zijn met antidepressiva.

Er zijn verschillende soorten stress die tot afgifte van het stresshormoon cortisol kunnen leiden. De twee bekendste zijn: Emotionele stress en lichamelijke inspanning. Daarnaast is het zo dat een infectie of ontstekingsreactie in het lichaam leidt tot activatie van de HPA-as met als gevolg de afgifte van cortisol door de bijnier. Dit komt omdat diverse ontstekings-mediatoren de HPA-as activeren.

Cortisol is zeer belangrijk voor een goede energiehuishouding in het lichaam en verhoogt de activatiestaat van het lichaam. Cortisol zorgt er dus voor dat het lichaam beter om kan gaan met stressvolle omstandigheden.

Een andere belangrijke rol van cortisol is het in de hand houden van ontstekingsreacties. Als er een ontsteking optreedt door bijvoorbeeld een infectie, dan wordt via diverse ont-stekingsmediatoren de HPA-as geactiveerd. Het cortisol dat dan gemaakt wordt remt weer de ontstekingsreacties, waardoor deze ontsteking niet uit de hand loopt. Daarom worden synthetische corticosteroïden in de kliniek gebruikt als ontstekingsremmers en als onder-drukkers van het afweersysteem om afstoting van getransplanteerde organen te voorkomen.

Verder is aangetoond dmv proefdieronderzoek en in diverse humane studies, dat de mate waarin het lichaam in staat is om cortisol te maken als gevolg van een ontstekingsproces, bepalend is voor de gevoeligheid voor diverse autoimmuunziekten als reuma en multiple sclerose en of een ontstekingsproces veel sneller uit de hand kan lopen.

Doel van de studie

Ondanks het feit dat het is vastgesteld dat de verschillende hormonen die onder invloed staan van de HPA-as sterke regulerende functies hebben in het afweersysteem, is het tot op dit moment onduidelijk tot op welke hoogte de afwijkingen gevonden in het afweersysteem van CVS-patiënten gerelateerd zijn aan de gevonden endocrinologische verstoringen.

In dit proefschrift heb ik vooral de regulerende eigenschappen van corticosteroïden in het afweersysteem onderzocht. Ik heb onderzocht hoe de productie van belangrijke signaalstoffen

genaamd cytokinen, die een belangrijke rol spelen in het functioneren van het afweersysteem, door corticosteroïden worden gereguleerd. Dit is gedaan om een beter inzicht te krijgen in de effectiviteit van corticosteroïden in het algemeen, zodat een beter behandelingsprotocol kan worden opgesteld, waardoor er minder bijwerkingen kunnen ontstaan. Verder is een goed inzicht nodig in de effecten van corticosteroïden op het afweersysteem. Dit is noodzakelijk, omdat het doel van het onderzoek bij CVS-patiënten is om na te gaan in hoeverre de afwijkingen gevonden in het afweersysteem bij deze patiënten gerelateerd zijn aan een verstoorde gevoeligheid voor corticosteroïden.

Hieronder zal ik kort uitleggen wat de belangrijkste bevindingen, conclusies en bijdragen aan de wetenschap zijn van het onderzoek beschreven in dit proefschrift. Daar ik deze Nederlandse samenvatting voor een zo breed mogelijk publiek wil schrijven verwijs ik voor alle wetenschappelijke details naar het Engelstalige gedeelte van mijn proefschrift.

In **hoofdstuk 2** wordt beschreven dat een type witte bloedcel, die belangrijk is voor het goed functioneren van het immuunsysteem, in CVS-patiënten niet goed functioneert. Dit type bloedcel wordt de T helper cel genoemd. Deze cel maakt in CVS-patiënten minder van de stof interferon gamma in vergelijking met gezonde controles. Voor de rest functioneert deze cel goed. De stof interferon gamma is nodig voor een goed functioneren van het cellulaire immuunsysteem. De resultaten in dit hoofdstuk suggereren dus dat een tekort aan deze stof in CVS-patiënten de oorzaak zou kunnen zijn van de verminderde cellulaire immuniteit in CVS.

Voor een goede interferon gamma productie van de T helper cel is een juiste balans van een aantal belangrijke signaalstoffen in het afweersysteem noodzakelijk. Een tweetal zeer belangrijke van deze stoffen zijn het interleukine-10 en het interleukine-12. Het interleukine-12 is zeer belangrijk voor een goede productie van interferon gamma door de T helper cel. Het interleukine-10 daarentegen remt de productie van interferon gamma direct, maar ook indirect door de remming van interleukine-12.

In **hoofdstuk 4** wordt beschreven dat CVS-patiënten minder interleukine-12 en meer interleukine-10 produceren dan gezonde controles.

Samenvattend is de balans in het afweersysteem van CVS-patiënten dus verstoord. De verminderde interferon gamma productie door de T helper cellen van CVS-patiënten zou daarom veroorzaakt kunnen worden door een tekort aan het interleukine-12 en een overschot aan interleukine-10.

Waarom deze balans verstoord is, is dus de grote vraag

Het onderzoek beschreven in dit proefschrift laat zien dat een verstoorde gevoeligheid van de witte bloedcellen voor stresshormonen en dan met name de corticosteroïden in CVS-patiënten één van de belangrijkste oorzaken kan zijn.

Zoals hierboven al was beschreven hebben CVS-patiënten een verstoorde functie van hun hersenstam hypofyse bijnier-as systeem. Deze verstoring uit zich in chronisch verlaagde niveaus van de corticosteroïd cortisol. Daar het hormoon cortisol van groot belang is voor het functioneren van het afweersysteem, is in dit proefschrift de gevoeligheid van de witte bloedcellen van CVS-patiënten en gezonde controles voor corticosteroïden onderzocht.
Ik maak nu even een kort uitstapje naar het gedeelte van het proefschrift dat de effecten van corticosteroïden op het afweersysteem in het algemeen beschrijft.

In hoofdstuk 3 en de appendix van hoofdstuk 3 wordt beschreven dat corticosteroïden de productie van de cytokinen interleukine-10 en interleukine-12 heel verschillend beïnvloeden. In dit onderzoek is aangetoond dat corticosteroiden stimulerend zijn voor de productie van interleukine-10 en remmend zijn voor de productie van interleukine-12 en TNF-alfa. Het interleukine-10 zoals boven al beschreven is een lichaamseigen remmer van de interferon-gamma productie. Verder is dit cytokine ook een sterke remmer van andere ontsteking bevorderende cytokinen als het interleukine-12 en TNF-alfa. Het interleukine-12 en TNF-alfa zijn ontsteking bevorderende stoffen. Normaal zijn deze stoffen belangrijk om infecties snel op te kunnen ruimen. Echter de bijkomende ontstekingsprocessen mogen niet uit de hand lopen en daarvoor zorgt onder andere het interleukine-10. Het interleukine-10 kan dus beschouwd worden als een lichaamseigen ontstekingsremmer.

Het onderzoek beschreven in hoofdstuk 3 laat zien dat corticosteroïden die in de kliniek worden gebruikt als onderdrukkers van het afweersysteem, de lichaamseigen ontstekings-remmer interleukine-10 min of meer met rust laten. Verder geeft dit onderzoek een basis voor

combinatietherapie met synthetisch interleukine-10 en corticosteroïden. Er kunnen dan in de toekomst lagere dosis van corticosteroïden worden gebruikt, waardoor de bijwerkingen van deze corticosteroïden zoals het vasthouden van vocht kunnen worden voorkomen. In de internationale literatuur zijn recent publikaties verschenen dat zo'n combinatietherapie succesvol kan zijn in de behandeling van darmontstekingen.

Zoals eerder vermeld, produceren de T helper cellen van CVS-patiënten minder interferon-gamma en is in volbloed kweken aangetoond dat CVS-patiënten minder IL-12 en meer IL-10 produceren dan gezonde controle personen. Daar corticosteroïden de productie van deze cytokinen sterk kunnen beïnvloeden, veronderstelden we dat de verstoorde balans in de productie van de cytokinen in CVS-patiënten het gevolg kon zijn van een verstoorde gevoeligheid voor corticosteroïden.

Inderdaad laat het onderzoek in de **hoofdstukken 2, 4 en 5** zien dat witte bloedcellen van CVS-patiënten gevoeliger zijn voor corticosteroïden dan de witte bloedcellen van controle personen.

Deze verhoogde gevoeligheid voor corticosteroïden is alleen in het laboratorium in geïsoleerde cellen van patiënten aangetoond. Als we veronderstellen dat dit ook in het lichaam het geval is, dan kan de verhoogde gevoeligheid voor corticosteroïden een verklaring zijn voor gevonden subtiele verstoringen binnen het afweersysteem en het hormoonsysteem van CVS-patiënten.

Wat het onderzoek beschreven in hoofdstuk 5 verder laat zien, is dat de verhoogde gevoeligheid voor corticosteroïden in CVS-patiënten niet gepaard gaat met een verhoogde affiniteit en een toename van het aantal receptoren in de cellen voor de corticosteroïden. Dit betekent dat de verhoogde gevoeligheid van de witte bloedcellen van CVS-patiënten het gevolg is van moleculaire processen die niet betrokken zijn bij de binding van de corticosteroïden met de specifieke receptoren.

Wat verder ook in deze studie is aangetoond, is het feit dat de verstoringen in het afweersysteem van CVS-patiënten subtiel van aard zijn. Ook was het in deze studie het geval dat de samenstelling van de patiëntengroep een groot effect had op de mate van deze verstoringen. Alle verschillen die zijn aangetoond waren verschillen op basis van groepen.

Daarom zijn metingen aan het afweersysteem niet geschikt om als diagnostisch criterium te dienen voor het vaststellen van CVS. Dit is jammer, want dat was onder meer een van de doelen van het onderzoek.

Echter de verschillen in gevoeligheid voor corticosteroïden tussen CVS-patiënten en gezonde controle personen is veel minder afhankelijk van de samenstelling van de patiënten groep. Bestudering van de processen die de reactie op corticosteroïden reguleren kan mogelijkheden bieden voor het ontwikkelen van een diagnostische test en eventuele therapieën.

Een studie van Dr. Strauss in de Verenigde Staten dat recent gepubliceerd is in het vooraanstaande medische tijdschrift "The Journal of the American Medical Association (JAMA)" was niet succesvol en veroorzaakte zelfs bijnierinsufficiëntie. Het onderzoek beschreven in dit proefschrift kan deze bevindingen verklaren, omdat door de verhoogde gevoeligheid voor corticosteroïden de situatie bij CVS-patiënten alleen maar zal verslechteren wanneer ze cortisol krijgen toegediend. Er zal eerder gedacht moeten worden aan het ingrijpen op de interactie van de effecten van de corticosteroïden om een succesvolle therapie te ontwikkelen.

Wat het onderzoek in dit proefschrift heeft aangetoond, is dat er een verband lijkt te bestaan tussen de endocrinologische verstoringen en de verstoringen in het afweersysteem van CVS-patiënten. Er is echter meer wetenschappelijk onderzoek noodzakelijk naar het CVS om het hoe en waarom van deze ziekte te verklaren en een therapie te ontwikkelen.

Nawoord

Het onderzoek dat in dit proefschrift is beschreven kon natuurlijk nooit voltooid worden zonder de hulp van veel mensen.

Ik wil dan ook alle medewerkers van de afdeling I & I van TNO-pg en Medische Farmacologie van het LACDR hartelijk bedanken voor de prettige werksfeer gedurende mijn AIO-periode. Ook de stagiaires die ik mocht begeleiden wil ik bedanken.

Zonder patiënten was er geen onderzoek mogelijk geweest en daarom wil ik de medewerkers van het Leids Instituut voor Huisartsgeneeskunde bedanken voor hun goede inzet voor de rekrutering van de patiënten.

Zonder de steun van mijn vader en moeder, mijn broer en zus, Natalie, Henk en Grietje had ik nooit kunnen bereiken wat ik nu heb bereikt.

Curriculum vitae

Jeroen Visser werd op 8 december 1967 geboren aan de Grote Zomerdijk te Wognum. Na het succesvol doorlopen van de lagere school en het halen van het VWO diploma aan de Rijksscholengemeenschap West-Friesland te Hoorn in 1987, werd in datzelfde jaar aangevangen met de studie Biologie aan de Universiteit van Amsterdam. Na het behalen van de propaedeuse Biologie in augustus 1988, koos Jeroen voor de specialisatie Medische Biologie. In augustus 1993 werd het doctoraal examen Medische Biologie afgelegd. Jeroen had als specialisaties Immunologie (stage bij Celbiologie en Histologie AMC, onder leiding van Dr. M. Mevissen en Dr. C. de Groot) en Genetica (stage bij Antropogenetica, AMC, onder leiding van Dr. J. Hoovers).

Verder had Jeroen zijn standaard programma uitgebreid met extra examenonderdelen in Neurobiologie, Milieukunde en Milieurecht. Ook behaalde hij een didactische aantekening.

In september van dat jaar vervulde Jeroen als een van de laatste lichtingen zijn militaire dienstplicht bij de geneeskundige troepen. In augustus 1994 werd Jeroen op vrijwillige basis uitgezonden naar Afrika als lid van de VN-missie "Providing Care" in Goma Zaire, het tegenwoordige Congo.

Na beëindiging van zijn militaire dienstplicht startte Jeroen met het promotie-onderzoek dat beschreven is in dit proefschrift. Hij deed dat bij TNO Preventie en Gezondheid, afdeling Immunologische en Infectieziekten te Leiden in samenwerking met de divisie Medische Farmacologie van het LACDR te Leiden.

Onder begeleiding van promotor Prof. Dr. Ronald de Kloet en co-promotor Dr. Lex Nagelkerken mondde het onderzoek uit in een een aantal internationale publicaties die mede de basis vormen van dit proefschrift.

Sinds juni 1999 is Jeroen als Post-Doc verbonden aan de Rijksuniversiteit Groningen. Hij verricht daar in samenwerking met Dr. Jan Rozing en Dr. Herman Groen onderzoek naar de vroege fase in het ontstaan van diabetes.

Wat de verre toekomst Jeroen nog gaat brengen is ongewis, maar in ieder geval heeft de inhoud van dit proefschrift de kennis omtrent het Chronisch Vermoeidheid Syndroom en de werking van corticosteroïden op het functioneren van het afweersysteem doen toenemen.

List of publications

Overview of abstracts, presentations and publications regarding the research of Jeroen T. J. Visser

ABSTRACTS

J. Visser, B. Blauw, B. Hinloopen, C. Kluft, E. Brommer, E.R. de Kloet and L. Nagelkerken. 1995.
Decreased IFN-γ production and increased sensitivity to dexamethasone of CD4 positive T lymphocytes from patients with Chronic Fatigue syndrome.
Joint congress of the British and Netherlands Societies for Immunology. 6-8 December 1995, Brighton, UK. Immunology 1995; 86 (S 1): 63. Abstract number: 9.41.

J. Visser, B. Blauw, D. Methorst, C. Kluft, E. Brommer, E.R. de Kloet and L. Nagelkerken. 1996.
Modulation of cytokine production by glucocorticoids in patients with Chronic Fatigue Syndrome.
The third international congress of the International Society of Neuroimmunomodulation (ISNIM). 13-15 November 1996, National Institutes of Health, Bethesda, Maryland.

J. Visser, B. Blauw, D. Methorst, C. Kluft, E. Brommer, E.R. de Kloet and L. Nagelkerken. 1996.
Modulation of cytokine production by glucocorticoids in patients with Chronic Fatigue Syndrome.
Najaarsvergadering Nederlandse Vereniging Voor Immunologie (NVVI), 12-13 December 1996, Noordwijkerhout, Nederland.

J. Visser, D. Methorst, E.R. de Kloet and L. Nagelkerken. 1996.
The differential regulation of IL-10 and IL-12 by glucocorticoids.
Najaarsvergadering Nederlandse Vereniging Voor Immunologie (NVVI), 12-13 December 1996, Noordwijkerhout, Nederland.

J. Visser, B. Blauw, B. Hinloopen, C. Kluft, E. Brommer, E.R. de Kloet and L. Nagelkerken. 1997.
Altered cytokine profiles in patients with Chronic Fatigue Syndrome and differential sensitivity to glucocorticoids.
13th European Immunology Meeting. 22-25 June 1997, Amsterdam, The Netherlands. Immunology letters 1997; Volume 56/1-3: 470.

J. Visser, D. Methorst, T. Brunt, E.R. de Kloet and L. Nagelkerken. 1997.
Differential regulation of IL-10 and IL-12 by glucocorticoids and glucocorticoid receptor antagonists.
13th European Immunology Meeting. 22-25 June 1997, Amsterdam, The Netherlands. Immunology letters 1997; Volume 56/1-3: 367.

J. Visser, B. Blauw, T Brunt, E.R. de Kloet and L. Nagelkerken. 1997.
Glucocorticoids differentially regulate cytokine production in patients with chronic fatigue syndrome.
Najaarsvergadering Nederlandse Vereniging Voor Immunologie (NVVI), 18-19 December 1997, Noordwijkerhout, Nederland.

J. Visser, B. Blauw, W. Graffelman, T. Brunt, E.R. de Kloet and L. Nagelkerken. 1998.
Altered cytokine production in whole blood cultures of Chronic Fatigue Syndrome patients and a disturbed regulation by glucocorticoids.
Fifth international congress of the international society of neuroimmunology, 23-27 August 1998, Montreal Canada.
J. Neuroimmunology, volume 90/1 (1998). Abstract number: 242.

J. Visser, E. Siemssen, L. Hendriks, E.R. de Kloet and L. Nagelkerken. 1998.
Regulation of IL-12p35, IL-12p40 and IL-10 mRNA expression by glucocorticoids.
Najaarsvergadering Nederlandse Vereniging Voor Immunologie (NVVI), 17-18 December 1998, Noordwijkerhout, Nederland.

PRESENTATIONS

J. Visser, B. Blauw, D. Methorst, C. Kluft, E. Brommer, E.R. de Kloet and L. Nagelkerken. 1996.
Modulation of cytokine production by glucocorticoids in patients with Chronic Fatigue Syndrome.
Najaarsvergadering Nederlandse Vereniging Voor Immunologie (NVVI), 12-13 December 1996, Noordwijkerhout, Nederland.

J. Visser, D. Methorst, E.R. de Kloet and L. Nagelkerken. 1996.
The differential regulation of IL-10 and IL-12 by glucocorticoids.
Najaarsvergadering Nederlandse Vereniging Voor Immunologie (NVVI), 12-13 December 1996, Noordwijkerhout, Nederland.

J. Visser, B. Blauw, B. Hinloopen, C. Kluft, E. Brommer, E.R. de Kloet and L. Nagelkerken. 1997.
Altered cytokine profiles in patients with Chronic Fatigue Syndrome and differential sensitivity to glucocorticoids.
4th International Symposium on Clinical Immunology, 20-22 June 1997, Amsterdam, The Netherlands.

J. Visser, D. Methorst, T Brunt, E.R. de Kloet and L. Nagelkerken. 1997.
Differential regulation of IL-10 and IL-12 by glucocorticoids and glucocorticoid receptor antagonists.
13th European Immunology Meeting. 22-25 June 1997, Amsterdam, The Netherlands.

W. Graffelman, J. Visser, A. Knuistingh Neven, H. Petri en L. Nagelkerken. 1997.
Het chronisch vermoeidheidsyndroom (CVS) in de huisartspraktijk.
Referatendag 1997. Onderzoek in de huisartsgeneeskunde. 30 Mei 1997, Rotterdam, Nederland

J. Visser en L. Nagelkerken. 1997.
Immunologische aspecten van het chronisch vermoeidheidsyndroom.
Medische Faculteit der Leidse Studenten (M.F.L.S) Avondsymposium, 22 Mei 1997, Leiden, Nederland.

J. Visser, B. Blauw, T Brunt, E.R. de Kloet and L. Nagelkerken. 1997.
Glucocorticoids differentially regulate cytokine production in patients with Chronic Fatigue Syndrome.
Najaarsvergadering Nederlandse Vereniging Voor Immunologie (NVVI), 18-19 December 1997, Noordwijkerhout, Nederland.

J. Visser, B. Blauw, W. Graffelman, T. Brunt, E.R. de Kloet and L. Nagelkerken. 1998.
Altered cytokine production in whole blood cultures of Chronic Fatigue Syndrome patients and a disturbed regulation by glucocorticoids.
Fifth international congress of the international society of neuroimmunology, 23-27 August 1998, Montreal Canada.

J. Visser, E. Siemssen, L. Hendriks, E.R. de Kloet and L. Nagelkerken. 1998.
Regulation of IL-12p35, IL-12p40 and IL-10 mRNA expression by glucocorticoids.
Najaarsvergadering Nederlandse Vereniging Voor Immunologie (NVVI), 17-18 December 1998, Noordwijkerhout, Nederland.

ARTICLES

J. Visser, B. Blauw, B. Hinloopen, E. Brommer, E.R. de Kloet, C. Kluft, and L. Nagelkerken. 1998.
CD4 T lymphocytes from patients with the Chronic Fatigue Syndrome display a decreased IFN-γ production and an increased sensitivity to dexamethasone.
Journal of Infectious Diseases; 177: 451-4.

J. Visser, A. van Boxel-Dezaire, D. Methorst, T. Brunt, E.R. de Kloet and L. Nagelkerken. 1998.
Differential regulation of IL-10 and IL-12 by glucocorticoids in vitro.
Blood, vol 91, No 11 (june 1), 4255-4264.

J. Visser, W. Graffelman, B. Blauw, I Haspels, E. Lentjes, E.R. de Kloet and L. Nagelkerken.
Altered cytokine production in whole blood cultures of Chronic Fatigue Syndrome patients and a disturbed regulation by glucocorticoids.
Submitted.

J Visser, E. Lentjes, I Haspels, W. Graffelman, B. Blauw, E.R. de Kloet and L. Nagelkerken..
Glucocorticoid receptors and glucocorticoid responsiveness in Chronic Fatigue Syndrome.
Submitted.

J. Visser, E.R. de Kloet and L. Nagelkerken.
Altered glucocorticoid regulation of the immune response in the Chronic Fatigue Syndrome.
Submitted.

H. Petri, W. Graffelman, A. Knuistingh Neven, L. Mearin, M. von Blomberg, J. Visser and M. Springer.
No increased prevalence of serologic coeliac disease in persons with chronic fatigue syndrome.
Submitted.