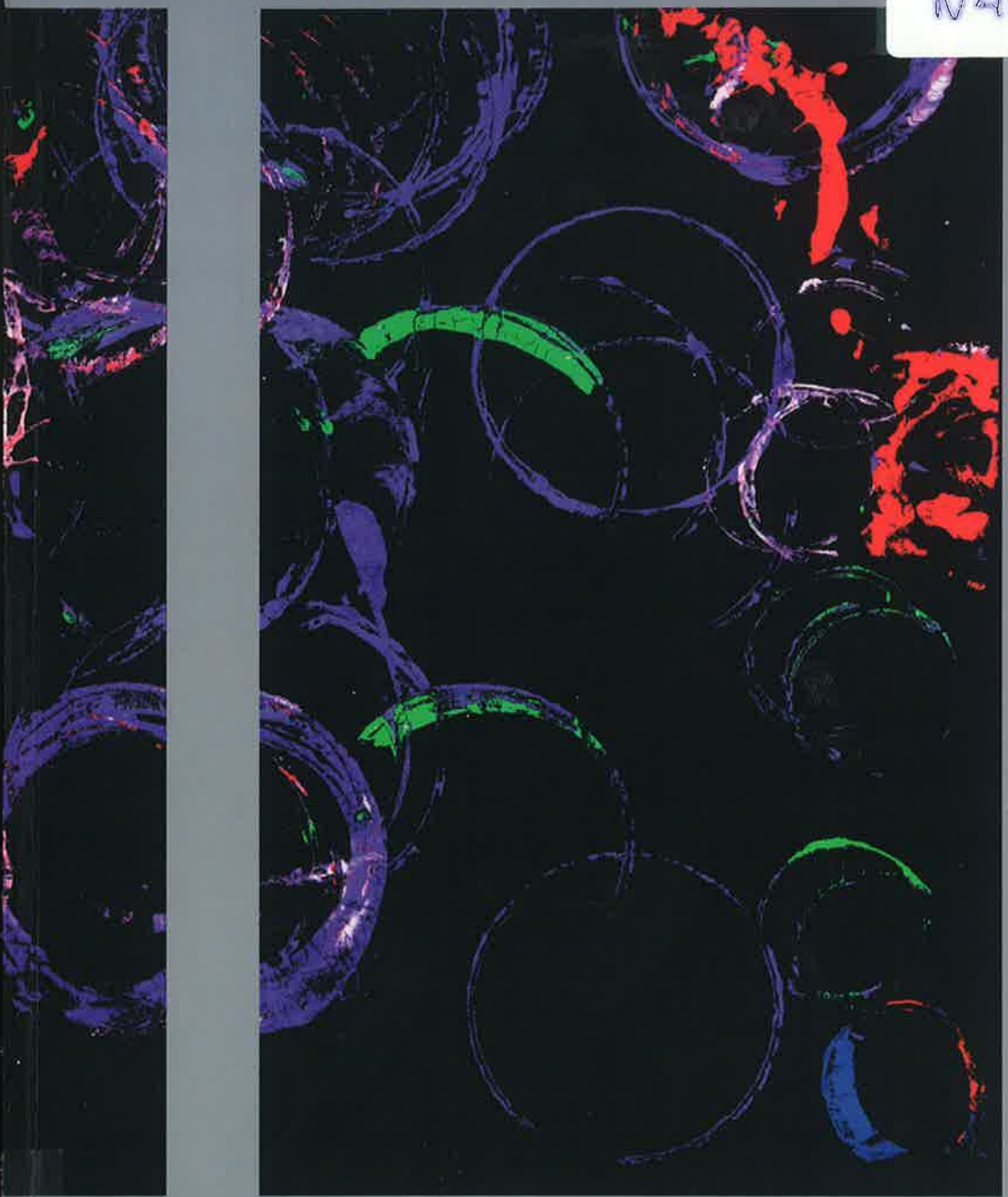
REGULATION OF T CELL ACTIVATION BY GLUCOCORTICOIDS IN HEALTHY DONORS AND PATIENTS WITH COGNITIVE DISORDERS



Erik Nijhuis

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STELLINGEN

behorende bij het proefschrift

Regulation of T cell activation by glucocorticoids in healthy donors and patients with cognitive disorders

1. Het is zeer de vraag of de *in vitro* vastgestelde leeftijdsgerelateerde veranderingen in het immuunsysteem klinisch relevant zijn.
   Dit proefschrift

2. Een verschil in gevoeligheid voor dexamethason van naieve en memory $CD4^+$ T cellen suggereert een leeftijdsgerelateerde verandering in respons op glucocorticoiden.
   Dit proefschrift

3. De veronderstelling dat een verhoging van het absolute aantal $CD4^+$ T cellen door middel van IL-2 toediening zal bijdragen aan een vermindering van opportunistiche infecties is prematuur.
   Kovacs et al., NEJM 1995, 332(9);567.

4. De inductie van IFN-γ in T_H2 klonen door het recente ontdekte T-cel activatie molekuul SLAM betekent een verdere vervaging van het onderscheid tussen T_H1 en T_H2 type $CD4^+$ T cellen
   Cocks et al., Nature 1995, 376;260.
   Kelso et al., Immunol Today 1995, 16(8);374.

5. Voor adequate diagnostiek is een optimaal contact tussen clinicus en patholoog onontbeerlijk. Deze open deur kan niet vaak genoeg worden ingetrapt.

6. Het houden van (stads)referenda heeft geen zin als niet van te voren vaststaat welke consequenties aan de uitslag verbonden zullen worden.

7. Politici wekken vaak ten onrechte de indruk dat hun beleid ingegeven wordt door partijpolitieke opvattingen, in plaats van door een beperkt budget.

8. De inspanningen die een beroepsvoetballer in Nederland zich moet getroosten om zich op het hoogste internationale niveau te handhaven vallen in het niet vergeleken bij die van een atleet.

9. De wegen van de computergestuurde verwerking van particuliere gegevens zijn ondoorgrondelijk en zelden aangenaam.
   vrij naar Bob den Uyl

10 Het wordt tijd om te erkennen dat de heren Jagger en Richard het ruim op punten hebben gewonnen van de heren Lennon en McCartney.

11. De Franse kernproeven geven het begrip 'De Stille Zuidzee' helaas een geheel nieuwe betekenis.

Erik Nijhuis, 15 februari 1996.

REGULATION OF T CELL ACTIVATION BY GLUCOCORTICOIDS IN HEALTHY DONORS AND PATIENTS WITH COGNITIVE DISORDERS

PROEFSCHRIFT

ter verkrijging van de graad van Doctor
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Erik Wilhelm Pieter Nijhuis

geboren te Den Haag in 1960

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In the dreary Sunday daylight
reality takes shape
Whose reality, yours or mine
No, I'm never ever sure

(Willy DeVille, Sportin' Life 1985)

Ter herinnering aan Rina

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

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GENERAL INTRODUCTION

1. THE IMMUNE SYSTEM

1.1 Introduction

Vertebrates are equipped with an immune system that protects them against pathogens like viruses, bacteriae, parasites and tumour cells. The interaction between the immune system and an antigen leads to a regulated immune response, involving different cell types and an extensive array of cell products, which are all part of an integrated effort of the organism to eliminate the pathogen.
The immune system can be divided into a non-specific and a specific component. The non-specific immune system consists of cells (macrophages, granulocytes and NK cells) which are able to eliminate the antigen by phagocytosis or non-specific cell-mediated cytotoxicity. The specific component of the immune system is characterised by T- and B-cells which recognize antigen through an antigen-specific receptor, the T-cell receptor (TCR) or membrane bound immunoglobulin, respectively. T- and B- cells are functionally distinct, in that T-cells provide not only for the regulation of the immune response but also for cellular immunity whereas B-cells are responsible for the humoral immune response.
T-cells can be divided phenotypically and functionally in $CD4^+$ and $CD8^+$ T-cells. $CD4^+$ T-cells, also known as T helper cells, are generally considered to play a central regulatory role in both cellular and humoral immune responses. $CD8^+$ T-cells are primarily referred to as cytotoxic T-lymphocytes. This introduction will be further focussed on $CD4^+$ T-cells since this thesis deals primarily with the influence of age and glucocorticoids on this T-cell subset.

1.2. Antigen-specific recognition and subsequent events

T-cells recognize specific antigens by a TCR, which consist of either a $\alpha\beta$ [1,2] or a $\gamma\delta$ disulfide linked heterodimer [3,4], which is in close association with a pentameric protein complex, termed CD3 [5].
After intracellular processing of pathogens by an antigen-presenting cell (APC),

antigens are presented as small peptides in the context of self components, *i.e.* the Major Histocompatibility Complex Class (MHC) I or II molecules. Presentation of peptide by these molecules is the result of distinct processing pathways. [6,7]. $CD4^+$ and $CD8^+$ T-cells recognize their antigen in conjunction with MHC class I and II molecules respectively [8,9]. This phenomenon is commonly referred to as MHC restriction.

Recognition of the peptide-MHC complex by the TCR-CD3 complex results in activation of the T-cell through a series of enzymatic reactions. The association of CD4 and TCR during T-cell activation, combined with the linkage of the protein-tyrosine kinases (PTK) $p56^{lck}$ to CD4 and $p59^{fyn(T)}$ to the TCR [reviewed in 10], leads to a phosphorylation of the γ, δ, ϵ, and ξ chains of CD3 [11] and subsequent phosphorylation and activation of phospholipase C-γ1 (PLC-γ1) [12] via an intermediate PTK, ZAP 70 [13,14]. Moreover, interaction of the TCR with a guanosine-tri-phosphate (GTP) binding protein and GTP probably leads to activation of β-isoforms of PLC [15]. The isoforms of PLC are capable of hydrolizing phosphatidyl-inositol-4,5 biphosphate (PIP-2) into inositol-triphosphate (IP-3) and diacylglycerol (DAG) [16]. IP-3 mediates the release of calcium from intracellular stores [17,18], resulting in activation of calmodulin regulated enzymes, like calcineurin [19,20]. DAG activates protein kinase C (PKC), an enzyme that is capable of activating other enzymes through phosphorylation of serine and threonine residues. It has been demonstrated that Ca^{2+} increase and PKC activation are two ultimate events required for T-cell activation.

T-cells can also be activated through triggering of the CD28 membrane molecule [reviewed in 21]. CD28 is a disulfide linked homodimer, which is expressed on $CD4^+$ T-cells and a subset of $CD8^+$ T-cells [22-24]. In contrast to TCR mediated T-cell activation, an increase in intracellular calcium concentrations is not consistently found after CD28 triggering [25,26]. Moreover CD28 triggering does not lead to PKC activation [27]. Recently it has been demonstrated that binding of the lipid kinase phosphatidyl-inositol-3-OH kinase to a specific site on the cytoplasmic tail of CD28 is important in CD28 mediated signalling [28,29]. It is suggested that interaction with PTK's like $p56^{lck}$, $p59^{fyn(T)}$ or ZAP 70 provide for such a binding site [reviewed in 30].

1.2.1. Transcription factors involved in IL-2 gene transcription

The target of the calmodulin regulated enzyme calcineurin, which has

phosphatase activity, is probably NFAT, an important transcription factor in the regulation of IL-2 gene expression [reviewed in 31,32]. Of all the lymphokines the control of IL-2 gene expression has been studied most intensively. So far several nuclear transcription factors have been described which play a role in IL-2 gene transcription. Apart from NFAT these include AP-1, AP-3, NFκB and octamer binding factors. Deletion or mutation of any of their DNA binding sites leads to a decrease in IL-2 gene transcription. How all these factors interact with respect to IL-2 gene expression is not exactly known, information becomes increasingly available regarding their activation requirements. NFAT and AP-1 expression for instance require two separate mitogenic signals when T-cells are concerned *in vitro* (PHA, anti-CD3, or anti-CD28 together with PMA) whereas NFκB, AP-3 and OCT-1 only required one signal [33]. It has been shown that distinct modes of T-cell activation give rise to the expression of distinct transcription factors which bind to specific DNA binding sites. CD28 mediated induction of IL-2 gene transcription for instance is accompanied by binding of members of the NFκB family to a unique CD28-responsive element in the IL-2 promoter [34–36]. CD28 mediated signals result in enhancement of T-cell proliferation and lymphokine production [25,37–42] probably caused by the stabilization of lymphokine mRNAs [43].
The identification of the transcription factors involved in IL-2 gene transcription have lead to new insights into the mechanisms of action of several immunotherapeutic agents. Corticosteroids primarily interfere with AP-1 activity, whereas the target of Cyclosporine A (CsA) is calcineurin, which is the primary activator of NFAT [31]. Accordingly CD28 mediated T-cell activation is largely CsA resistant, since this mode of activation AP-1 and NFAT expression does not seem to be essential [44].

1.3. $CD4^+$ T-cell subsets and their function

The acquisition of T-cell memory is an important example of the dynamic processes that take place within the immune system. Secondary immune responses ensure an efficient eradication of pathogens that are encountered for the second time. In the recent past, cell surface markers have become available that discriminate memory T-cells from naive T-cells. $CD4^+$ T-cells can be divided into subsets on the basis of the expression of CD45 isoforms on their membrane [reviewed in 45]. The $CD4^+CD45RA^+$ subset represents the naive

$CD4^+$ T-cell population whereas the $CD4^+CD45RO^+$ T-cell population is claimed to represent memory T-cells, because it is responsible for the response to recall antigens [46]. All CD45 isoforms are members of a family of glycosylated transmembrane proteins expressed on nucleated cells of the haematopoietic system [reviewed in 47]. The different isoforms (CD45RA, CD45RB, CD45RC and CD45RO) are the result of alternative splicing of CD45 mRNA, resulting in different extracellular domains [47–50]. CD45 is thought to have a regulatory role in T-cell activation through its cytoplasmic domain which has tyrosine phosphatase activity [51,52]. Despite an identical intracytoplasmic part, different natural ligands for the CD45 isoforms may lead to distinct intracellular effects. One natural ligand which has been identified is CD22 (a B-cell adhesion molecule), which specifically binds to CD45RO on T-cells [53]. In man, the CD45RA isoform is expressed on naive T-cells, whereas the presence of the CD45RO isoform characterizes the memory T-cells [54,55]. CD45RA and CD45RO separate the $CD4^+$ T-cell population also with respect to their activation requirements *in vitro* [56–59]. Naive $CD4^+$ T-cells for instance are preferentially activated by phytohaemagglutinin (PHA), whereas memory $CD4^+$ T-cells are more responsive to stimulation with anti-CD3 or the combination of anti-CD2 and anti-CD28 [56,57,60]. That naive and memory $CD4^+$ T-cells represent a different stage of differentiation is evident from the fact that $CD45RA^+CD4^+$ T-cells produce predominantly IL-2, whereas memory $CD4^+$ T-cells have acquired the capacity to produce lymphokines like interleukin-4 (IL-4) and interferon-γ (IFN-γ) in addition to IL-2. [60,61]

In the murine system it has been demonstrated at the clonal level that $CD4^+$ T-cells can be divided into subtypes on the basis of the interleukins they produce. T_H1 cells produce IL-2, IFN-γ and lymphotoxin, whereas T_H2 cells produce IL-4, IL-5, IL-6 and IL-10 [reviewed in 62]. The production of IL-3, GM-CSF, and TNF-α is unrestricted. T_H0 cells are characterized by the production of all interleukins mentioned above [63–65]. It is believed that T_H0 cells are the precursor cells for T_H1- and T_H2- like cells [66], and that the development from one (T_H0) into the other (T_H1 or T_H2) is influenced by a variety of factors amongst which are cytokines [45], the type of APC [67], the duration of antigenic exposure [68] and the presence of steroid hormones [69]. T_H1- and T_H2- like cells are different with respect to second messengers that are generated following activation [70–72]. This dichotomy in the $CD4^+$ T-cell subset is of considerable *in vivo* relevance as illustrated by the protective effects of a T_H1- like immune response in mice infected with Leismania major [73,74]. A T_H2- like immune response is

fatal on such an occasion probably because IL-4 supresses the required T_H1 induced inflammatory response [75].
Evidence is now accumulating that in the human system also a T_H1 and T_H2 subdivision of the $CD4^+$ T-cell population is justified. For instance, individuals allergic to house-dust respond in a T_H2- like manner upon activation with the allergen [76], whereas the immune system responds in a T_H1 like manner in persons with multiple sclerosis (MS) [77] or leprosy [78].
Despite the similarities between the phenotypical and functional dichotomy in the $CD4^+$ T-cell population there is no unequivocal evidence that the expression of a particular CD45 isoform is associated with a T_H1- or a T_H2- like immune response.
The distinguishing of different $CD4^+$ T-cell populations on the basis of phenotypical and functional criteria has led to a novel understanding of the development of immunological memory. The acquisition of immunological memory, as it occurs during the ageing process, is evident from decreasing numbers of naive $CD45RA^+CD4^+$ T-cells and increasing numbers of memory $CD45RO^+CD4^+$ T-cells. Functionally, this implicates that cells that produce IL-2 only differentiate into cells that produce IL-4 and/or IFN-γ. This differentiation process is highly influenced by the neuroendocrine system. In recent years evidence has become available that regulatory elements like hormones, *e.g.* corticosteroids, play a significant part in the regulation of the immune response, the T_H1/T_H2 balance in particular [reviewed in 79]. The effect of ageing and changes in homeostasis on the immune system are discussed in the following paragraphs in more detail.

2. AGEING AND THE IMMUNE SYSTEM

2.1. Introduction

Susceptibility to infectious agents is increased in elderly individuals; this is reflected in the mortality within the elderly population due to infections [80–82]. Age-related changes in several elements of the immune system, *i.e.* B-cells, T-cells, macrophages and natural killer cells, have been suggested as playing a role in decreased immune reactivity in the elderly. Since $CD4^+$ T-cells play a central regulatory role in the induction of an adequate immune response

to foreign antigens, this part of the introduction will focus on the age-related changes that occur in this subset particularly.

2.2. Thymic involution

One of the first effects of ageing on the immune system is the process of thymic involution which is accompanied by a decrease in the thymic output [83–86]. The influence of such a decline in the generation of new naive T-cells on the composition of the T-cell compartment in the periphery, will depend on the ability of naive T-cells to survive in the absence of antigenic stimulation. It has been demonstrated that peripheral expansion and preservation of such T-cells may occur without the acquisition of a memory phenotype, and that this process is possibly influenced by homeostatic control [87,88]. Despite the preservation of naive T-cells in the periphery, it is not likely that the naive T-cells have an infinite life-span [89]. A decline in the generation of new naive T-cells is therefore expected to be ultimately reflected by a decrease in the number of these cells in the periphery. Indeed, in mice and in humans, several studies have demonstrated a decline in the immune reponse to a variety of neoantigens *in vivo* and *in vitro* [90–93]. Other studies have shown that this is at least in part due to a decline in the frequency of antigen-specific T-cells in the periphery [94–97]. Therefore, an age-related decrease in immune responses might partly be due to lower numbers of antigen specific cells.

2.3. Age-related changes in the phenotype of T-cells

In humans most studies have been restricted to the phenotypic analysis of peripheral blood mononuclear cells (PBMC), in addition to functional studies *in vitro*. Regarding the absolute numbers of the $CD3^+$ T-cells and the $CD4^+$ and $CD8^+$ subpopulations, data from the literature suggest a slight decrease in the number of $CD3^+$ T-cells with age [99,99, reviewed in 100], whereas a minor increase in the CD4/CD8 ratio [98] has been reported. However, studying absolute numbers of $CD3^+$ T-cells (or the subsets thereof) may be irrelevant if a decrease in the generation of new naive T-cells is compensated by antigen-specific expansion of T memory cells. Therefore, it is relevant to interpret age-related changes in immune reactivity in view of the changes that occur within

the T-cell compartment, in particular the contribution of naive and memory cells to the total T-cell population.

In human neonatal cord blood – reflecting a condition of low antigenic exposure – the majority of the $CD4^+$ T-cells is $CD45RA^+$ [101]. During ageing a decrease in the expression of CD45RA and an increased expression of CD45RO on $CD4^+$ T-cells is found, which suggests a decrease in the number of naive $CD4^+$ T-cells in favor of memory cells [reviewed in 102,103,104; Figure 1]. In the mouse, naive and memory $CD4^+$ T-cells can be defined on the basis of CD45RB and MEL-14 expression (markers for naive $CD4^+$ T-cells) as well as the expression of Pgp-1 (a marker for memory $CD4^+$ T-cells], a cell-adhesion molecule the expression of which increases after cellular activation [105 – 107]. During murine ageing the fraction of $CD45RB^{high}Pgp\text{-}1^-$ $CD4^+$ T-cells decreases in favour of $CD45RB^{low}Pgp\text{-}1^+CD4^+$ T-cells [108]. Most likely the age-related increase in the number of $CD4^+$ T-cells with a memory phenotype is a consequence of previous antigenic exposure, whereas a decline in the number of naive $CD4^+$ T-cells is caused by thymic involution. A decrease in the size of the naive T-cell pool in the periphery will at least in part be responsible for the

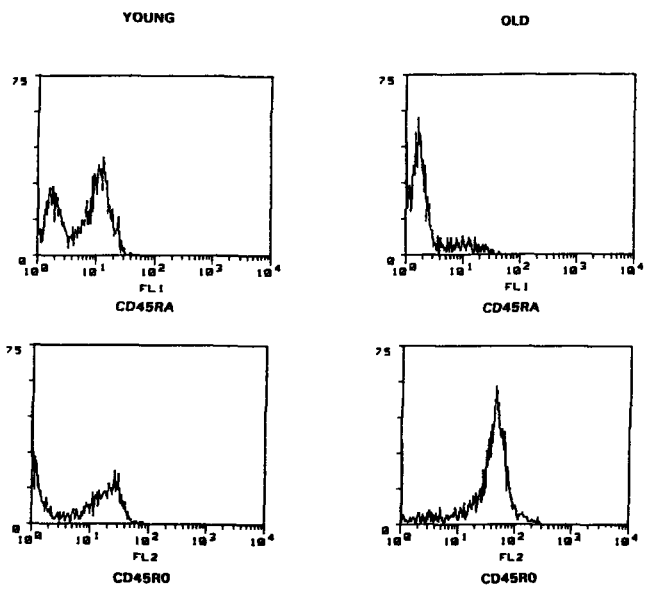

Figure 1. CD45RA and CD45RO expression on $CD4^+$ T-cells from a young and elderly donor.

age-related decline in the response to neoantigens, and as such one of the primary causes of a diminished immune response in the elderly. In addition – as described below – qualitative changes may contribute to the effficacy of an immune response in these individuals.

2.4. Age related changes in T-cell function

2.4.1. T-cell proliferation *in vitro*

Most insight into changes in the functional capacity of T-cells during ageing has been obtained through *in vitro* studies. It is well established that apart from a decline in antigen-specific T-cell responses the capacity of these cells to respond properly to polyclonal stimuli declines with age. Plant lectins such as Concanavalin A and Phytohaemagglutinin (PHA) have been widely used to demonstrate impaired proliferative responses in a variety of species [100,109]. It can be concluded that PBMC from elderly human donors respond to a lesser degree to mitogen than PBMC from young donors [102,103,110,111]. These differences may be related to a lower density of CD3 (and as a consequence the TCR) as demonstrated in two studies for T-cells from ageing mice and humans [112,113]. Accordingly, in the mouse also the mitogenic response to anti-CD3 antibodies is impaired [113,114]. In humans, results obtained by stimulation with anti-CD3 are, however, contradictory [102,103,115,116]. Using purified T-cell subsets and different (co)stimuli it has been demonstrated that the $CD8^+$ subset as well as the $CD4^+$ subset [103,110,117] may display a proliferative defect. As yet it is unclear whether these defects reside exclusively in one of these T-cell subsets or in both subsets. Lack of consistency in the published observations may be explained by the use of different (co)stimuli in these studies together with the differences in activation requirements between naive and memory T-cells.

2.4.2. Generation of second messenger signals

Because T-cells depend for their growth on IL-2, a variety of studies has focussed on the influence of age on the ability of T-cells to produce IL-2. It is generally found that T-cells from aged individuals have a diminished capacity to

produce IL-2 [99,102,118]. Because the mobilization of intracellular calcium is one of the key events in IL-2 gene expression [119] several groups noted the ability of 'old' T-cells to increase cytoplasmic calcium after T-cell receptor triggering or after stimulation with ionomycin. In this way, it was demonstrated that calcium mobilization in T-cells declines with age [110,120, 121]. In addition it has been demonstrated that protein kinase C activity is lower in 'old' cells [122]. These findings thus suggest that intrinsic defects contribute to a diminished T-cell proliferation. When T-cells are stimulated with the combination of the PKC activator Phorbol-12-myristate-13-acetate (PMA) and a calcium ionophore to bypass any possible T-cell receptor defects, proliferative responses and IL-2 production are considerably improved but still lower in the elderly population [113,117,123]. Although differences between young and old cells with regard to the generation of second messengers may be due to intrinsic defects accumulated during ageing, it might be that these results again reflect differences in signal transduction between naive and memory T-cells. Recently it was demonstrated in the mouse that the age-related decline in calcium mobilization may rely on differences between naive and memory cells, in that memory cells are poor responders [124]. Therefore, also the age-related change in calcium mobilization and possibly other intracellular events can in part be explained by a different composition of the $CD4^+$ T-cell population. An age-related decrease in calcium mobilization in unseparated populations of $CD4^+$ T-cells may thus partly be related to decreasing numbers of naive cells. On the other hand when $CD4^+$ T-cells with an identical phenotype are compared, old cells also appear to respond less vigorously [125].

2.4.3. Interleukin production in relation to phenotype

That the age-related decline in IL-2 production does not necessarily reflect a defect is evident from the observation that this decline is accompanied by an increased production of IL-4 and IFN-γ [114,124,126]. In humans, an age-related increase in the fraction of $CD4^+CD45RO^+$ T-cells may thus explain the age-related increase in IFN-γ production [126]. Both in humans and in mice it has been demonstrated that this decrease in IL-2 reflects a change in the lymphokine profile of the $CD4^+$ T-cells and that this is related to an increase in memory cells.
Although a changed composition in the $CD4^+$ T-cell subset may explain a lower

calcium mobilization and IL-2 production, this does not explain why T-cell proliferation is defective in the elderly. Again, it might be that naive and memory cells differ in their proliferative response. Memory T-cells respond better to anti-CD3 or to the combination of two anti-CD2 antibodies than naive T-cells [56,57]. Probably this is partly due to the increased expression of several adhesion molecules on memory cells, since several of these molecules are capable of giving costimulatory signals [55,127,128]. On the basis of these observations it would be expected that proliferative T-cell responses increase during ageing. The fact that the contrary is true points to age-related defects.
Beckman *et al.* were the first to relate the age-related change in the human T-cell phenotype with functional data [103]. They found that both the naive and the memory $CD4^+$ T-cells from elderly individuals responded poorly to PHA. Using several co-stimulation protocols they concluded however that the most profound defect, in terms of *in vitro* proliferation, in the T-cells from elderly individuals resided in the $CD4^+CD45RA^+$ population. In the mouse, Philosophe and Miller demonstrated poor responsiveness to polyclonal mitogens in $Pgp\text{-}1^+$ T-cells as compared to $Pgp\text{-}1^-$ T-cells [121]. In addition, Nagelkerken *et al.* found that $CD4^+CD45RB^+$ T-cells proliferate better than $CD4^+CD45RB^-$ T-cells, regardless of whether the cells were derived from young or ageing mice [124]. A proliferative defect was most evident for the naive $CD4^+$ T-cells, even when T-cell growth factors such as IL-2 and IL-4 were added to the culture.
As IL-2 is regarded as an essential factor in T-cell proliferation attempts have been undertaken both in man and mouse to establish whether proliferative responses could be restored by the addition of exogenous IL-2 [113,118,129,130]. Although in these studies IL-2 could enhance proliferative T-cell responses these were not restored to levels equal to those found with young cells. An explanation for this observation might be the fact that an important step in T-cell activation, *i.e.* the expression of the IL-2 receptor (IL-2R), the high affinity IL-2R in particular, is subject to an age-related decline [97,131 – 133]. Whether the IL-2R expression is lower on a per cell basis or that fewer cells express equal levels of IL-2R is still a topic of debate. An alternative possibility is that lymphokines preferentially produced by memory cells are inhibitory for naive cells. In the mouse it has been demonstrated at the clonal level that IL-10, as a product of T_H2 cells, inhibits the cytokine production by T_H1 cells [134].
Although many age-related changes in T-cell function observed *in vitro* can be related to differences in activation requirements of naive and memory cells, it is

likely that other changes contribute also to the net efficacy of the immune system. In this regard also the profile of cytokines produced, the affinity of cytokine receptors and changes in homeostatic control play a major role.

3. INTERACTIONS BETWEEN THE CENTRAL NERVOUS SYSTEM AND THE IMMUNE SYSTEM

3.1. Introduction

Communication between the central nervous system (CNS) and the immune system may occur in several ways. Firstly, the CNS may influence the immune system directly by innervation of lymphoid tissues like the thymus, spleen and lymph-nodes. Secondly, The CNS may have an indirect effect by inducing the release of soluble factors like neuroendocrine hormones and neurotransmitters. Expression of one or more of receptors for such substances on cells from the immune system is essential for such an interaction. Furthermore, the production of interleukins by cells from the CNS, might illustrate that these growth factors, which play a well-established role in the immune system, also have biological effects on cells in the CNS.
The way by which the immune system might influence the CNS is through the production of a vast array of soluble factors, called interleukins, which have already been mentioned above. Moreover, given the mobility of leukocytes, interaction may occur through receptor-ligand interaction between cells from both systems.

3.2. Central nervous system – immune system interactions

3.2.1 Innervation of lymphoid tissues

Lymphatic tissues like the thymus, spleen and lymph-nodes are mainly innervated by the autonomic nervous system, the sympathetic part in particular [reviewed in 135]. In the thymus it has been demonstrated that innervation is largely confined to the perivascular plexuses and that this is noradrenergic in nature [136]. In addition it has been shown that developing thymocytes express

β-adrenoreceptors, which makes these cells putative targets for noradrenalin [137]. Also several areas of lymph nodes are innervated by noradrenergic fibres [135,138]. The innervation of the spleen, also noradrenergic in nature, is associated with the course of the branches of the central artery, the periarteriolar lymphatic sheath, the marginal sinus and the parafollicular zone [135]. In several of these regions individual cells make contact with adjacent nerve fibres, indicating a direct interaction between the two systems through the noradrenalin released [135,139].
Evidence that these anatomical relations between both systems have functional implications, has been obtained by denervation studies. Denervation of the spleen, but also chemical sympathectomy, leads to an enhancement in the number of plaque-forming cells [140]. Moreover, after antigenic exposure a marked decrease in spleen noradrenaline levels have been demonstrated [140]. Furthermore, lesions in the anterior hypothalamus have been shown to be inhibitory for mitogen induced lymphocyte proliferation as well as antigen specific antibody titres [141,142]. In contrast, lesions in the mamillary bodies, hippocampus and amygdaloid complex enhanced lymphocyte proliferative responses [141].

3.2.2. Neuroendocrine – immune system interaction

Neuro- and neuroendocrine- hormones, may play a role in the interaction between the CNS and the immune system. A number of specific receptors for these substances have been described on cells from the immune system among which receptors are for ACTH [reviewed in 143], vasoactive intestinal peptide (VIP) [144], substance P [145], prolactin [reviewed in 146], growth hormone [147], catecholamines [148], steroid hormones [149,150] and opioid peptides [reviewed in 151].
Interaction of these hormones with their specific receptors on leukocytes leads to activation of intracellular enzyme systems and generation of second messenger signals. For instance, it is well established that activation of the β-adrenergic receptor by catecholamines stimulates adenylate cyclase and subsequently formation of cAMP [reviewed in 152]. Eventually, modulation of properties of cells from the immune system occurs. For example, ACTH inhibits antibody production of B-cells [143]. β-Adrenergic agonists and VIP suppress *in*

vitro T-cell proliferation [152, reviewed in 153], whereas it can be enhanced by substance P, prolactin, growth hormone and opioid peptides [145,154–156]. NK cell activity [152] and inhibition of IL-2 production of T-cells are inhibited by corticosteroids and β-adrenergic agonists [152,157].
Recently, evidence has become available indicating that several cell types present in the CNS are capable of producing cytokines. For instance cultured astrocytes and microglia been demontrated to produce IL-1β, IL-6 and TNF-α, [158–160]. These cytokines also have biological effects on these cells. TNF-α increases MHC class I expression on astrocytes and shares with IL-1 the capacity to induce ICAM-1 and VCAM-1 expression on astrocytes and neural cells [161–163].
The abovementioned effects of neuroendocrine hormones on the immune system might not only be mediated by CNS originated hormones but also by local production of these hormones by the leukocytes themselves. For a great number of these substances production by leukocytes has already been described [164–175]. Evidence is available that some of these hormones (*i.e.* ACTH, and endorphins), if produced by leukocytes, indeed play a role in a neuro-immuno-endocrine network and may act as a signal transmitter from the immune system to other organs [reviewed in 176].

3.2.2.1. Steroid hormones

Corticosteroids play an important role in the immune system both physiologically and therapeutically. Their immunosuppressive properties are best illustrated by their effect on T-cells. After binding to their specific intracytoplasmic receptor, the glucocorticoid receptor [177], they inhibit IL-2 expression by interfering with the nuclear transcription factor AP-1 [178,179], which is involved in IL-2 gene expression. Glucocorticoids also inhibit the production of other lymphokines like IL-4, IL-6 and IFN-γ [157,180,181]. The physiological immunoregulatory role of glucocorticoids *in vivo* is substantiated, inspired by data from Daynes *et al.*, who showed that DEX exerted differential effects on IL-2 and IL-4 producing cells [182]. In fact, GC inhibited IL-2 production and enhanced IL-4 production.
These studies, among others, suggest that GC may play a role in the shift from a T_H1- like immune response to a T_H2- like immune response. Since GC are a link in the HPA axis, the CNS may be able to alter the course of an immune

response and perhaps vice versa.

3.2.3. The role of interleukins in the CNS

Choi showed that haematogenously derived cells are ubiquitous in neural tissue during embryonic development [183]. In fact, it has been suggested that these cells may act as pluripotent stem cells in the adult [184]. Furthermore MHC class II bearing cells in the CNS have been shown to be derived from a bone marrow precursor [185]. That immune system-derived soluble factors like interleukins may influence cells from the CNS, is therefore not entirely unexpected.

IL-1 is believed to play a role in tissue repair in the CNS, since IL-1 promotes growth of microglial cells and astrocytes [186,187]. Moreover, the glial maturation factor (GMF), produced by astrocytomas, induces IL-1 production, suggesting an autocrine mechanism for growth and differentiation of astrocytes [188]. IL-1 also induces ICAM-1 expression on astrocytes, enhancing its antigen-presenting potential [162].

IL-6 has a mitogenic effect on astrocytes [189], and might play a role in neural differentiation by inducing nerve growth factor synthesis in astrocytes [158]. In relation to an immune response, IL-6 synthesized in the CNS play an important role in the intrathecal immunoglobulin synthesis, that has been described by several authors [190–192]. Furthermore, TNF-α promotes astrocytic growth, and is cytotoxic for oligodendrocytes [189,193].

It has already been discussed to some extent that cells from the CNS can be responsive to certain lymphokines (see 3.2.2), and can in some cases be a source of interleukins as well. T-lymphocytes have also the capacity to produce glial stimulating factor (GSF), and glial-growth promoting factor (GGPF) [194–196]. GSF has a proliferative effect on astrocyte precursors [188], whereas GGPF is specific for oligodendrocytes [196].

The T-cell product IL-2 has been shown to be stimulatory for oligodendrocyte growth [197], which is consistent with the observation that these cells express the IL-2R [198]. IFN-γ greatly enhances the expression of MHC class I and II molecules on astrocytes [199–201] as well as ICAM-1 expression on astrocytes [162]. These cells can in some instances be demonstrated to act as bonafide antigen presenting cells in a MHC class I or II restricted manner [202,203].

That actual cell-cell interaction is possible between cells from the CNS and the immune system is not only suggested by the presence and induction of MHC class I and II antigens in the CNS. Also, the induction of several adhesion molecules on astrocytes and neural cells by interleukins like IL-1, TNF-α, and IFN-γ points to the ability of these cells to interact with leukocytes. Moreover, Akiyama *et al.* showed that microglia cells constitutively express β-2 integrins, among which are LFA-1, CD11b, CD11c, and CD18 [204].

3.3. Central nervous system – immune system interaction and disease

Alzheimer's disease as a cause of cognitive impairment and consequently disability in the elderly is an important health care problem. Its neuropathological hallmarks are the presence of neurofibrillary tangles and β-amyloid containing senile plaques [205,206]. The deposition of β-amyloid in the CNS is considered to be specific for the disease. This peptide is derived from its precursor protein the Amyloid precursor protein (APP). APP is a transmembrane protein that can be intracellularly processed in two different ways. Firstly, it can be cleaved by a secretase, within the β-amyloid sequence, through a process that does not result in β-amyloid deposition. Secondly APP can be degraded by a endosomal-lysosomal pathway which results in carboxyl-terminal APP fragments which contain the β-amyloid fragment, and which may eventually lead to the deposition of this peptide in the CNS [reviewed in 207]. That β-amyloid may indeed be causally involved in the development of AD is illustrated by its apoptotic effect on cultured neurons and its neurotoxicity [208,209]. Mutations in the APP gene cause hereditary early onset disease, as well as 'Dutch type' hereditary cerebral haemorrhage with amyloidosis [210,211]. The precise physiological role off APP is still unknown. Parts of the molecule appear identical to the protease nexin II, a potent anti-chymotrypsin, which is a Kunitz-type protease inhibitor which inhibits the coagulation factor IXa [212,213]. The source of APP in the serum and the senile plaques might be shedding by platelets or, as Mönning *et al.* demonstrated, by peripheral blood T-cells [214].
Despite the absence of a putative (auto)antigen, several lines of evidence suggest that interactions between the CNS and the immune system occur during this disease process, that might be relevant for the pathogenesis and course of the disease. For instance, Vandenabeele and Fiers suggested that APP expression in the brain forms part of a cerebral acute phase response and as

such is present as β-amyloid in senile plaques like α_1-antichymotrypsin. This suggests that expression of these proteins forms part of an immune system mediated inflammatory response [215]. Furthermore, $CD4^+$ and $CD8^+$ T-cells have been demonstrated in affected brain tissue in addition to MHC class II positive reactive microglia cells [216–218]. Therefore, sufficient opportunities for antigen presentation to T-cells in the CNS in AD are available. Thirdly, IL-1 immuno-reactivity is increased in the brain of patients with Alzheimer's disease [219]. The importance of this observation is illustrated by the fact that IL-1 promotes APP mRNA expression in endothelial cells *in vitro* [220]. Fourthly, the neuroendocrine hormone regulation via the HPA axis appears to be disturbed [221–223], which might influence the immune system. Finally, *in vitro* data on proliferation, cytokine production and generation of second messenger signals of peripheral blood mononuclear cells in AD, revealed a decline with respect to these parameters in some studies [224–226], although this could not be confirmed by other investigators [227–229].

This thesis partly focusses on the abovementioned disturbance of the neuroendocrine hormone regulation in AD in relation to the immune system. The GC, which are regulated by ACTH, are of particular interest. As has been mentioned earlier in section 3.2.2.1. of this introduction these hormones may exert differential effects on cells from the immune system in that they can inhibit IL-2 production while enhancing IL-4 production [182]. In fact, it has been suggested that these hormones play an important regulatory role in the immune system [79]. Therefore it is conceivable that impairment of the GC homeostasis leads to altered properties of the immune system. *In vitro* studies in that respect might for instance reveal a change in cytokine production profile or a different response to GC.

Another GC mediated mode of regulation in the immune system might be via the induction of apoptosis. GC have recently been shown to be capable of inducing apoptosis in mature T-cells [229]. A possibly related molecule in this process might be the cytoplasmic protein bcl-2, which protects the cell from apoptosis [230]. Therefore, in this thesis experiments were undertaken which try to relate disturbances in the GC homeostasis in AD to altered susceptibility of cells from the immune system with respect to apoptosis. To this end the level of bcl-2 expression was evaluated.

4. Scope of the thesis

Experiments were undertaken to evaluate the hypothesis that a disturbed HPA axis in AD leads to alterations in properties of cells from the immune system. In this respect the effects of the synthetic GC dexamethasone (DEX) on different parameters was studied on PBMC both from AD patients and age-matched controls. In connection with these experiments, data were obtained in order to gain more insight into the immunoregulatory properties of GC in general, and DEX in particular, using PBMC from healthy young donors. The effect of DEX on $CD4^+$ T-cells, or the naive and memory subset thereof, was studied employing different routes of T-cell activation. Finally, the possibility was evaluated that impairment of the HPA-axis in AD patients might be associated with a different susceptibility to apoptosis, a process that can be induced by DEX in mature T-cells. This lead to experiments where the level of bcl-2 expression, a cytoplasmic protein that protects the cell from apoptosis, in PBMC from AD patients and controls was evaluated.

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CHAPTER 2

DECREASED SENSITIVITY TO DEXAMETHASONE IN LYMPHOCYTES FROM PATIENTS WITH ALZHEIMER'S DISEASE

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SUMMARY

Cortisol levels in patients with Alzheimer's disease (AD) are relatively unaffected by a challenge with dexamethasone (DEX) *in vivo*. In the present study it is demonstrated that DEX is less inhibitory for phytohaemagglutinin (PHA) induced T cell proliferation in AD patients as compared to age-matched controls. Since no significant differences were found between AD patients and age-matched controls with regard to the fraction of $CD45RA^+$ or $CD45RO^+$ $CD4^+$ T cells nor the ability of peripheral blood mononuclear cells to produce IL-2 or IL-4, it is unlikely that the difference in DEX-sensitivity is due to a changed lymphokine profile or changed composition of the $CD4^+$ T cell population. Sensitivity to DEX was negatively correlated with the ability to produce IL-2 and IL-4 in the controls but not in AD patients. This suggests that IL-2 and IL-4 synthesis in AD patients is less sensitive to regulation by glucocorticocids.

INTRODUCTION

The pathogenesis of Alzheimer's disease (AD) is still largely unknown. The neuropathological hallmarks of this disease, namely the senile plaques, containing the β-amyloid protein [1], and the neurofibrillary tangles [2] have still not been proven to be causative in the pathogenesis of the disease, although a mutation found in the amyloid precursor protein (APP) gene [3] suggests that in some cases β-amyloid might cause AD. Evidence is becoming available indicating that defects observed in AD are not confined to the central nervous system but can also be observed in other organ systems, for instance the immune system. Compatible with an impaired immune system in AD are clinical data showing that – compared to age-matched controls – life expectancy of AD patients is reduced, the primary cause of death being infections of the respiratory tract [4]. Furthermore, there is evidence that treatment with anti-inflammatory drugs may reduce the rate of progression of the dementia in AD patients [5]. Moreover, in contrast to vascular dementia, infection-related mortality in AD patients increases with the severity of dementia [6].

The fact that APP has been identified in PHA stimulated peripheral blood mononuclear cells (PBMC) of normal healthy individuals [7], also suggests that the deposition of the β-amyloid protein may be related to an immunological or inflammatory process. This is supported by the recent observation that Epstein-

Barr virus transformed B cell lines from an AD patient produce antibodies which are reactive with the ß-amyloid protein [8].
In addition, pro-inflammatory cytokines like interleukin-1 (IL-1) and IL-6 have been suggested to play a role in the amyloidogenesis in AD [9]. Arguments in favour of this hypothesis are that microglial cells and astrocytes can produce IL-1 and IL-6, respectively [10,11] and that IL-1 is able to increase APP mRNA expression in cultured human endothelial cells [12]. However, plasma levels of IL-6, which are increased in various diseases with an inflammatory compound, are unchanged in AD patients [13].
With regard to the properties of the cells of the immune system in AD, there are several *in vitro* studies showing a diminished proliferative response of PBMC [14-15]. However, other studies showed no differences with respect to this parameter [16-18].
In vivo studies have indicated that the cortisol levels of AD patients were less inhibited by a dexamethasone (DEX) challenge as compared to controls, pointing to an imbalance of the hypothalamic pituitary adrenal axis (HPA) in AD [19-21]. Since glucocorticoids are widely known for their potent immuno-suppressive effect, we studied whether also the sensitivity of PBMC from AD patients to DEX was different from that of age-matched controls. Our present study shows that the suppressive effect of DEX on T cell proliferation is significantly less in AD patients as compared to the controls and that this difference is unrelated to the capacity of the cells to produce IL-2 or IL-4.

MATERIAL AND METHODS

Patients

Patients (n = 30; mean age 69 years, 20 female and 10 male) and controls (n = 30; mean age 70 years, 19 female and 11 male) were derived from an epidemiological study of risk factors of clinically diagnosed early onset (diagnosis ≤ 70 years) AD [13]. This population includes cases with familial as well as sporadic AD. AD was considered familial if there was at least one first degree relative with AD. All patients met the NINCDS-ADRDA criteria for probable AD [22]. For this study, the clinical diagnosis of AD was confirmed by two independent experts [13].

Cell cultures

PBMC were obtained by Percoll (Biochrom KG, Berlin, Germany) density centrifugation (ρ = 1.077 g/cm^3) and cryopreserved in culture medium containing 20 % (v/v) Foetal Calf Serum (FCS, Seralab, Crawley Down, UK) and 10 % (v/v) DMSO, using a software-directed programme for the cryopreservation of lymphocytes as described elsewhere [23]. Per experiment, cell suspensions from an equal number of patients and controls were thawed by dropwise dilution (at least ten-fold) in ice-cold culture medium containing 20 % FCS. The preservation time of PBMC from all donors used in this study was approximately 2 years. Viable cells ($\geq$ 80 %) were enumerated by trypan blue exclusion. In agreement with Bom-van Noorloos et al. [23], we found no changes in the phenotype of the cells after this procedure, with the exception of a slight decrease in monocytes. With respect to functional data a positive correlation was found between data obtained with cryopreserved cells as compared to fresh PBMC from identical donors (data not shown). The slight decrease in the percentage monocytes is probably responsible for the fact that peak responses with cryopreserved cells are found at day 5, instead of a peak at day 4 found with fresh PBMC. All cultures were performed in Iscove's modified Dulbecco's medium (Biochrom KG) supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamin (2 mM) and β-mercaptoethanol (50 μM).

PBMC ($4x10^4$/well) were cultured in flat bottom microtiter plates (Costar, Cambridge, MA). Cells were stimulated with 0.5 μg/ml PHA (Murex, Dartford, UK) in the absence or in the presence of 10^{-7} M DEX (Sigma, St. Louis, MO). This PHA concentration was optimal for proliferation of PBMC, but suboptimal for the induction of IL-2. This approach allows a sensitive discrimination between individual AD patients and controls with respect to differences in DEX sensitivity of PBMC. Where indicated exogenous human recombinant IL-2 (Cetus, Emeryville, CA; kindly provided by Dr. E. Braakman, Dr. Daniel den Hoed Cancer Center, Rotterdam, The Netherlands) was added to a concentration of 50 U/ml.

Cell proliferation was measured by adding 0.25 μCi methyl-tritium-thymidine ([^{3}H]TdR) (specific activity 2 Ci/mmol; Radiochemical Centre, Amersham, UK) during the last 6 hours of culture. Cells were harvested onto glass fibre filter paper (Packard Instrument Company, Meriden, CT). Filters were counted using a Matrix 96 β-counter (Packard Instrument Company). This procedure has an

efficiency of about 20 % as compared to standard liquid scintillation counting.
IL-2 production was induced by 10 μg/ml PHA. For the induction of IL-4, anti-CD2 (CLB-T11.2/1 and CLB T11.1/2) and anti-CD28 (CLB-CD28/1) monoclonal antibodies (mAbs) were used in a 1:2000 dilution of murine ascites and 2 μg/ml of purified antibody respectively. These antibodies were a generous gift from Dr. R.A.W. van Lier from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (Amsterdam, The Netherlands). All supernatants were harvested at day 3 and stored at -20 °C until assay.

IL-2 and IL-4 assays

IL-2 was measured by ELISA, according to the instructions of the manufacturer (Genzyme, Cambridge, MA). Alternatively, IL-2 activity was assessed with the use of the CTLL-2 cell line. In the CTLL-2 bio-assay, five thousand cells were cultured in the presence of serially diluted supernatants. Human recombinant IL-2 was used as a standard. During the last 4 hours of the 24 hour culture period cells were pulsed with 0.25 μCi [^{3}H]TdR. IL-4 levels in the supernatant were assessed using an ELISA technique as described elsewhere [24] employing reagents generously provided to us by Dr. T. van der Pouw-Kraan, CLB, Amsterdam, The Netherlands. Plates were read using a Biorad Microplate reader (Biorad, Richmond, CA). Human recombinant IL-4 was used as a standard (Genzyme). The detection limit of the assay was 100 pg/ml.

Phenotypic analysis

For phenotypic analysis, $5x10^4$ cells were incubated with a saturating amount of FITC- or PE- conjugated mAbs. Thereafter, cells were washed and analyzed on a FACScan (Becton & Dickinson). Gates were set on the basis of forward and sideward scatter, including all viable leucocytes.
Seven thousand gated events were analyzed for green and red fluorescence. To determine the expression of CD45RA, CD45RO or CD27 within the $CD4^+$ T cell population, only viable cells stained with anti-CD4-PE were included. The following reagents were used: 2H4-FITC (anti-CD45RA; Coulter, Hialeah, FL), UCHL-1-FITC (anti-CD45RO; DAKO, Glostrup, Denmark) and anti-CD27 (CLB-CD27/1), which was a kind gift from Dr. RAW van Lier (CLB, Amsterdam, The

Netherlands). Anti-CD3-FITC, anti-CD4-PE, anti-CD8-PE and anti-CD14-FITC were all purchased from Becton & Dickinson.

Expression of glucocorticoid receptors

PBMC were fixed and permeabilized as described elsewhere [25] with some modifications. Briefly, cells were washed twice with PBS to remove the FCS from the culture medium. Thereafter the cells were fixed (5 minutes, 4 °C) with a 2 % paraformaldehyde in PBS. Subsequently, the cells were permeabilized by adding an equal volume of 0.4 % Triton X-100 for an additional 5 minutes. Finally, the cells were washed twice with PBS containing 0.5 % BSA. $5x10^5$ permeabilized PBMC were incubated with 5 μg/ml mAb number 7, kindly provided by Dr. A.C Wikström, Karolinska Institutet, Huddinge, Sweden [26], a mouse antibody crossreactive with the human glucocorticoid receptor (GCR). Staining occurred with Goat-anti-Mouse-FITC (CLB, Amsterdam, The Netherlands) as second step antibody. Double staining for $CD4^+$ T cells was performed with a CD4PercP conjugate (Becton & Dickinson, Mountain View, CA) in 5 % (v/v) normal mouse serum. After each incubation step, cells were washed twice and 15,000 PBMC were analyzed on a FACScan (Becton & Dickinson). An irrelevant mouse IgG2a antibody was used as an isotype control. Results for each individual patient or control are expressed as relative fluorescence intensity, *i.e.* mean fluorescence intensity found with anti-GCR divided by the mean fluorescence intensity of the isotype control.

Statistics

Statistic analysis was performed using the two-tailed Mann-Whitney U-test. Differences were considered to be statistically significant at a confidence level of 95 % or higher ($p < 0.05$).

RESULTS

Decreased sensitivity of T cell proliferation to dexamethasone in AD patients

In contrast to several other studies [14,15], the present study does not confirm the observation that T cell proliferation in response to PHA (0.5 μg/ml) is impaired in AD patients. Stimulating PBMC from a well-defined panel of AD patients (n = 30) and a panel of age-matched controls (n = 30), lead to a mean [^{3}H]TdR incorporation by PBMC of 6504 and 6265 cpm, respectively (p = 0.59; data not shown).

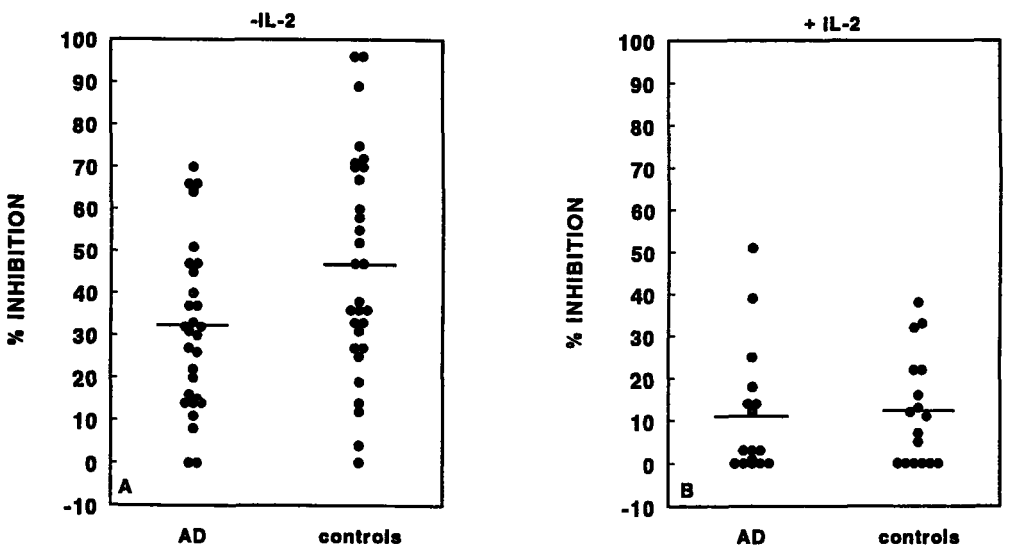

Figure 1. **PBMC of AD patients display a diminished sensitivity for dexamethasone.**
PBMC were stimulated with 0.5 μg/ml PHA in the presence of 10^{-7} M DEX. For each individual donor the percentage inhibition of the response at day 5 is shown (Figure 1A). The effect of exogenous IL-2 (50 U/ml) on DEX induced inhibition was studied with PBMC from 17 individuals of each group (Figure 1B). Mean proliferative responses in the absence of DEX were 6265 (AD patients) and 6504 (controls) cpm in the absence of exogenous IL-2 and 7026 (AD patients) and 7457 (controls) cpm in the presence of exogenous IL-2. Background proliferation was less than 50 cpm.

Since AD patients are less sensitive to a DEX challenge in vivo [19–21], we studied whether this phenomenon was also true for the sensitivity of PBMC in vitro. As can be seen in Figure 1A, the proliferative response of PBMC from AD patients to an optimal concentration of PHA (0.5 μg/ml) was less inhibited by 10^{-7} M DEX (32.7 % of the control response on average) than that of controls (46.5 % of the control response on average). Despite the heterogeneity of the response in both groups this difference was significant ($p = 0.03$). Addition of a saturating amount of exogenous IL-2 (50 U/ml) to the cultures reduced the inhibitory effect of DEX and the differences between the two groups (Figure 1B; $p = 0.90$). PBMC from familial and sporadic cases of AD were equally sensitive to DEX (data not shown).

As shown in Figure 2 – for a random selection of 11 patients and 12 controls – PBMC stimulated with a suboptimal PHA concentration (0.125 μg/ml), were almost completely inhibited by 10^{-7} M DEX (mean inhibition 75.7 % and 87.9 %

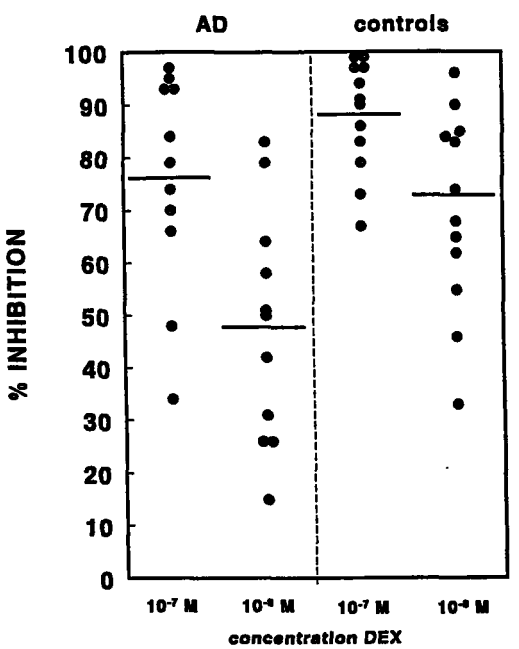

Figure 2. **Differences in dexamethasone sensitivity of PBMC from AD patients and controls at suboptimal PHA concentrations.**
PBMC from 11 AD patients and 12 controls were stimulated with 0.13 μg/ml PHA. Mean proliferative responses in the absence of DEX were 3053 and 2465 cpm for AD patients and controls, respectively. Shown is the percentage of inhibition at day 5 in the presence of 10^{-7} M and 10^{-8} M DEX for each individual donor.

in AD and controls, respectively; p = 0.12). Partial inhibition was now found at 10^{-8} M DEX and a significant difference between the two groups was again found (mean inhibition 47.7 % and 70.1 % in AD and controls, respectively; p = 0.02).

We subsequently excluded the possibility that the difference in sensitivity to DEX between the two groups was due to a difference in growth kinetics of the cells. This was done for 15 patients and 14 age-matched controls, randomly chosen from the panel used in Figure 1A. As can be seen in Figure 3A there was no difference in growth kinetics of PBMC from AD patients and controls. As shown in figure 3B, the decreased sensitivity for DEX in AD was already evident at day 4 of the culture (p = 0.01, versus p = 0.04 on day 5). Again, the addition of a saturating amount of exogenous IL-2 (50 U/ml; figure 3C) to the cultures reduced the differences between the two groups.

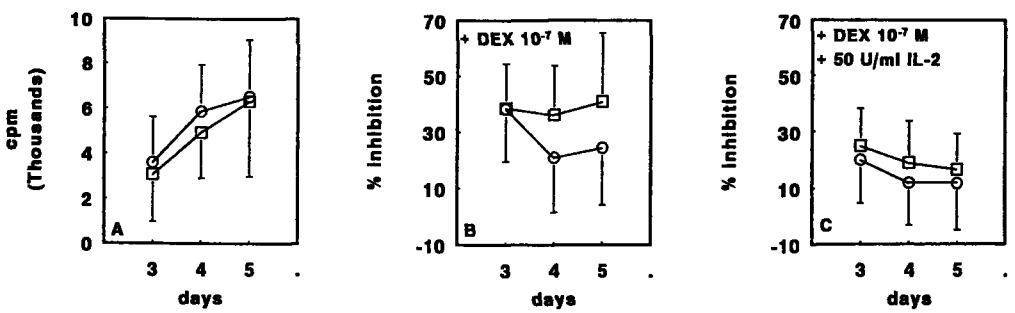

Figure 3. **Growth kinetics of PBMC from AD patients and controls are comparable.**
PBMC from 15 patients and 14 controls were stimulated with 0.5 μg/ml PHA. Shown is the mean of the individual data. Mean proliferative responses in the absence of exogenous IL-2 on day 3, 4 and 5 are shown in Figure 3A. The extent of inhibition by 10^{-7} M DEX in the absence (Figure 3B) and in the presence (Figure 3C) of 50 U/ml exogenous IL-2 is shown. The means of the proliferative responses in the presence of exogenous IL-2 on day 3,4 and 5 were 3372, 5192 and 6357 cpm for PBMC from AD patients and 3463, 4833 and 6255 cpm for PBMC from control donors. Proliferation in the absence of mitogen was less than 50 cpm.

Phenotype of PBMC from AD patients and controls

In the mouse, it has been demonstrated that DEX, while inhibiting IL-2 may enhance IL-4 production [27]. In the human situation, IL-2 is the main product of naive $CD4^+CD45RA^+$ T cells whereas IL-4 is mainly produced by $CD4^+CD45RO^+$ T cells [28]. We therefore evaluated whether the diminished DEX-sensitivity of PBMC from AD patients was related to an altered composition of the $CD4^+$ T cell population. As depicted in Table 1, there were however no significant differences with regard to the fraction of $CD45RA^+CD4^+$ T cells or $CD45RO^+CD4^+$ T cells between the two groups, although there was a tendency towards a higher percentage of $CD4^+CD45RA^+$ T cells in the AD group. Moreover, there was no significant correlation in either group between the extent of inhibition by DEX and the percentage of $CD45RA^+$ or $CD45RO^+$ $CD4^+$ T cells (data not shown). Also the percentage of $CD4^+$ T cells expressing CD27 (a marker absent from a subset of the $CD4^+CD45RO^+$ 'memory' T cells) [29] was comparable in patients and controls.
In addition, no differences were observed with respect to the relative number of $CD3^+$ and $CD8^+$ T cells or $CD14^+$ monocytes in the PBMC of the two groups.

Table 1. **The relative number of cells in a total PBMC population.**

Phenotype of PBMC from AD patients and controls

	% of PBMC				% of $CD4^+$ T cells		
Subjects	CD3	CD4	CD8	CD14	CD45RA	CD45RO	CD27
AD	48.6	30.5	20.1	15.4	42.8	60.1	90.6
SD	13.9	9.6	11.1	7.5	18.3	16.1	5.9
n	31	31	31	31	31	31	22
Controls	47.7	27.4	22.0	13.6	36.8	63.4	87.4
SD	14.0	9.6	10.5	6.3	15.8	14.6	8.8
n	31	34	31	34	32	32	20
p value	0.87	0.33	0.46	0.43	0.14	0.36	0.27

Note. The relative number of cells in a total PBMC population. The percentage of $CD4^+$ T cells expressing $CD45RA^+$, $CD45RO^+$, and $CD27^+$ cells was calculated using gated $CD4^+$ (PE-positive) T cells. n and SD represent the number of donors tested and standard deviation, respectively.

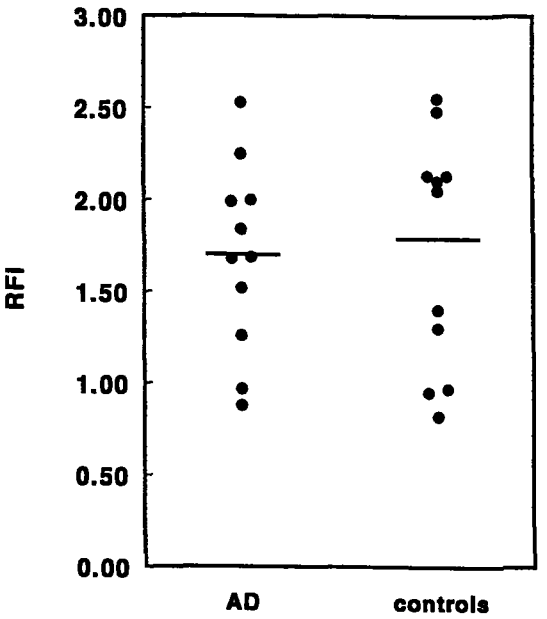

Figure 4. **Comparable expression of the glucocorticoid receptor in CD4$^+$ T cells from AD patients and age-matched controls.**
For the measurement of the GCR, cells from 11 AD patients and controls were fixed and permeabilized (see material and methods) and indirectly stained with an anti-GCR antibody. The density of GCR in PBMC is depicted as the relative fluorescence intensity, *i.e.* the ratio between the mean fluorescence intensities found with anti-GCR antibody and an irrelevant isotype-matched control antibody.

Comparable expression of the glucocorticoid receptor in CD4$^+$ T cells from AD patients and age-matched controls

The lower sensitivity of PBMC in AD patients for DEX as compared to that of age-matched controls might be due to a difference in expression of the cytoplasmic GCR. Therefore, we determined for 11 AD patients and 11 controls, randomly chosen from the panel in Figure 1A, the level of GCR expression in CD4$^+$ T cells by flowcytometry. Results are expressed as relative fluorescence intensity, i.e. the mean fluoresence intensity found with anti-GCR divided by the mean fluorescence intensity of the isotype control. As can be seen in Figure 4, AD patients expressed comparable levels of GCR as controls (mean relative fluorescence intensity for AD and controls, 1.71 and 1.79 respectively; $p = 0.49$).

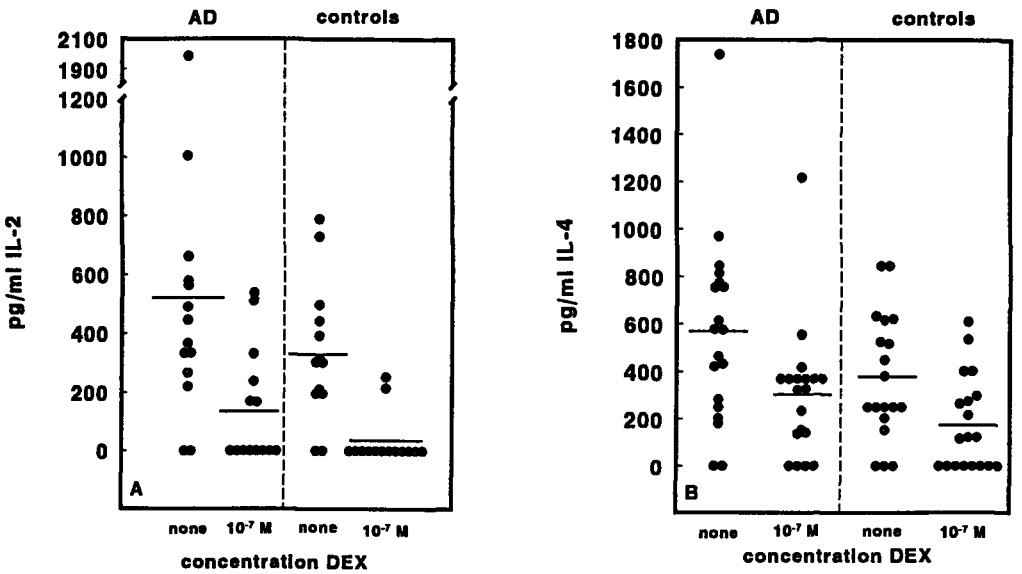

Figure 5. **IL-2 and IL-4 production in AD patients and controls are comparable and equally sensitive to DEX.**
For the measurement of IL-4, PBMC from 19 AD patients and controls were stimulated with the combination of anti-CD2 and anti-CD28 (see material & methods). For the measurement of IL-2, PBMC from 14 patients and 14 controls were stimulated with 10 μg/ml PHA. Supernatants were harvested at day 3 and the interleukins were determined by ELISA.
Background values for IL-2 and IL-4 were lower than 100 pg/ml.

Diminished sensitivity to dexamethasone in AD is unrelated to IL-2 or IL-4 production

Despite the fact that we did not find differences in the composition of the $CD4^+$ T cells on the basis of their phenotype, PBMC from AD patients may differ from control cells with respect to the amount of IL-2 and/or IL-4 produced. Alternatively, the production of these cytokines may be differentially affected by DEX. In Figure 5A it is shown that higher levels of IL-2 were detected in the patient group than in the control group (519 and 327 pg/ml in AD and controls, respectively), although this observation was not statistically significant (p =

0.18). The same tendency towards higher levels of IL-2 was observed when we determined the biological activity of the IL-2 present in the supernatants: in AD patients (n = 20) the mean IL-2 production was 25.5 U/ml and in controls 17.6 U/ml (n = 21; p = 0.25) (data not shown). In the presence of 10^{-7} M DEX, 6 out of 14 supernatants from patients showed detectable levels of IL-2 in the ELISA whereas in the control group only 2 out of 14 donors showed detectable amounts of IL-2. The mean inhibition of IL-2 production in AD patients and controls was 73 % and 93 % respectively (p = 0.18).

Stimulation with PHA does not result in detectable levels of IL-4 in the supernatant of PBMC (data not shown). We therefore stimulated PBMC with a combination of anti-CD2 and anti-CD28. As can be seen in Figure 5B, the mean IL-4 production in AD patients was 561 pg/ml and in controls 371 pg/ml. Again, these differences between the two groups were not significant (p = 0.15). When the cultures were performed in the presence of 10^{-7} M DEX, the mean inhibition of IL-4 in AD patients and controls was 50 % and 61 %, respectively (p = 0.26).

Donors that produced high levels of IL-2 or IL-4 in the absence of DEX also produced the highest levels in the presence of DEX ($p < 0.005$ for both IL-2 and IL-4 using linear regression).

By comparing the extent of inhibition of IL-2 and IL-4 in the total population (pooled data from AD patients and controls), it appeared that at a concentration of 10^{-7} M DEX the IL-2 production was more sensitive to DEX than the IL-4 production (data from Figure 5 were used; mean inhibition of IL-2 and IL-4 production was 83.1 % and 55.3 %, respectively). Thus, although DEX does not enhance IL-4 production – as described for the mouse – it may affect the production of IL-2 and IL-4 differently.

Since DEX inhibits the production of IL-2 as well as IL-4, it might be that the extent of inhibition by DEX in the proliferative response is correlated with the extent of interleukin production. As can be seen in Figure 6, this was indeed the case in the control group (Fig. 6A and 6B), i.e. high producers of IL-2 or IL-4 were relatively insensitive to DEX.

By contrast, there was no relation between IL-2 or IL-4 production and DEX-sensitivity in the AD group (Fig. 5C and 5D). This suggests that the diminished sensitivity to DEX in AD is not exclusively related to an effect on the synthesis of IL-2 or IL-4.

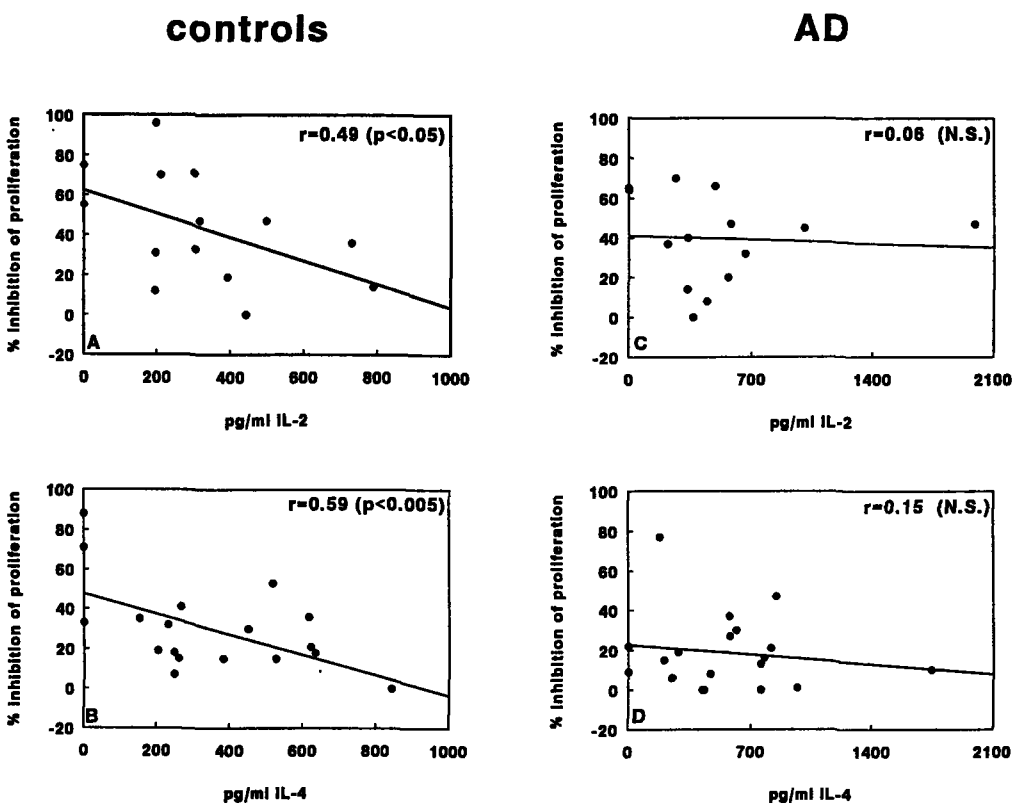

Figure 6. **Inhibition of proliferation by dexamethasone correlates with the production of IL-2 and IL-4 in controls, but not in AD patients.** Inhibition of proliferation by DEX in the PHA response was plotted against the amount of IL-2 produced by the same cells in the absence of DEX for each individual control (A) and AD patient (C). In a similar fashion, DEX-mediated inhibition of the anti-CD2/anti-CD28 response was plotted against the amount of IL-4 produced by controls (B) and AD patients (D). Lymphokine data were derived from the experiments shown in Fig. 4. Correlation-coefficients and p-values were obtained through linear regression.

DISCUSSION

In the present study, we investigated the possibility that the functioning of the immune system in AD patients is altered as a consequence of a disturbed HPA

axis. Previous **in vivo** studies have indicated that cells from the pituitary gland in these patients, as compared to controls, are less sensitive to the influence of glucocorticoids, in that cortisol levels in plasma are less decreased by an **in vivo** challenge with DEX, although this appeared not to be specific for Alzheimer's disease [19 – 21].

Our experiments show that the same is true for the sensitivity of T cells **in vitro**. This might be caused by the fact that these cells were already suppressed **in vivo**, e.g. by occupancy of the intracytoplasmic GCR. However, we obtained no evidence for a suppressed T cell population **in vivo**, since proliferative responses of T cells from AD patients and controls **in vitro** were comparable, and cytokine production was rather higher than lower in AD patients. The fact that some reports [14-15] show a diminished proliferative response in AD might in our view be caused by the lack of uniformity in AD with respect to diagnosis and the heterogeneity among AD patients.

A second possibility is that a diminished DEX sensitivity of T cells in AD is related to an altered composition and function of the $CD4^+$ T cell population. Daynes & Araneo showed in the murine system that DEX can enhance IL-4 production while inhibiting IL-2 production, indicating a differential effect of this hormone on IL-2 and IL-4 producing cells [27]. Given the fact that IL-4 is produced by $CD45RO^+CD4^+$ T cells and not by $CD45RA^+CD4^+$ T cells [28], it might thus be that also in the human situation the effect of DEX is dependent on the phenotype and functional characteristics of the $CD4^+$ T cells. In this regard it is worthwile to remark that the process of aging is accompanied by a decrease in the relative number of $CD4^+CD45RA^+$ T cells [30,31], most likely as a consequence of thymic involution [32]. Moreover, patients with Down's syndrome – who frequently develop AD at a young age – have lower numbers of $CD4^+CD45RA^+$ T cells in their peripheral blood [33]. However, we were not able to show a statistically significant difference in phenotype between AD patients and controls : comparable numbers of naive and memory $CD4^+$ T cells were found. Also with regard to IL-2 and IL-4 production we found no significant difference between the two groups.

Our data did not show that DEX stimulates IL-4 production by human PBMC as was found in the murine system by Daynes and Araneo [27]. It might however be that IL-2 and IL-4 production by human $CD4^+$ T cells are differentially affected by DEX : 10^{-7} M DEX caused in 83 % inhibiton of IL-2 production and 55 % inhibiton of IL-4 production. It should be taken into account that different activation pathways were used for the induction of these lymphokines and that

the effect of DEX may depend on the activation pathway.
The reasons for the lower sensitivity of the proliferative response in AD are as yet unclear, but are possibly related to quantitative differences in the expression of the GCR. This possibility would explain the lack of correlation between the extent of inhibition by DEX and interleukin production in AD, in contrast to the significant correlation that was found in controls. However, the expression of the GCR receptor, as determined by immunofluorescence, was comparable between patients with early onset AD and controls (Figure 4). Therefore it might be that the affinity of the GCR in PMBC from AD patients is lower. Alternatively, the production of growth factors different from IL-2 and IL-4 may play a role in the lower sensitivity to DEX in AD patients.
The observation that in AD both PBMC and cells from the pituitary gland are less sensitive to DEX points to a more general phenomenon. More insight into the mechanism of the diminished DEX sensitivity may provide valuable information concerning the etiology of the disease. PBMC are a useful and easily accessible tool to study such putative generalized cellbiological defects in AD.

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CHAPTER 3

AGE-RELATED INCREASE IN THE FRACTION OF CD27$^-$ CD4$^+$ T CELLS AND IL-4 PRODUCTION AS A FEATURE OF CD4$^+$ T CELL DIFFERENTIATION IN VIVO

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SUMMARY

The influence of ageing on phenotype and function of $CD4^+$ T cells was studied by comparing young (19–28 years of age) and aged (75–84 years of age) donors that were selected using the SENIEUR protocol to exclude underlying disease. An age-related increase was observed in the relative number of memory cells, not only on the basis of a decreased CD45RA and increased CD45RO expression, but also on the basis of a decrease in the fraction of $CD27^+CD4^+$ T cells. Our observation that the absolute number of $CD45RO^+CD4^+$ T cells was increased, while absolute numbers of $CD27^-CD4^+$ T cells remained unchanged in aged donors, indicates that the latter subset does not merely reflect the size of the $CD45RO^+CD4^+$ T cell pool. The increased fraction of memory cells in the aged was functionally reflected in an increased IL-4 production and T cell proliferation, when cells were activated with the combination of anti-CD2 and anti-CD28, whereas IL-2 production was comparable between both groups. No differences were observed with respect to proliferative T cell responses or IL-2 production using plate-bound anti-CD3 or PHA. The observation that IL-4 production correlated with the fraction of memory cells in young donors but not in aged donors suggests different functional characteristics of this subset in aged donors.

INTRODUCTION

Ageing is accompanied by an increased susceptibility to infections [1–3]. For instance, the mortality due to influenza infection is by far the greatest in the elderly population [4]. It is suggested that these findings are related to an impaired function of the immune system, because it has been demonstrated that the proliferative response of T cells to the mitogen phytohaemagglutinin (PHA) *in vitro* is lower in aged compared with young individuals [5–8].

Whether these diminished proliferative responses are related to intrinsic defects like diminished calcium responses [9] or to a changed subset composition and/or cytokine synthetic capacity of the T cell population is uncertain. Furthermore, it has been reported in mouse and in man that ageing is accompanied by a decline in the production of IL-2 [5,10–12], that is accompanied by an increase in the production of IL-4 and IFN-γ [12,13]. These phenomena are probably a

reflection of an age-related shift from a naive to a memory phenotype in the CD4$^+$ T cell compartment [14]. Similar phenotypic changes have been suggested to occur in man on the basis of a decrease in the size of the fraction of the CD45RA$^+$ 'naive' CD4$^+$ T cell population, accompanied by an increase in the size of the fraction of the CD45R0$^+$ 'memory' CD4$^+$ T cell population [5,6,15]. Recently it has been suggested that long lived memory cells are characterised by the absence of CD27 expression [16]. However, no data with respect to the expression of this marker on CD4$^+$ T cells from aged donors have been available until now.

Changes in the functional properties of T cells observed in *in vitro* studies in the elderly may thus be due to different activation requirements of naive and memory CD4$^+$ T cells [17–20]. To address this possibility, we employed different activation pathways with a preference for naive or memory CD4$^+$ T cells to assess the capacity of T cells to proliferate and to produce IL-2 and IL-4. To exclude the influence of underlying diseases in this study, young as well as aged donors were selected according to the 'SENIEUR' criteria for immunogerontological studies [21]. The present study shows that an age-related increase of the fraction of 'memory' CD4$^+$ T cells is evident on the basis of the expression of CD45RA, CD45R0 and CD27. Functionally, age-related changes were only observed when the cells were stimulated with the combination of anti-CD2 and anti-CD28, in that an increased proliferation and IL-4 production was observed in the elderly population.

MATERIALS AND METHODS

Donors

Young (19–28 years of age) and aged (75–84 years of age) donors were selected according to the EURAGE SENIEUR protocol as described by Ligthart *et al.* [21]. This protocol contains exclusion criteria on clinical information, laboratory data and use of pharmaceutical agents. After informed consent was obtained, 23 young (22 ± 3 years old (mean ± S.D.) and 41 aged (mean age 78 ± 3 years old) individuals were included in the study. All subjects were recruited in the city of Leiden, The Netherlands.

Isolation of PBMC and $CD4^+$ T cell subsets

Peripheral blood mononuclear cells (PBMC) were obtained by Percoll (Biochrom KG, Berlin, Germany) density centrifugation (ρ = 1.077 g/cm^3), cryopreserved and thawed according to standard procedures [22]. Viable cells were enumerated by trypan blue exclusion. In agreement with Bom-Van Noorloos *et al.*, we found no changes in the phenotype of the cells after this procedure, with the exception of a slight decrease in monocytes. With respect to functional data a positive correlation was found between data obtained with cryopreserved cells as compared to fresh PBMC from identical donors (data not shown). $CD45RA^+CD4^+$ and $CD45RO^+CD4^+$ T cells were enriched by depletion of $CD8^+$ T cells, B cells, NK cells and monocytes by incubation with: anti-CD8 (CLB-T8/4), anti-CD14 (CLB-mon/1), anti-CD16 (CLB-FcR gran/1) and anti-CD19 (CLB-B4/1), which were all purchased from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (Amsterdam, The Netherlands). Thereafter cells were washed twice to remove unbound monoclonal antibodies (MoAbs) and subsequently incubated with magnetic goat-anti-mouse-Ig (Advanced Magnetics Inc, Cambridge, MA) beads. The adherent fraction was removed using a Dynal MPC-6 Magnetic Particle Concentrator (Dynal, Oslo, Norway). The $CD4^+$ T cell population was split into two fractions and incubated with 2H4 (anti-CD45RA; Coulter, Hialeah, FL) or UCHL-1 (anti-CD45RO; DAKO, Glostrup, Denmark) to remove $CD45RA^+$ and $CD45RO^+$ cells respectively, using magnetic beads conjugated with goat-anti-mouse-Ig as described above. By this method $CD45RA^+CD4^+$ and $CD45RO^+$ $CD4^+$ T cells were enriched to a purity of at least 90 %. The percentage of monocytes was less than 1 % on the basis of forward versus sideward scatter pattern and indirect staining of CD14.

Phenotypic analysis

For phenotypic analysis, $5x10^4$ cells were incubated with a saturating amount of FITC- or PE- conjugated MoAbs. Thereafter, cells were washed and 7000 cells were analysed on a FACScan (Becton Dickinson, Mountain View, CA). The following MoAbs were used: CD19 and CD27 (CLB-B4/1) and CLB-CD27/2 respectively; CLB, Amsterdam,The Netherlands). CD3-FITC, CD4-PE, CD8-PE, CD14-FITC, CD45RA-FITC, CD45RO-PE and TCR$\gamma\delta$-PE MoAbs were purchased

from Becton & Dickinson. The absolute numbers of leucocytes and mononuclear cells were determined on a Coulter counter (Coulter Electronics, Hialeah, FL) in combination with a Haemalog D (Technicon Instruments, Tarrytown, NY). The absolute numbers of cells with a particular phenotype were calculated on the basis of the total number of mononuclear cells.

Proliferation assays

Cultures were performed in Iscove's Modified Dulbecco's Medium (Biochrom KG, Berlin, Germany) supplemented with 10% Foetal Calf Serum (FCS, Seralab, Crawley Down, UK), penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamin (2 mM) and β-mercaptoethanol (50 μM). Unseparated PBMC ($4x10^4$/well) were cultured in flat bottom microtiter plates (Costar, Cambridge, MA) and stimulated with PHA (Murex, Dartford, UK; 0.5μg/ml). Alternatively, plate-bound anti-CD3 (CLB-T3/3; 1:500 dilution of murine ascites; CLB, Amsterdam, The Netherlands), prepared as described elsewhere [23], was used as stimulator. The alternative pathway of T cell activation was studied using a combination of anti-CD2 and anti-CD28. To this end, two anti-CD2 MoAbs (CLB-T11.1/1 and CLB T11.2/1; CLB, Amsterdam, The Netherlands), each in a dilution of 1:2000 of murine ascites, were used in combination with anti-CD28 mAb (CLB-CD28/1; 2 μg/ml of purified MoAb; CLB, Amsterdam, The Netherlands). Cell proliferation was measured by adding 0.25 μCi methyl-tritium-thymidine (methyl-[^{3}H]TdR) (specific activity 2 Ci/mmol; Radiochemical Center, Amersham, UK) during the last 6 hours of culture. Cells were harvested onto glass fibre filter paper (Packard Instrument Company, Meriden, CT). Filters were counted using a Matrix 96 β-counter (Packard Instrument Company). This procedure has an efficiency of about 20 % as compared to standard liquid scintillation counting.

IL-2 and IL-4 assays

For the induction of IL-2 production PBMC ($4x10^4$/well) were stimulated with 10 μg/ml PHA or with the combination of anti-CD2 and anti-CD28. Supernatants were harvested at day 3 and kept at -20 °C until assay. IL-2 activity was assessed using the CTLL-2 cell line as described elsewhere [24]. Human recombinant IL-2 was used as a standard (Cetus, Emeryville, CA). IL-4

production was induced by the combination of anti-CD2 and anti-CD28 ($4x10^4$ PBMC/well). IL-4 levels in the supernatant were assessed using an ELISA technique as described elsewhere [25]. Plates were read using a Biorad Microplate reader (Biorad, Richmond, CA). Human recombinant IL-4 (Genzyme, Cambridge, MA) was used as a standard.

Statistical analysis

Statistical analysis was performed using the two-tailed Mann-Whitney U-test. Differences with a confidence level of 95 % or higher were considered to be statistically significant ($p < 0.05$).

RESULTS

Age-related changes in the phenotype of $CD4^+$ T cells

No differences between young and aged donors were found in the relative or the absolute numbers of $CD4^+$ or $CD8^+$ T cells, $CD19^+$ B cells or $CD14^+$ monocytes (Table 1). Both the relative and the absolute numbers of $CD3^+$ T cells were marginally decreased in the aged compared with the young. In the aged group a significant decrease was also found in the fraction and absolute number of TCR$\gamma\delta$ bearing T cells ($p \leq 0.0001$). With respect to several markers which distinguish naive from memory $CD4^+$ T cells, major changes were found: an increased fraction of $CD45RO^+$ cells and decreased fraction of $CD45RA^+$ cells was found in the aged. Similar changes were evident from the absolute cell numbers. To determine whether an age-related increase in the number of memory $CD4^+$ T cells could also be observed on the basis of a decreased expression of CD27, we performed a double staining for CD4 and CD27. It was indeed found that the fraction of $CD27^+$ $CD4^+$ T cells was decreased in aged donors as compared to $CD4^+$ $CD27^+$ T cells from young donors (80.2 % and 87.4 %, respectively ; $p < 0.001$). However, no changes were observed in the absolute numbers of $CD4^+$ $CD27^+$ T cells between the two age groups.

Table 1. **Phenotype of peripheral blood mononuclear cells (PBMC) in young and aged individuals.**

Marker	Young (n = 23) % of positive cells (absolute number (10^9/l))	Aged (n = 41) % of positive cells (absolute number (10^9/l))	p
CD3	52.8 ± 9.3	47.2 ± 11.3	0.05
	(1.09 ± 0.34)	(0.92 ± 0.33)	0.05
CD4	25.0 ± 6.4	25.8 ± 8.5	0.98
	(0.50 ± 0.15)	(0.50 ± 0.22)	0.72
CD8	22.6 ± 8.6	19.8 ± 9.4	0.21
	(0.48 ± 0.26)	(0.39 ± 0.22)	0.15
CD14	20.4 ± 10.2	17.9 ± 6.3	0.46
	(0.42 ± 0.25)	(0.34 ± 0.12)	0.15
CD19	12.5 ± 4.4*	12.1 ± 5.2**	0.48
	(0.25 ± 0.07)*	(0.24 ± 0.14)**	0.18
TCR$\gamma\delta$	5.2 ± 3.4	3.1 ± 3.4	0.0001
	(0.11 ± 0.08)	(0.06 ± 0.07)	<0.0001
	% of CD4$^+$ T cells (absolute number of CD4$^+$ T cells (10^9/l))	% of CD4$^+$ T cells (absolute number of CD4$^+$ T cells (10^9/l))	
CD45RA	52.4 ± 13.4	32.5 ± 17.5	<0.0001
	(0.26 ± 0.10)	(0.16 ± 0.12)	<0.0001
CD45RO	45.7 ± 10.4	64.6 ± 15.4***	<0.0001
	(0.23 ± 0.09)	(0.33 ± 0.14)***	0.007
CD27	87.4 ± 4.9	80.2 ± 9.3	0.0007
	(0.41 ± 0.18)	(0.39 ± 0.20)	0.18

Shown are the relative (as a percentage of the total PBMC population) and absolute numbers of cells. The relative number of CD45RA$^+$, CD45RO$^+$ and CD27$^+$ cells is expressed as a percentage of the CD4$^+$ T cell population.
n, the number of donors tested. * n = 13; ** n = 22; *** n = 40.

Proliferative responses of PBMC from aged individuals are not impaired

Given the changed composition of the $CD4^+$ T cell subset and the fact that naive and memory cells display different activation requirements [17–20], we studied the responsiveness of unseparated PBMC, from donors of different ages, using three distinct stimulation routes. In agreement with literature data, under our culture conditions PHA and plate bound anti-CD3 favoured the response of naive and memory $CD4^+$ T cells respectively [17,18,20,26] (Fig. 1). The fact that we could measure a PHA response despite the depletion of accessory cells is probably explained by the observation that as little as 0.25 % contamination of monocytes is sufficient for a PHA response [18]. Furthermore, memory $CD4^+$ T cells were more responsive to the combination of anti-CD2 and anti-CD28, a pathway that bypasses the T-cell receptor (Fig. 1).

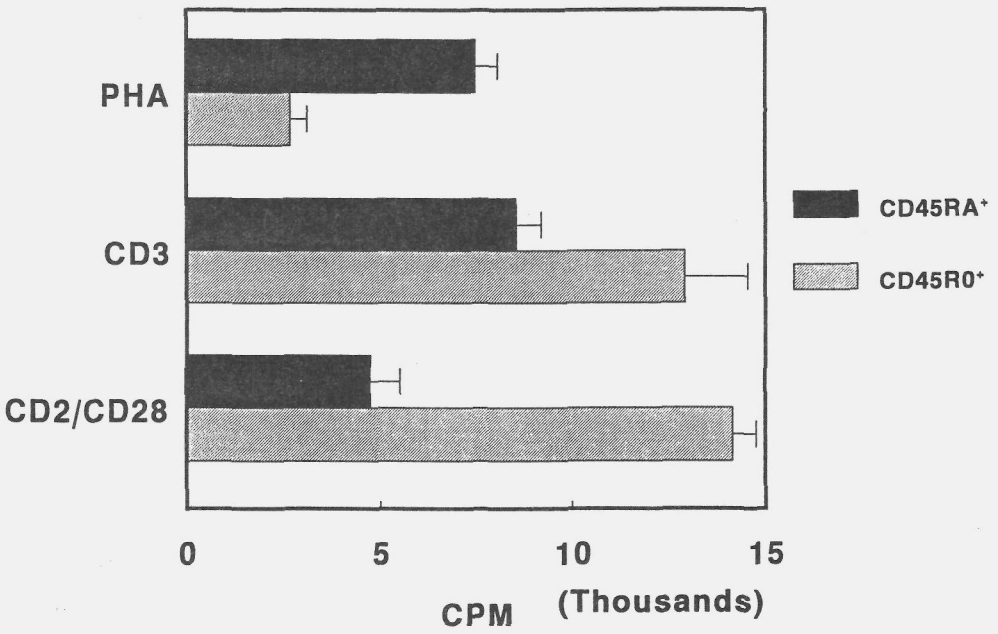

Figure 1. **Different activation requirements for naive and memory $CD4^+$ T cells.** $CD45RA^+CD4^+$ or $CD45R0^+CD4^+$ T cells from a representative young donor were stimulated with plate-bound anti-CD3, the combination of anti-CD2 and anti-CD28, or phytohaemagglutinin (PHA) (see Material and Methods). [^{3}H]TdR incorporation (ct/min as mean of triplicate cultures ± s.d.) at day 4 is shown. Background proliferation was less than 10 ct/min.

In contrast to previous studies [5–8], PBMC from young donors and aged donors that were stimulated with an optimal concentration of PHA showed a comparable proliferative response (Fig. 2A; mean [^{3}H]-TdR incorporation by PBMC from young donors 4425 ct/min and aged donors 4269 ct/min). Also no differences were found when the cells were stimulated with anti-CD3 (Fig. 2B; mean [^{3}H]-TdR incorporation 4856 and 4848 ct/min for young and aged donors, respectively). When the alternative pathway of T cell activation was used (Fig. 2C), PBMC from aged donors (mean [^{3}H]-TdR incorporation 6043 ct/min) proliferated significantly better than PBMC from young donors (mean [^{3}H]-TdR incorporation 5136 ct/min; $p < 0.01$), an observation consistent with an increased fraction of memory cells.

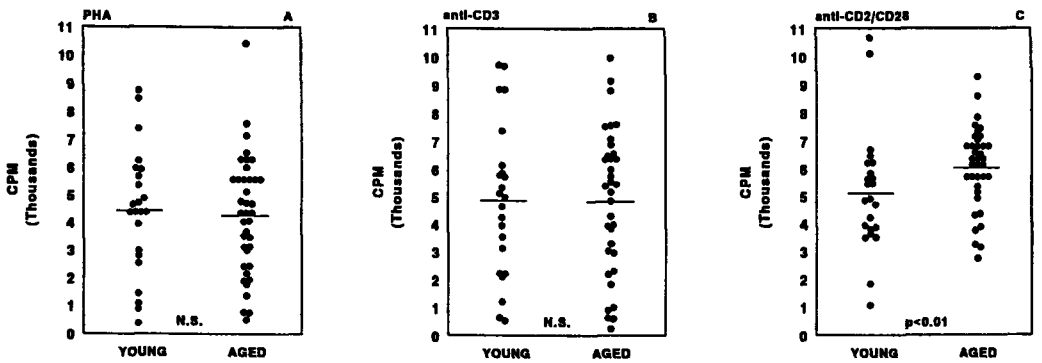

Figure 2. **Proliferative responses in the elderly are not impaired.**
Peripheral blood mononuclear cells (PBMC) from 23 young donors and 37 aged donors were stimulated with phytohaemagglutinin (PHA) (a), plate-bound anti-CD3 (b) and the combination of anti-CD2 and anti-CD28 (c) (see Material and Methods). [^{3}H]TdR incorporation (ct/min ± s.d.) was measured after 5 days of culture. NS, not significant.

Age-related increase in IL-4 production

In humans, IL-2 is the main cytokine product of naive CD4⁺CD45RA⁺ T cells, whereas CD4⁺CD45RO⁺ T cells produce IL-2 and have acquired the capacity to

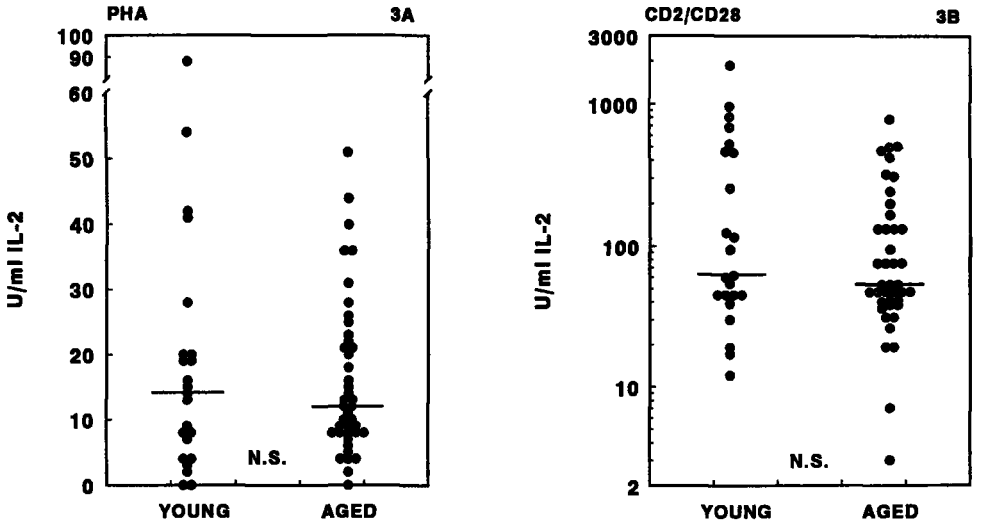

Figure 3. **Interleukin-2 production in young and aged individuals is comparable.** Peripheral blood mononuclear cells (PBMC) from 23 young and 41 aged donors were stimulated with the combination of anti-CD2 and anti-CD28 (b), or phytoheamagglutinin (PHA) (10 μg/ml) (a). Supernatants were harvested at day 3 and IL-2 was measured using CTLL-2 cells. NS, not significant. Median values are indicated.

produce IL-4 [27]. In view of the age-related increase in the fraction of the memory $CD4^+$ T cells, we were interested whether these phenotypical changes were reflected by changes at the level of IL-2 and/or IL-4 production. IL-2 production in response to anti-CD3 was below the detection limit in both groups (data not shown). PHA induced IL-2 production was comparable in both groups (young: median production 14 U/ml; aged: mean production 12 U/ml; $p = 0.80$; Fig. 3A). In response to anti-CD2 and anti-CD28 no differences were found with regard to IL-2 production (young: median production 62 U/ml; aged: mean production 53 U/ml; $p = 0.81$; Fig. 3B).

Stimulation with PHA or anti-CD3 did not result in detectable levels of IL-4 in the supernatant of PBMC from young and aged donors (data not shown). However, considerable levels of IL-4 were found after stimulation with the combination of anti-CD2 and anti-CD28. As shown in Fig. 4, the mean IL-4 production by PBMC from young donors was lower than in aged donors (mean production 302

pg/ml and 478 pg/ml for the young and aged, respectively; $p < 0.005$).
Thus, the shift from naive to memory $CD4^+$ T cells is reflected by an increased IL-4 production but not by significant changes at the level of IL-2. Analysing individual donors (in the young as well as in the aged group) showed that high IL-4 production correlated with low IL-2 production (Fig. 5A and B).
Because the production of IL-4 is strongly associated with $CD45RO^+CD27^-CD4^+$ T cells [16], we evaluated whether there existed a correlation between the production of IL-4 and the percentage of $CD45RO^+CD4^+$ or $CD27^-CD4^+$ T cells. As can be seen in Fig. 5C and 5E, there was a significant correlation between IL-4 production and the fraction of $CD45RO^+CD4^+$ T cells and $CD27^-CD4^+$ T cells in young donors ($p < 0.025$ for CD45RO and $p < 0.05$ for CD27, respectively). By contrast, no correlation was found in the aged group (Fig. 5D and F).

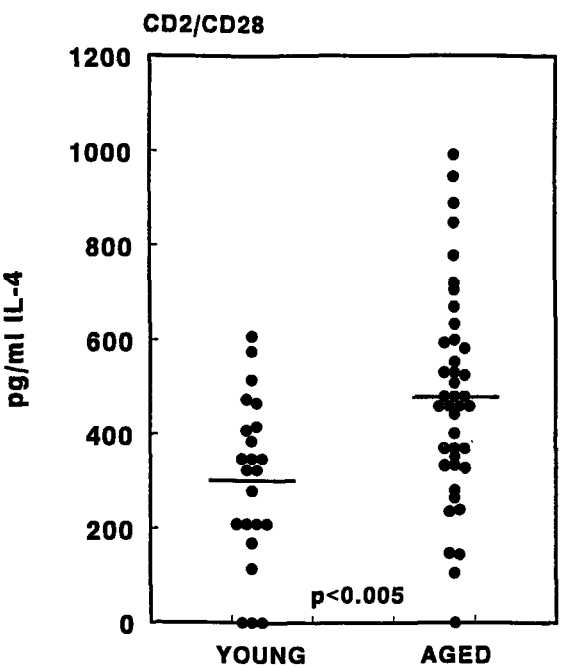

Figure 4. **Interleukin-4 production is increased in the elderly.**
Peripheral blood mononuclear cells (PBMC) from 23 young and 41 aged donors were stimulated with the combination of anti-CD2 and anti-CD28. Supernatants were harvested at day 3 and IL-4 was measured by ELISA.

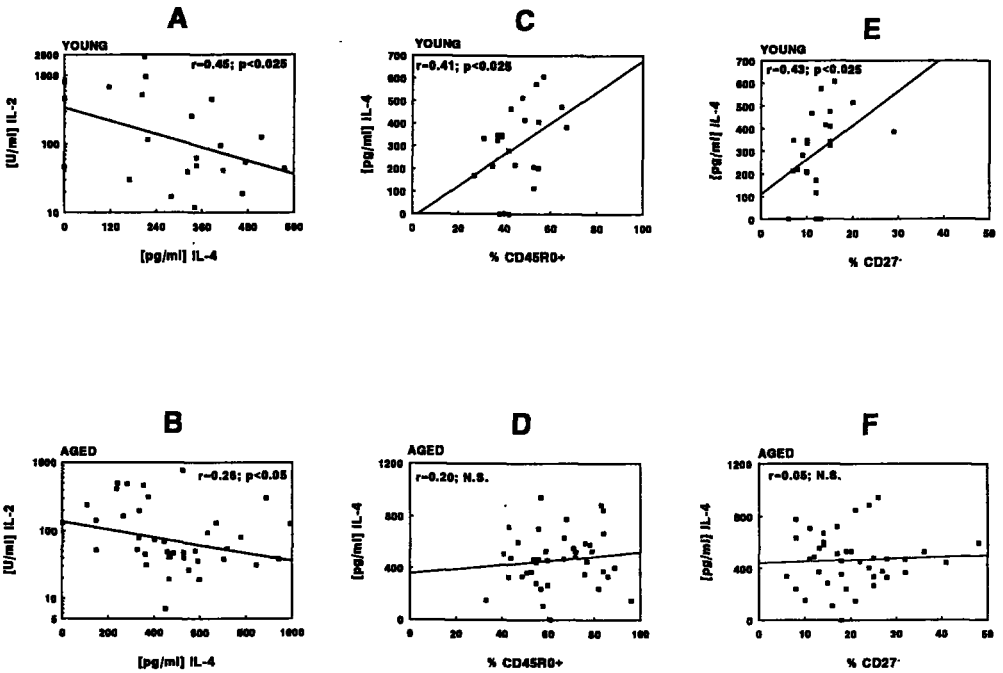

Figure 5. **IL-4 production correlates with the fraction of memory CD4$^+$ T cells in *young but not in aged donors*, and is inversely correlated with IL-2 production.**

Data concerning IL-2 and IL-4 production were derived from the results obtained after stimulation with the combination of anti-CD2 and anti-CD28 as depicted in Figs 3 and 4. Individual data from Table 1 were used for the percentages of CD45RO$^+$ and CD27$^-$ CD4$^+$ T cells. Correlation was determined by linear regression. NS, not significant.

DISCUSSION

Our present data extend previous studies [5,6,15] that show that ageing is accompanied by a conversion of a naive (expression of the CD45RA isoform) to a memory (expression of the CD45RO isoform) phenotype of the CD4$^+$ T cells. This was done under conditions that excluded the influence of underlying

disease, *i.e.* employing the SENIEUR protocol not only for the aged but also for the young donors. The increase in the fraction of memory cells was substantiated by an increase in the fraction of CD27$^-$CD4$^+$ T cells. This subset comprises about 18 % of the memory CD4$^+$ T cells and is thought to be primarily responsible for the response to recall antigens and the production of IL-4 [16]. The fact that the absolute number of CD27$^-$CD4$^+$ T cells was comparable in both groups, in contrast to the age-related increase in the absolute number of CD45RO$^+$CD4$^+$ T cells, indicates that the number of CD27$^-$CD4$^+$ T cells does not merely reflect the size of the CD45RO$^+$CD4$^+$ T cell pool. Possibly, the absence of CD27 expression represents a state of recent antigenic stimulation *in vivo* [16]. It is likely that the age-related shift from a naive to a memory phenotype is to a major extent due to a decrease in the number of CD45RA$^+$CD4$^+$ T cells. This might be explained by the fact that during ageing the thymic output of new naive T cells declines [28–30]. An impaired function of the thymus in the elderly could also responsible for lower numbers of T cells expressing the $\gamma\delta$ T cell receptor. As an alternative to a decreased thymic output, thymic emigrants in aged individuals may have a lower repopulating capacity. That peripheral T cells are able to repopulate the T cell pool has been demonstrated by Miller and Stutman, who showed that expansion of injected splenic T cells occurs in mice depleted of T cells by thymectomy and lethal irradiation [31]. The age-related decrease in the number of naive CD4$^+$ T cells is in agreement with observations in mice concerning a decrease in the frequency of antigen-specific T cells, *e.g.* with specificity for keyhole limpet hemocyanin [32]. This phenomenon might also be explanatory for the fact that the elderly are less responsive to neo-antigens such as influenza, evident fron a higher virus-related mortality [4]. In a previous study, we showed that this was also evident from a lower IgG response in the elderly to a standard dose of influenza vaccine [33]. However, this response was improved by increasing the dose of vaccin. Simultaneously, it was found that the IgA response to the vaccine was higher in the aged. Although we did not perform a phenotypic and functional analysis of T cells in this group of individuals it is tentative to speculate that the increased IgA response is due to increased numbers of memory cells and the related cytokines. The relation between the composition of the CD4$^+$ T cell population, their functional capacities and the nature of the *in vivo* response to influenza vaccine is currently under investigation.

We further investigated whether the age-related phenotypical changes were reflected in different functional capacities of the PBMC from both groups.

Several studies indicate that lectin induced T cell proliferation is declined in aged individuals [5–8]. This would be in accordance with observations that PHA preferentially activates naive $CD4^+$ T cells [20,26]. However, despite the fact that our culture conditions also favoured the proliferation of naive $CD4^+$ T cells using PHA, we did not find differences in proliferation between the aged and the young, when unseparated PBMC were studied.

Stimulation with anti-CD3, a condition that favours the proliferation of memory $CD4^+$ T cells [17,18], also did not show differences in proliferation between young and aged donors. This lack of difference might be due to the fact that accessory cells, through the secretion of the co-stimulatory cytokines IL-1 and IL-6, have augmented naive T cell responses to comparable levels of proliferation as memory T cells. However, the alternative pathway of T cell activation by simultaneously triggering of CD2 and CD28 molecules on the T cells, resulted in a significant higher response in the aged as compared to the young. In view of own data and data from Horgan *et al.* [18], regarding the lower responsiveness of naive $CD4^+$ T cells to anti-CD2 and anti-CD28 as compared to memory $CD4^+$ T cells, this finding is not unexpected. This might be explained by the observation that $CD4^+CD45RO^+$ T cells express CD2 in a higher density and therefore are possibly more responsive to triggering by anti-CD2 MoAbs [34].

In humans IL-2 is produced by $CD4^+CD45RA^+$ T cells as well as $CD4^+$ $CD45RO^+$ T cells [26,27,35]. In contrast to several other studies we did not observe an age-related decline in IL-2 production [5,10,11]. This finding was irrespective of the way of induction of IL-2 production (PHA, or anti-CD2 in combination with anti-CD28). The lack of an age-related decline in IL-2 production in the aged group in this study is compatible with the fact that both $CD4^+$ T cell subsets can produce IL-2 and that no age-related defects in proliferative responses are observed. Moreover, recent studies in the murine system have demonstrated that the preferential activation of memory $CD4^+$ T cells with plate-bound anti-CD3 even results in an age-related increase in IL-2 production (R. Dobber *et al.*, submitted for publication).

A change in IL-4 production, as would be expected from the conversion of $CD4^+$ T cells to a memory phenotype, was evident in the aged as compared to the young. Since IL-4 production has been attributed to $CD45RO^+CD4^+$ T cells and more specifically to the $CD27^-$ fraction thereof, we studied whether there was a relation between the relative number of $CD27^-CD4^+$ cells and the IL-4 levels. In young donors we confirmed the correlation between these two parameters. The fact that we found no correlation between IL-4 production and

the relative number of CD27$^-$CD4$^+$ T cells in the aged might be explained by the possibility that in aged donors, in contrast to young donors, IL-4 is only produced by a population of effector memory CD27$^-$CD4$^+$ T cells that has recently been activated *in vivo*, and not by the total CD27$^-$CD4$^+$ T cell population. Recent data indeed suggest that antigenic restimulation of memory CD4$^+$ T cells results into a substantial upregulation of IL-4 production by these cells [36]. Moreover, it appeared that the aged donors with a high percentage of CD27$^-$CD4$^+$ T cells produced only moderate amounts of IL-4 (Fig. 5F). We cannot exclude however that intrinsic cellular defects also play a role during ageing [7,9,14], and are responsible for a loss of IL-4 production in a subpopulation of memory CD4$^+$ T cells. Since IL-4 is considered to be an important cytokine for the induction of the IgE isotype, one might expect that an age-related increase in IL-4 production *in vivo* leads to elevated IgE levels and consequently to an increased susceptibility to allergic reactions in response to recall antigens. However, IgE levels in the elderly are rather lower than higher [37] and the onset of allergic symptoms in the aged have not been reported to increase with age. This might be explained by the age-related increase in IFN-γ production [38], which cytokine counteracts some of the biological effects of IL-4, among which the switching of B cells to IgE secretion [39].

In contrast to other studies on age-related changes of the immune system we could not demonstrate a decline in PHA responsiveness nor a significant decline in IL-2 production induced by PHA. A reason for this might be the differences in selection of the donors: several other investigators [5,7,8] have not applied the SENIEUR protocol, and may not have excluded the influence of underlying disease and medication. However, Beckman *et al.* [6] applying the SENIEUR criteria, showed a diminished response to PHA (but not to anti-CD3) in the elderly, which could be restored by exogenous IL-2, suggesting a decline in IL-2 production in the elderly.

In conclusion we would like to stress that age-related changes in the function of the CD4$^+$ T cells, are not necessarily due to intrinsic cellular defects but rather to the differentiation towards a population of cells consisting of a larger fraction of memory CD4$^+$ T cells.

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CHAPTER 4

ABROGATION OF THE SUPPRESSIVE EFFECTS OF DEXAMETHASONE BY PKC ACTIVATION OR CD28 TRIGGERING

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SUMMARY

The suppressive effect of the glucocorticoid dexamethasone (DEX) on purified $CD4^+$ T cells was found to depend on the activation pathway. In contrast to anti-CD3- or PHA-induced T cell proliferation, the alternative pathway of T cell activation, *i.e.*, through anti-CD2 and anti-CD28 appeared largely resistant to DEX. By titrating anti-CD28 or the protein kinase C (PKC) activator PMA in the DEX-sensitive systems, it was demonstrated that inhibition by DEX could be abrogated by enhancing the CD28 signal or by stimulation of the PKC-dependent pathway. Supraoptimal concentrations of PMA were inhibitory for proliferation and this effect was partly prevented by DEX. These data suggest that the outcome of the effect of DEX on $CD4^+$ T cells is dependent on the activation pathway, in particular the role and composition of the transcription factor AP-1.

INTRODUCTION

Glucocorticoids (GC) are widely known for their suppressive effects on cells of the immune system and are therefore frequently used for therapeutic purposes in a variety of diseases, *e.g.*, allograft rejection and autoimmune diseases.
Although many cell types are influenced by the action of GC [1,2], the inhibition of the function of T cells by GC is thought to be the primary cause of suppression of immune responses [3,4]. The inhibition of the production of interleukin-2 (IL-2) by T cells is an important characteristic of GC-induced immunosuppression [5]. GC, including the synthetic glucocorticoid dexamethasone (DEX), are thought to bind to an intracytoplasmic glucocorticoid receptor (GCR) [1,6]. This complex regulates IL-2 gene transcription in a negative fashion through interference with the transcription factor AP-1 [2,7,8]. The effect of DEX appears to be dependent on the composition of AP-1, which may be composed of cJun homodimers or of cJun-cFos heterodimers; interaction of the AP-1 homodimer with the GCR enhances the transcription of the murine proliferine gene, whereas interaction with the heterodimer suppresses gene transcription [9]. It may be that the effects of GC on IL-2 production and proliferation also depend on the composition of AP-1, which in turn may be related to the pathway of T cell activation. When $CD4^+$ T cells are activated by

perturbation of CD2 and CD28 molecules on the cell membrane, this results in a strong IL-2 and IL-4 production in a process that does not involve activation of protein kinase C (PKC) [10–12]. IL-2 gene transcription in this essentiallly different pathway of T cell activation is probably regulated by a different set of transcription factors [13–16].

Although it is likely that GC interfere with AP-1 induced by PKC activation [2,7,8,17], it is unclear whether and how GC regulate the gene transcription of IL-2 and other cytokines induced by triggering of the CD2 and CD28 membrane molecules. Interestingly, it has been demonstrated in the murine system that DEX may even stimulate the production of IL-4 [18]. Therefore, we investigated whether the effects of DEX on $CD4^+$ T cells depend on the pathway of T cell activation and the cytokines produced. To this end, we compared PKC-dependent proliferation of $CD4^+$ T cells induced by plate-bound anti-CD3 or by phytohaemagglutinin (PHA) [19,20] with the alternative pathway by perturbation of the CD2 and CD28 membrane molecules. The present study shows that the effect of DEX on $CD4^+$ T cell proliferation depends on the mode of activation and that this effect can even be stimulatory rather than inhibitory.

MATERIAL AND METHODS

Purification of $CD4^+$ T cells

PBMC from young healthy blood bank donors were obtained by Percoll (Biochrom KG, Berlin, Germany) density centrifugation (ρ = 1.077 g/cm^3) and subsequently cryopreserved. Cryopreserved PBMC were thawed according to standard procedures, and viable cells were enumerated by trypan blue exclusion and brought to the desired concentration for proliferation assays.

$CD4^+$ T cells were enriched by depletion of $CD8^+$ T cells, B cells, NK cells and monocytes by incubation with: anti-CD8 (CLB-T8/4), anti-CD14 (CLB-mon/1), anti-CD16 (CLB-FcR gran/1) and anti-CD19 (CLB-B4/1), which were all purchased from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB, Amsterdam, The Netherlands). Thereafter, cells were incubated with magnetic goat-anti-mouse-Ig (Advanced Magnetics Inc., Cambridge, MA) beads. The adherent fraction was removed using a Dynal MPC-6 Magnetic

Particle Concentrator (Dynal, Oslo, Norway). By this method $CD4^+$ T cells were enriched to a purity of at least 85 %.

Proliferation assays

All cultures were performed in Iscove's modified Dulbecco's medium (Biochrom KG) supplemented with 10% foetal calf serum (FCS, Seralab, Crawley Down, UK), penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamin (2 mM) and β-mercaptoethanol (50 μM).
$CD4^+$ T cells ($4x10^4$/well) were cultured in flat bottom microtiter plates (Costar, Cambridge, MA). To study $CD4^+$ T cell proliferation PHA (Murex, Dartford, UK) was used in a concentration of 0.5 μg/ml. Alternatively, $CD4^+$ T cells were stimulated with plate-bound anti-CD3. To this end, 100 μl/well of anti-CD3 (CLB-T3/3; 1:500 dilution of murine ascites) in phosphate-buffered saline (PBS) were incubated at 4 °C in 96-well plates. After overnight incubation the supernatant was removed and the wells were washed twice with PBS.
The alternative pathway of T cell activation was studied using a combination of anti-CD2 and anti-CD28. To this end, two anti-CD2 mAbs (CLB-T11.1/1 and CLB T11.1/2), each in a dilution of 1:2000 of murine ascites, were used in combination with anti-CD28 mAb (CLB-CD28/1; 2 μg/ml of purified mAb). The CD2, CD3, and CD28 mAbs were all kindly provided by Dr. R.A.W. van Lier (CLB, Amsterdam, The Netherlands). Alternatively, $CD4^+$ T cells were stimulated with the combination of phorbol 12-myristate 13-acetate (PMA, Sigma, St. Louis, MO) and ionomycin (Sigma) in a concentration of 0.5 ng/ml and 300 nM, respectively. Dexamethasone (DEX, Sigma) was used as indicated. Cell proliferation was measured at day 4 by adding 0.25 μCi methyltritium-thymidine ([^{3}H]TdR) (sp act 2 Ci/mmol; Radiochemical Centre, Amersham, UK) during the last 6 hr of culture. Cells were harvested onto glass-fiber filter paper (Packard Instrument Co., Meriden, CT). Filters were counted using a Matrix 96 β-counter (Packard Instrument Co.). This procedure has an efficiency of about 20 % as compared to standard liquid scintillation counting.

IL-2 and IL-4 assays

All supernatants were harvested at day 3 and stored at −20 °C until assay. IL-

2 activity was assessed with the use of the CTLL-2 cell line. DEX concentrations of 10^{-9} M and lower had no influence on the CTLL-2 cell line. In the CTLL-2 bioassay, 5000 cells were cultured in the presence of serially diluted supernatants. Human recombinant IL-2 was used as a standard (Cetus, Emeryville, CA; kindly provided by Dr. E. Braakman of the Dr. Daniel den Hoed Cancer Center, Rotterdam, The Netherlands). During the last 4 hr of the 24-hr culture period cells were pulsed with 0.25 μCi [^{3}H]TdR. IL-4 levels were determined using an ELISA technique as described elsewhere [12] employing reagents generously provided by Dr. T. van der Pouw-Kraan, CLB, Amsterdam, The Netherlands. Plates were read using a Bio-Rad Microplate reader (Bio-Rad, Richmond, CA). Human recombinant IL-4 was used as a standard (Genzyme, Cambridge, MA).

Phenotypic analysis

To assess the purity of the CD4$^+$ T cell populations, 5 x 10^4 cells were incubated with a saturating amount of FITC- or PE-conjugated mAbs for phenotypical analysis. Thereafter, cells were washed and 7000 cells were analyzed on a FACScan (Becton-Dickinson, Mountain View, CA).

Statistical analysis

Statistical analysis was performed, unless otherwise indicated, using the two-tailed Wilcoxon test for matched pairs. Differences with a confidence level of 95 % or higher were considered to be statistically significant ($p < 0.05$).

RESULTS

The alternative pathway of CD4$^+$ T cell activation is relatively insensitive for dexamethasone

CD4$^+$ T cells were activated with optimal concentrations of plate-bound anti-

CD3 or with PHA, in a PKC-dependent manner. Alternatively, $CD4^+$ T cells were activated through simultaneous triggering of the CD2 and CD28 membrane molecules, which does not lead to PKC activation.

As shown in Figs. 1A and B, anti-CD3 or PHA-induced $CD4^+$ T cell proliferation are highly sensitive to DEX in a dose dependent manner. The addition of DEX over a range of 10^{-8} to 10^{-5} M resulted in 19 to 97 % inhibition in the case of the anti-CD3 response, whereas the same concentration range caused 82 to 99 % inhibition of the PHA response.

By contrast, $CD4^+$ T cell proliferation induced by the combination of anti-CD2 and anti-CD28 mAbs was considerably less sensitive; only a partial inhibition by DEX, ranging from 10 to 38 %, was found at concentrations from 10^{-8} to 10^{-5} M (Fig. 1C). Analyzing 5 different donors, we observed a heterogeneity in DEX-

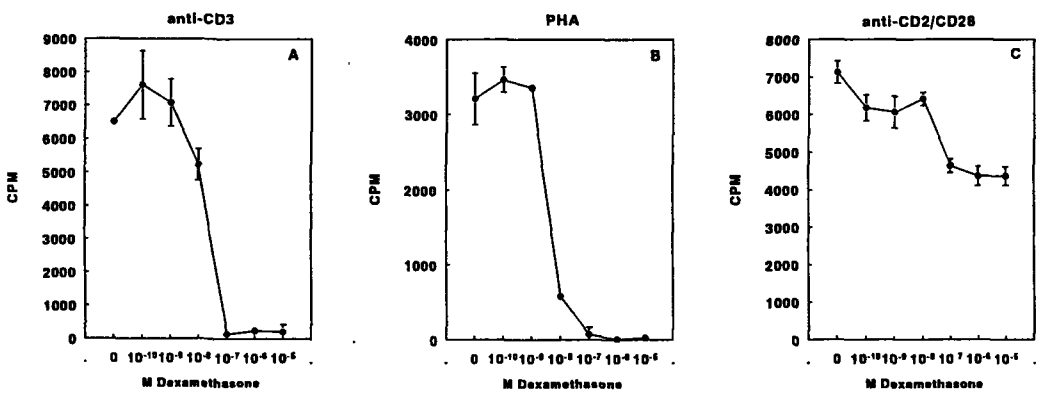

Figure 1. CD2- and CD28-mediated $CD4^+$ T cell proliferation is relatively resistant to dexamethasone.

$CD4^+$ T cells were activated with plate bound anti-CD3, PHA, or the combination of anti-CD2 and anti-CD28 (mean control responses 6498, 3208, and 7127 cpm, respectively) and inhibited with various concentrations of DEX. The percentage inhibition of the response is shown. Anti-CD3- and PHA-induced responses were significantly more inhibited by DEX than the response induced by anti-CD2/CD28 ($p < 0.02$ from 10^{-8} to 10^{-5} M DEX for anti-CD3; $p < 0.05$ from 10^{-7} to 10^{-5} M DEX for PHA) when $CD4^+$ T cells from five different donors were tested using the two-tailed Mann-Whitney *U* test. Results obtained with a representative donor are shown. [^{3}H]TdR incorporation (cpm as mean of triplicate cultures ± SD) was measured after 4 days of culture.

induced inhibition of $CD4^+$ T cell proliferation. The mean inhibition (± SD) at 10^{-7} M DEX was 88 ± 24 %, 64 ± 32 %, and 20 ± 23 % for anti-CD3, PHA, and anti-CD2/CD28, respectively.

PMA or anti-CD28 abrogate the dexamethasone induced inhibition of $CD4^+$ T cell proliferation

The higher sensitivity of anti-CD3 or PHA induced $CD4^+$ T cell proliferation for DEX, as compared to activation via CD2 and CD28, may be due to lack of perturbation of the CD28 membrane molecule. Alternatively, DEX-sensitivity might be dependent on the extent of PKC activation in these systems. Therefore, we studied the effect of anti-CD28 and PMA, a phorbol ester that directly activates PKC, on DEX-induced inhibition of $CD4^+$ T cell proliferation.

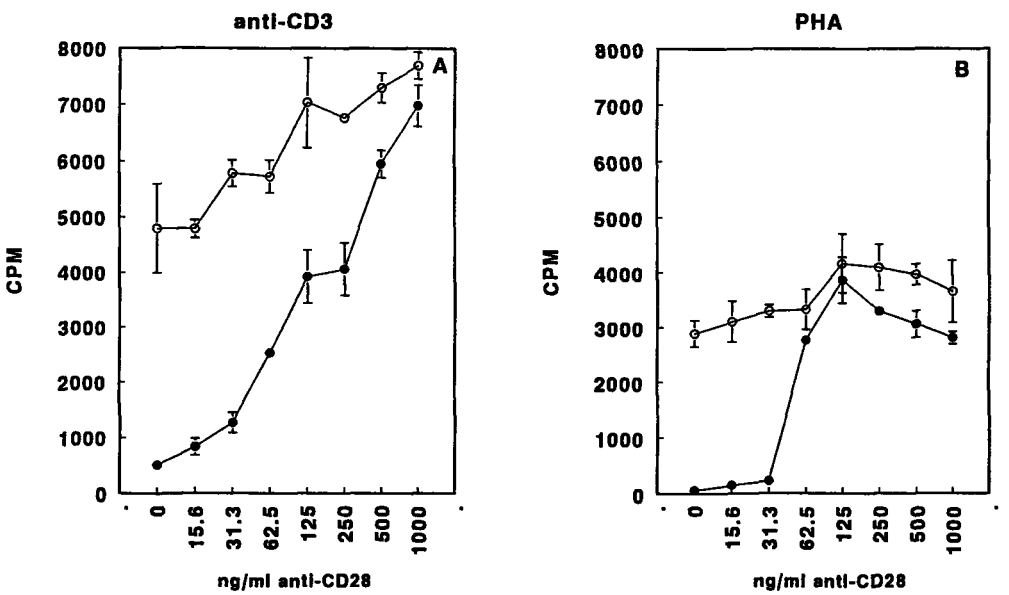

Figure 2. **Anti-CD28 abrogates dexamethasone induced inhibition of anti-CD3 or PHA induced $CD4^+$ T cell proliferation**
$CD4^+$ T cells were activated with plate-bound anti-CD3 or PHA and costimulated with various concentrations of anti-CD28 both in the presence (●——●) and absence (O——O) of 10^{-7} M DEX. Shown is a representative example of three different experiments with $CD4^+$ T cells from different donors. [^{3}H]TdR incorporation (cpm ± SD) was measured after 4 days of culture.

Figure 2 illustrates that anti-CD28 was dose-dependently able to abrogate DEX induced inhibition of $CD4^+$ T cell responses when these cells were stimulated with anti-CD3 or PHA (Figs. 2A and 2B, respectively). Whereas 10^{-7} M DEX caused 89.5 % (anti-CD3) and 98.1 % (PHA) inhibition in the absence of anti-CD28, only 9.3 % (anti-CD3) and 23.2 % (PHA) inhibition was observed in the presence of 1 μg/ml anti-CD28. This indicates that the introduction of a PKC independent pathway makes $CD4^+$ T cells less sensitive to DEX.
In addition, the extent of PKC activation was a determinant of the sensitivity of $CD4^+$ T cells for DEX. As shown in Fig. 3, anti-CD3-mediated $CD4^+$ T cell proliferation was costimulated by PMA up to 0.125 ng/ml PMA. Further increase of the PMA concentration led to inhibition of the response.

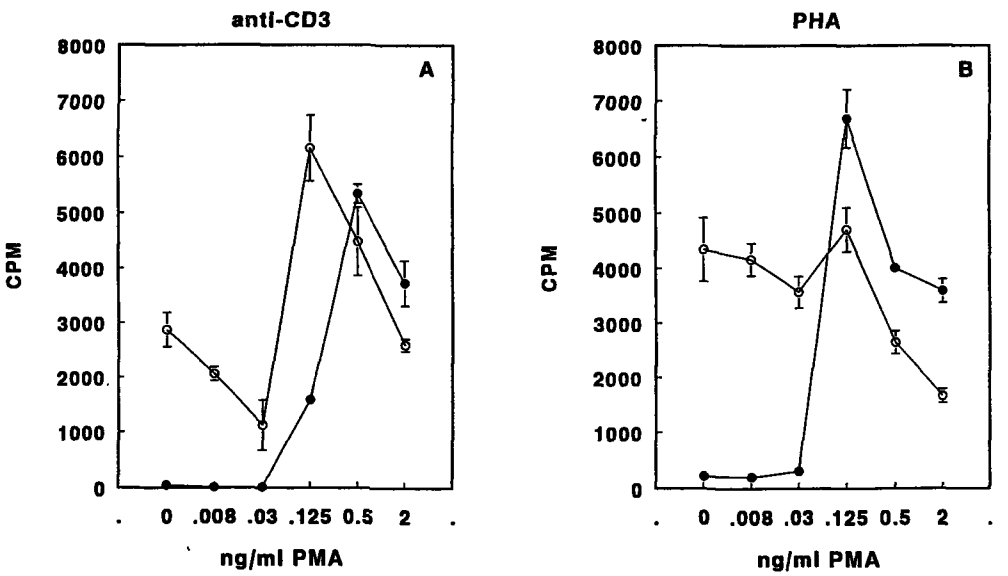

Figure 3. **PMA abrogates dexamethasone induced inhibition of anti-CD3 or PHA induced $CD4^+$ T cell proliferation**
$CD4^+$ T cells were stimulated with plate-bound anti-CD3 or with PHA, in the absence (O——O) or in the presence (●———●) of 10^{-7} M DEX. Various concentrations of PMA were studied for an effect on DEX induced inhibition. A representative example of five different experiments with $CD4^+$ T cells from different donors is shown. [^{3}H]TdR incorporation (cpm ± SD) was measured after 4 days of culture.

Costimulation by PMA of the PHA response was less evident but again higher PMA concentrations were also inhibitory in this pathway. This inhibitory action of PMA at higher concentrations was independent of the kinetics of the response (data not shown). As can be seen in Figs. 3A and 3B, PMA was dose-dependently able to abrogate the suppressive effect of 10^{-7} M DEX on proliferative responses induced by anti-CD3 and PHA. For instance, in the anti-CD3 response 10^{-7} M DEX lead to 100 % inhibition, whereas 0.5 ng/ml PMA resulted in full restoration of the response. These data illustrate that the inhibitory effect of DEX is also dependent on the extent of PKC activation.
The unexpected observation that DEX partly counteracted the inhibitory effect of supraoptimal PMA concentrations will be discussed in more detail below.
Abrogation of DEX induced inhibition by anti-CD28 or PMA was also evident at the level of IL-2 production. As shown in Table 1, both agents were able to increase the IL-2 production both in the presence and in the absence of 10^{-7} M DEX. Similar to PMA, anti-CD28 had only a marginal effect on the anti-CD3-induced IL-2 production by $CD4^+$ T cells (data not shown).

Table 1. Influence of dexamethasone on IL-2 production by $CD4^+$ T cells

	Anti-CD3[a]		PHA			PHA	
PMA (ng/ml)	None	+DEX[b]	None	+DEX	anti-CD28 (μg/ml)	None	+DEX
0	<1	<10	7.2	<10	0	<1	<10
0.008	<1	<10	12.1	<10	0.016	<1	<10
0.03	<1	<10	1.7	<10	0.032	<1	<10
0.125	<1	<10	21.0	<10	0.063	2.0	<10
0.5	1.7	<10	155.1	14.1	0.125	6.9	<10
2	2.4	<10	149.0	17.3	0.250	14.2	<10
8	1.8	<10	90.0	19.5	0.5	40.4	<10
					1	39.9	21.1

a $CD4^+$ T cells were activated with anti-CD3 (1:500 dilution of murine ascites) or PHA (0.5 μg/ml) and costimulated with increasing concentrations of PMA or anti-CD28, both in the presence and absence of 10^{-7} M DEX. IL-2 concentrations are presented in U/ml. The IL-2 values that are shown correspond with data from Fig. 2.

b Supernatants containing DEX were diluted at least 100-fold before testing in the CTLL bioassay to exclude the influence of DEX on this cell line. Therefore, the lower detection limit under these conditions is 10 U/ml.

In contrast to PMA or anti-CD28, costimulation with anti-CD2 or ionomycine was not able to abrogate DEX induced inhibition of proliferation, indicating that increasing the intracellular calcium concentration does not prevent DEX from being inhibitory (data not shown).

Dexamethasone is stimulatory for CD4$^+$ T cell proliferation at supraoptimal PMA concentrations

As shown in Fig. 3, 10^{-7} M DEX partly counteracts the PMA-induced inhibition of CD4$^+$ T cell proliferation at supraoptimal PMA concentrations ($p < 0.05$ at 0.5 ng/ml PMA for anti-CD3; $p < 0.05$ at 0.5 and 2 ng/ml PMA for PHA). To

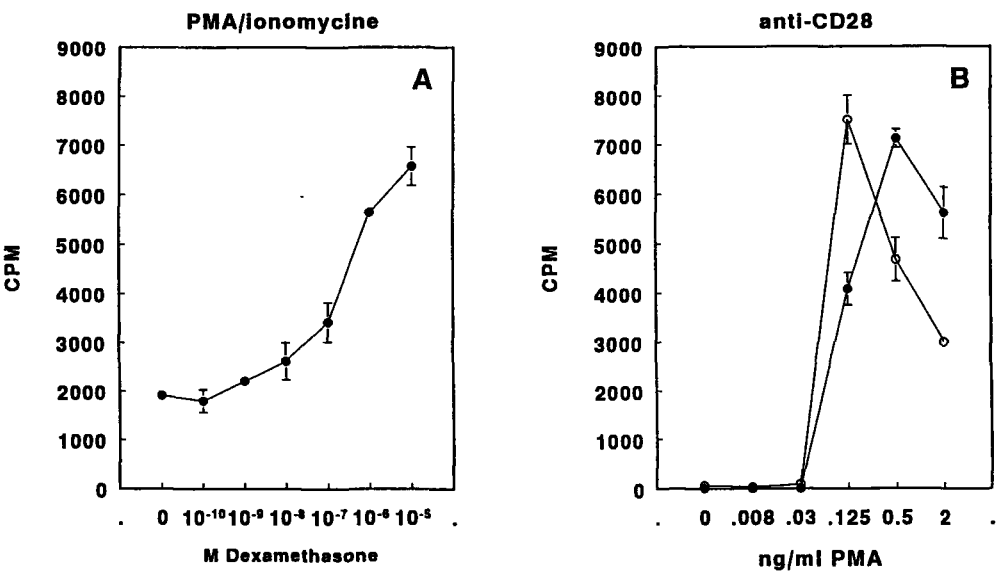

Figure 4. **Dexamethasone enhances CD4$^+$ T cell proliferation at supraoptimal PMA concentrations**
CD4$^+$ T cells were stimulated with the combination of 0.5 ng/ml PMA and 300 nM ionomycine in the presence of various concentrations of DEX (A). In B, CD4$^+$ T cells were stimulated with the combination of 2 μg/ml anti-CD28 and various concentrations of PMA in the absence (O——O) or in the presence (●———●) of 10^{-7} M DEX. Representative examples out of 5 experiments with CD4$^+$ T cells from different donors are shown. [^{3}H]TdR incorporation (cpm ± SD) was measured after 4 days of culture.

substantiate this finding, $CD4^+$ T cells were activated with the combination of 0.5 ng/ml PMA and 300 nM ionomycine, an activation route that generates excessive amounts IL-2. Also in this activation pathway, 0.5 ng/ml PMA is supra-optimal in that it inhibits $CD4^+$ T cell proliferation (data not shown). As shown in Fig. 4A, proliferation of $CD4^+$ T cells was stimulated in this supraoptimal system by DEX over the concentration range from 10^{-10} M to 10^{-5} M ($p < 0.05$ from 10^{-8} to 10^{-5} M DEX). This was irrespective of the time point when the proliferation was measured (data not shown). DEX also counteracted the inhibition of $CD4^+$ T cell proliferation by supraoptimal PMA concentrations in combination with anti-CD28 ($p < 0.05$ at 0.5 and 2 ng/ml PMA; Fig. 4B). For both activation pathways stimulatory effects of DEX on proliferation were observed despite inhibition of IL-2 production (data not shown).

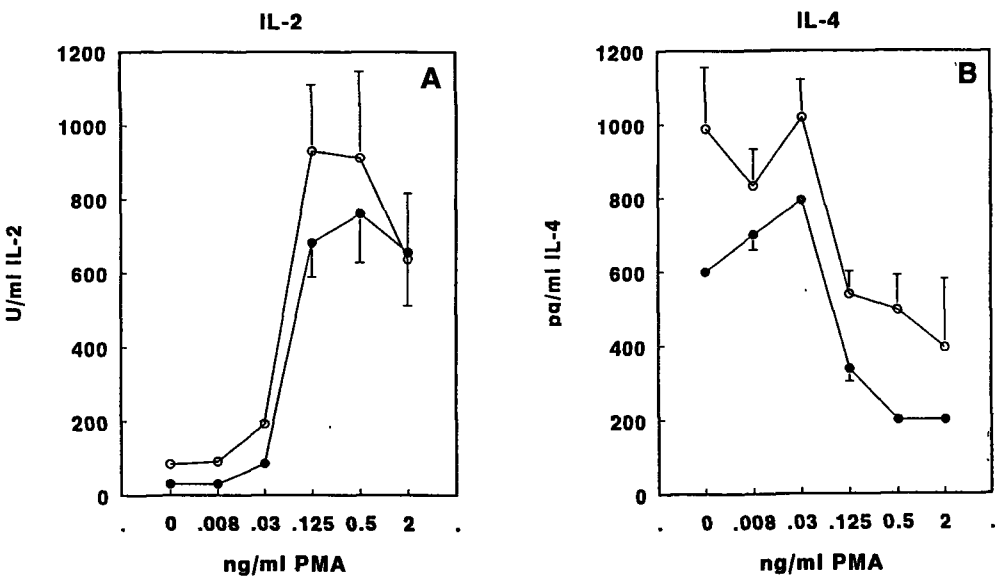

Figure 5. **Dexamethasone does not enhance IL-4 production at supraoptimal PMA concentrations**
$CD4^+$ T cells were stimulated with the combination of anti-CD2 (1:2000 dilution of murine ascites) and anti-CD28 (2 μg/ml) and were costimulated with increasing concentrations of PMA in the absence (O——O) or in the presence (●——●) of 10^{-7} M DEX. Results are the means of triplicates ± SD and representative for five experiments with $CD4^+$ T cells from different donors.

Dexamethasone does not enhance IL-4 production by $CD4^+$ T cells

It has recently been shown that PMA downregulates IL-4 production induced by triggering of the CD2 and CD28 membrane molecules [12]. Since DEX was found to enhance IL-4 production in the murine system [18] and IL-4 may act as an alternative growth factor [21–23], we investigated whether the counteracting effects of DEX on the PMA induced inhibition of $CD4^+$ T cell proliferation were due to an increased IL-4 production.
$CD4^+$ T cells were stimulated through perturbation of the CD2 and CD28 molecules on the membrane, a condition that results into considerable IL-4 production. As shown in Fig. 5, PMA had a strong enhancing effect on the production of IL-2, whereas it had an inhibitory effect on the IL-4 production. DEX was only marginally inhibitory for IL-2 production (Fig. 5A), suggesting that PMA-induced inhibition of proliferation is not mediated at the level of IL-2. Moreover, inhibition of IL-4 production could not be restored by DEX (Fig. 5B). Since exogenous IL-4 was also not able to restore the proliferative response induced by the combination of anti-CD2 and anti-CD28 (data not shown), IL-4 is probably not involved in the mechanism by which DEX partly restores the inhibition of $CD4^+$ T cell proliferation at supraoptimal PMA concentrations.

DISCUSSION

The aim of the present study was to investigate whether the immunosuppressive effect of glucocorticoids on $CD4^+$ T cells, in this case the synthetic glucocorticoid DEX, is dependent on the pathway of T cell activation that was used. Recent literature data indicate that the transcription factor AP-1 is an important factor in PKC-induced T cell activation [17] and probably the primary target for DEX induced suppression of IL-2 production [7]. Therefore, we evaluated T cell activation pathways that are PKC dependent (CD3- and PHA-mediated T cell activation) [10,19,20] with an activation pathway that is PKC independent, that is, via triggering of the CD2 and CD28 membrane molecules [10,11], with respect to their sensitivity to DEX. In contrast to the potent inhibitory effect of DEX on anti-CD3- and PHA-induced $CD4^+$ T cell responses, it appeared that stimulation of $CD4^+$ T cells via perturbation of the CD2 and CD28 molecules led to a relative resistance to the suppressive action of DEX

(Fig. 1). Literature data suggested that CD28-mediated $CD4^+$ T cell activation involves a different set of nuclear transcription factors compared to PKC-mediated activation. For instance Lu *et al.* suggested that for IL-2 gene transcription induced by CD28 triggering, NFAT and AP-1 binding activity is not essential and that a specific binding site for the CD28 pathway may be involved [24]. Furthermore, a novel nuclear binding factor has been proposed with apparent specificity for CD28 mediated T cell activation [14].

Therefore, our observation that costimulation with anti-CD28 makes activated $CD4^+$ T cells insensitive to DEX (Fig. 3) would be consistent with a minor involvement of AP-1 in this pathway. As an alternative to CD28 triggering, also the optimal activation of PKC by addition of the phorbol ester PMA abrogated the DEX induced inhibition of $CD4^+$ T cell responses (Fig. 2). This seems to be incongruent with data from Furue and Ishibashi [25] and Almawi *et al.* [26] who showed that the combination of PMA with CD3-, CD28- or PHA-induced proliferation of total T cells or PBMC is dose dependently inhibited by DEX. Possibly, the mitogenic combination of a different anti-CD28 mAb (CLB-CD28/1 instead of 9.3) with PMA in our experiments results into an optimal proliferative response that is insensitive to DEX. We cannot exclude, however, that the absence of $CD8^+$ T cells and accessory cells in our cultures accounts for the differences in results.

Abrogation of DEX induced inhibition of $CD4^+$ T cell proliferation by anti-CD28 or PMA might, at least in the PHA system, be due to their costimulatory effect on IL-2 production (Table 1). By contrast, these substances only marginally increased the IL-2 production in response to anti-CD3. Still, inhibition of both responses by DEX was completely abrogated by PMA. This would implicate that (1) low levels of IL-2 are sufficient to restore DEX mediated inhibition of proliferation or that (2) IL-2 is not the only growth factor involved in the abrogation of DEX induced inhibition. Most likely, also other cytokines are involved in the process of DEX-induced immunosuppression. For instance Almawi *et al.* showed that the combination of IL-1, IL-6 and IFN-γ was able to abrogate DEX induced inhibition completely [26].

Unexpectedly, stimulation of $CD4^+$ T cells with the combination of PMA and ionomycine was dose dependently enhanced by DEX (Fig. 4). The fact that supraoptimal PMA concentrations for proliferation are not inhibitory for IL-2 production and that DEX inhibited rather than enhanced IL-2 production, indicates that the stimulatory effect of DEX is probably not mediated through IL-2. Enhancement of $CD4^+$ T cell proliferation by DEX could not be explained by

an increase in IL-4 production by DEX. In contrast to the murine system where IL-4 production could be enhanced by DEX [18], IL-4 production by DEX was inhibited in our experiments, which is in agreement with data from Wu *et al.* who demonstrated this for unseparated human T cells [2].
Since the primary known target of DEX is the transcription factor AP-1 [7], the question emerges how DEX could be able to enhance $CD4^+$ T cell proliferation through interaction with AP-1. First, the effect of DEX is dependent on the composition of AP-1 and the ratio of cJun and cFos expression. If AP-1 is composed of a homodimer of cJun proteins, then interaction with the GCR leads to enhancement rather than inhibition of gene transcription [9]. Although PMA induces both cFos and cJun expression [28,29], it might be that high PMA concentrations preferentially induce cJun expression giving rise to formation of relatively more AP-1 homodimers. Possibly, interaction of the GCR/DEX complex with the cJun homodimer is responsible for the stimulatory effect we observed in this study. Secondly, data from Rincón *et al.* suggest that persistent AP-1 expression is inhibitory for the CD3-dependent proliferation of T cells [30]. Possibly, by reducing the AP-1 binding activity, DEX may have a stimulatory effect under conditions of persistent AP-1 expression in our experiments. Altogether our data suggest that the effect of glucocorticoids on $CD4^+$ T cells are dependent on the extent of PKC activation and the involvement of CD28 triggering during T cell activation and under specific conditions may be stimulatory in nature.

ACKNOWLEDGEMENTS

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CHAPTER 5

DIFFERENTIAL SENSITIVITY OF HUMAN NAIVE AND MEMORY CD4$^+$ T CELLS FOR DEXAMETHASONE

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ABSTRACT

Human CD45RA$^+$ ('naive') and CD45RO$^+$ ('memory') CD4$^+$ T cells were compared with respect to their sensitivity to dexamethasone (DEX). In three different activation pathways, *i.e.* i) immobilized anti-CD3, ii) immobilized anti-CD3 + soluble anti-CD28 and iii) soluble anti-CD2 + soluble anti-CD28, naive CD4$^+$ T cells appeared more sensitive to DEX than memory CD4$^+$ T cells. In the anti-CD3 system this difference in sensitivity was apparent at a suboptimal DEX concentration. Addition of anti-CD28 rendered the cells largely insensitive to DEX, indicating that the CD28-pathway is less dependent of the DEX-sensitive transcription factor AP-1. However, the alternative pathway of T cell activation through CD2/CD28 triggering was highly sensitive to DEX when naive cells were studied; in the case of memory cells at least a 10-fold higher DEX-concentration was needed to achieve a comparable inhibition. The strong inhibitory effect of DEX on naive CD4$^+$ T cells stimulated via the alternative pathway was completely abrogated by activation of protein kinase C (PKC), with phorbol-12-myristate-13-acetate. Our data suggest that at least two different mechanisms contribute to DEX-resistance, *i.e.* CD28-triggering and PKC-activation, which are both more easily recruited in memory cells making them less sensitive to DEX.

INTRODUCTION

Human CD4$^+$ T-cells can be divided into two subsets on the basis of phenotypical and functional characteristics. The CD4$^+$CD45RA$^+$ T-cell subset consists of unprimed, naive cells whereas the CD4$^+$CD45RO$^+$ T-cell subset encompasses the primed, memory CD4$^+$ T-cell population which is able to respond to recall antigens *in vitro* [1-3]. These two CD4$^+$ subsets have different activation requirements *in vitro* when polyclonal stimuli are used [4-8]. Whereas memory CD4$^+$ T-cells are more responsive to stimulation with immobilized anti-CD3 or the combination of anti-CD2 and anti-CD28, naive CD4$^+$ T-cells are preferentially activated by phytohaemagglutinin (PHA) [1,5,9]. These subsets are also functionally different; the main cytokine produced by naive CD4$^+$ T-cells is IL-2, whereas memory CD4$^+$ T-cells have acquired the capacity to produce IL-4 and IFN-γ [10].

In previous studies, we and others have demonstrated that the sensitivity of T-cells to the immunosuppressive effect of dexamethasone (DEX) depends on the activation pathway employed [11,12]. It is however unclear whether these findings reflect a difference in sensitivity of naive and memory CD4$^+$ T-cells for glucocorticoids (GC). In the mouse indirect evidence for a subset specificity of GC has been obtained by Daynes *et al.*, who showed that IL-4 production could be enhanced by DEX whereas IL-2 production was inhibited [13].
In the present study we provide evidence that human naive CD4$^+$ T-cells are more sensitive to suppression by DEX than memory CD4$^+$ T-cells and that this is likely related to a difference in the activation state of these cells.

MATERIAL AND METHODS

Purification of naive and memory CD4$^+$ T cells

Peripheral blood mononuclear cells (PBMCs) from young healthy blood bank donors were obtained by Percoll (Biochrom KG, Berlin, Germany) density centrifugation ($\rho = 1.077$ g/cm^3) and subsequently cryopreserved and thawed according to standard procedures [14]. CD4$^+$ T-cells were enriched from PBMCs by depletion of CD8$^+$ T-cells, B cells, NK cells and monocytes by incubation with: anti-CD8 (CLB-T8/4), anti-CD14 (CLB-mon/1), anti-CD16 (CLB-FcR gran/1) and anti-CD19 (CLB-B4/1), which were all purchased from CLB, Amsterdam, The Netherlands. Thereafter, cells were incubated with magnetic goat-anti-mouse-Ig beads (Advanced Magnetics Inc, Cambridge, MA). The adherent fraction was removed using a Dynal MPC-6 Magnetic Particle Concentrator (Dynal, Oslo, Norway). The CD4$^+$ T-cell population was split into two fractions and incubated with anti-CD45RA (2H4; Coulter, Hialeah, FL) or anti-CD45RO (UCHL-1; DAKO, Glostrup, Denmark) to remove CD45RA$^+$ and CD45RO$^+$ cells respectively, using magnetic goat-anti-mouse-Ig beads as described above. By this method CD45RA$^+$ CD4$^+$ and CD45RO$^+$ CD4$^+$ T-cells were enriched to a purity of at least 90 % as assessed by flowcytometry.

Proliferation assays

Cultures were performed in Iscove's modified Dulbecco's medium (Biochrom KG, Berlin, Germany) supplemented with 10% foetal calf serum (FCS, Seralab, Crawley Down, UK), penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamin (2 mM) and β-mercaptoethanol (50 μM). $CD4^+CD45RA^+$ or $CD4^+CD45RO^+$ T-cells ($4x10^4$/well) were cultured in flat-bottomed microtitre plates (Costar, Cambridge, MA) and stimulated with plate-bound anti-CD3 (CLB-T3/3) or a combination of anti-CD2 (CLB-T11.1/1 and CLB-T11.2/1) and anti-CD28 (CLB-CD28/1) as described previously [12]. DEX (Sigma, St. Louis, MO) and phorbol myristate acetate (PMA; Sigma) were used as indicated. Cell proliferation was measured at day 4 by adding 0.5 μCi methyl-tritium-thymidine ([^{3}H]thymidine) (sp. act. 2 Ci/mmol; Radiochemical Centre, Amersham, UK) during the last 6 hours of culture. Cells were harvested onto glass fibre filter paper (Packard Instrument Company, Meriden, CT). Filters were counted using a Matrix 96 β-counter (Packard Instrument Company). This procedure has an efficiency of about 20 % as compared to standard liquid scintillation counting.

IL-2 assay

Supernatants were harvested at day 3 and stored at -20 °C until assay.
IL-2 activity was assessed with the use of CTLL-2 cells as described before [12], using human recombinant IL-2 (Cetus, Emeryville, CA) as a standard.

Statistical analysis

Statistical analysis was performed using the two-tailed Mann-Whitney *U*-test. Differences with a confidence level of 95 % or higher were considered to be statistically significant ($p < 0.05$).

RESULTS

Differential sensitivity of naive and memory CD4+ T-cells for inhibition by dexamethasone.

Naive and memory CD4+ T-cells were activated via different pathways in order to determine their relative sensitivity for DEX. As shown for a representative donor in Figure 1(A), the addition of 10^{-7} M DEX led to an almost complete inhibition of the proliferative response in naive and memory CD4+ T-cells induced by plate-bound anti-CD3 (100 % and 85 % inhibition, respectively). A

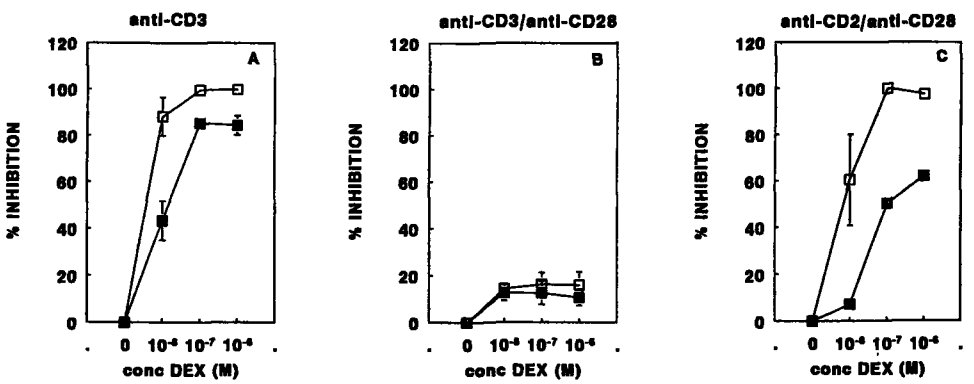

Figure 1. **Memory CD4+ T cells are less sensitive to dexamethasone than naive CD4+ T cells.**
Naive (□——□) and memory (■——■) CD4+ T cells were activated with plate-bound anti-CD3, the combination of plate-bound anti-CD3 with anti-CD28 or the combination of anti-CD2 and anti-CD28 (control responses were 8804, 18487, 7348 cpm, respectively for naive CD4+ T cells, and 12812, 13475, 12004 cpm for memory CD4+ T cells). Anti-CD2 or anti-CD28 alone induced background levels of proliferation (data not shown). Cells were inhibited with various concentrations of DEX. Representative titration curves out of three experiments with different donors are shown. [^{3}H]thymidine incorporation (mean cpm ± S.D.) was measured after 4 days of culture.

concentration of 10^{-8} M DEX caused 88 % inhibition in the case of naive $CD4^+$ T-cells but only partial (43 %) inhibition of memory $CD4^+$ T-cells. If the cells were stimulated with anti-CD3 and anti-CD28, both naive and memory $CD4^+$ T-cells were largely resistant to suppression by DEX over the entire concentration range (Fig. 1B). Triggering of CD28 as such was not sufficient for the induction of DEX-resistance since both naive and memory $CD4^+$ T-cells were sensitive to DEX when stimulated with anti-CD2 and anti-CD28 (Fig. 1C). In the sensitive systems at least a 10-fold higher concentration of DEX was required to inhibit memory $CD4^+$ T-cells to a similar extent as naive $CD4^+$ T-cells, indicating that memory cells were less sensitive.

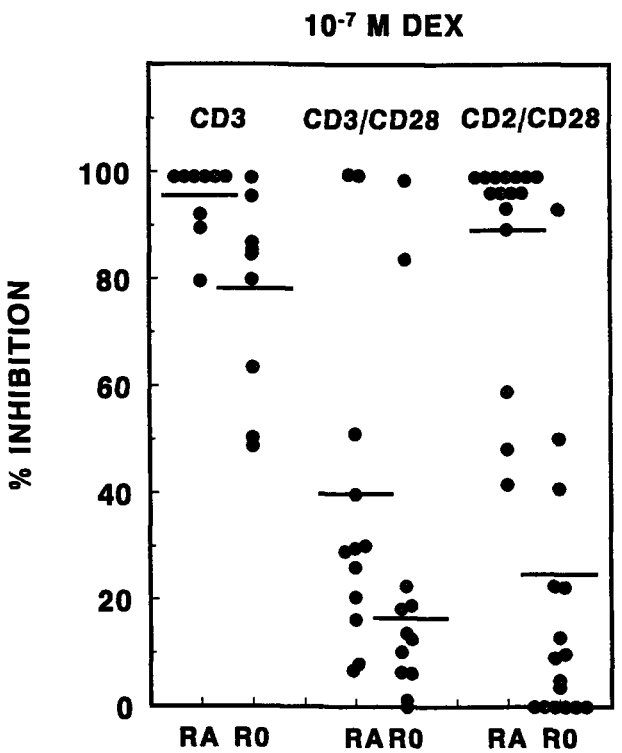

Figure 2. **Memory $CD4^+$ T cells are less sensitive to dexamethasone than naive $CD4^+$ T cells.**

Naive and memory $CD4^+$ T cells were stimulated as described in the legend of Figure 1 in the absence or in the presence of 10^{-7} M DEX. Each dot represents a different donor and both subsets from the same donor were always tested in at least one stimulation route. The percentage of inhibition is indicated.

These observations were substantiated in at least nine different donors, employing a concentration of 10^{-7} M DEX. Figure 2 shows once more that naive CD4$^+$ T-cells are highly sensitive to DEX when they are stimulated with anti-CD3 alone (mean inhibition 95 ± 7 %) or by the combination of anti-CD2 and anti-CD28 (mean inhibition 87 ± 21 %), but only partially sensitive (mean inhibition 40 ± 31 %) when stimulated with the combination of anti-CD3 and anti-CD28. These results suggest that naive CD4$^+$ T cells require the triggering of both CD3 and CD28 to become resistant to DEX. As compared to naive CD4$^+$ T-cells, memory CD4$^+$ T-cells were slighty more resistant to 10^{-7} M DEX when activated with anti-CD3 or the combination of anti-CD3 and anti-CD28 (mean inhibition 78 ± 18 % and 23 ± 29 %, respectively; $p < 0.05$). As compared with naive CD4$^+$ T-cells, memory CD4$^+$ T-cells were largely resistant to DEX when activated via the CD2 and CD28 membrane molecules (mean inhibition 17 ± 25 %; $p < 0.0001$).
Inhibition of naive and memory cells by DEX was primarily due to inhibition of IL-2 production since the addition of exogenous IL-2 (50 U/ml) completely abrogated the DEX induced inhibition (data not shown).

Attenuation of CD2 and CD28 triggering renders memory CD4$^+$ T-cells sensitive to dexamethasone

The relative insensitivity of memory CD4$^+$ T-cells to DEX in the CD2/CD28 system, might be due to a lower treshold of activation of these cells [4,5,7]. This idea is supported by the observation that memory cells produced significantly more IL-2 than the naive cells (respectively 58.4 ± 63.6 U/ml and 24.8 ± 37.1 U/ml; $n = 9$, $p < 0.01$; data not shown). In relation to this, endogenous levels of the DEX-sensitive transcription factor AP-1 may also be increased in memory cells which in turn would require higher DEX concentrations for an inhibitory effect. An alternative explanation might be that AP-1 plays only a minor role in this pathway of activation of memory CD4$^+$ T-cells [15]. As shown in Figure 3(A), lowering the concentration of anti-CD28 from 1000 to 111 ng/ml, at an optimal concentration of anti-CD2, hardly affected the proliferative response, whereas inhibition by 10^{-7} M DEX increased from 6 to 98 %. Decreasing the concentration of anti-CD2, at an optimal concentration of anti-CD28, slightly diminished the proliferative response in the absence of DEX, but again resulted in an almost complete inhibition by 10^{-7} M

DEX. Proliferative responses of naive CD4$^+$ T-cells were completely inhibited under all conditions shown in Fig. 3 (data not shown). That suboptimal stimulation conditions result in an increased DEX-sensitivity of memory CD4$^+$ T-cells is suggestive for a role for AP-1 in the alternative pathway of T cell activation. Possibly memory cells are more resistant as a consequence of higher endogenous levels of AP-1.

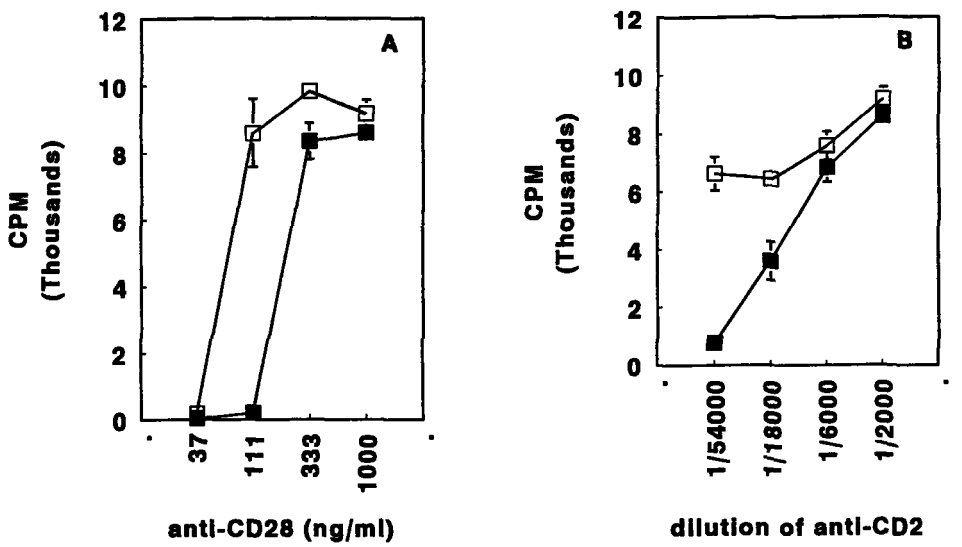

Figure 3. **Attenuation of CD2 or CD28 signaling leads to dexamethasone sensitivity of memory CD4$^+$ T cells.**
Memory CD4$^+$ T cells were activated with anti-CD2 in a dilution of 1:2000 and various concentrations of anti-CD28 (Figure 3A) or with 1000 ng/ml anti-CD28 and various concentrations of anti-CD2 (Figure 3B), both in the absence (□——□) and in the presence (■——■) of 10^{-7} M DEX. Representative titration curves out of three experiments with different donors are shown. [^{3}H]thymidine incorporation (mean cpm ± S.D.) was measured after 4 days of culture.

The sensitivity of naive CD4$^+$ T-cells for dexamethasone is abrogated by the activation of protein kinase C (PKC)

In contrast to memory CD4$^+$ T-cells, naive CD4$^+$ T-cells were highly sensitive to DEX when stimulated via CD2 and CD28, whereas combined triggering of these cells with anti-CD3 and anti-CD28 induced resistance to concentrations as high

as 10^{-6} M DEX (Fig. 1B). PKC activation is thought not to occur after perturbation of the CD28 molecule [16,17], but usually takes place when cells are activated by anti-CD3 [16,18]. Therefore, we investigated whether the sensitivity of naive CD4+ T-cells to DEX, stimulated by anti-CD2 and anti-CD28, could be abrogated by the activation of PKC through PMA.

As shown in Fig. 4, the addition of PMA resulted in a dose dependent co-stimulation of the proliferative response of naive CD4+ T-cells (open squares). Simultaneously, PMA was able to abrogate the DEX mediated inhibition of the response (filled squares). The mean inhibition declined from 99 % in the absence of PMA to 95, 29, and 0 % inhibition at 15, 30, and 60 pg/ml PMA, respectively (Fig. 4; open circles). These data suggest that - in contrast to memory CD4+ T-cells - endogenous levels of AP-1 are low in naive CD4+ T-

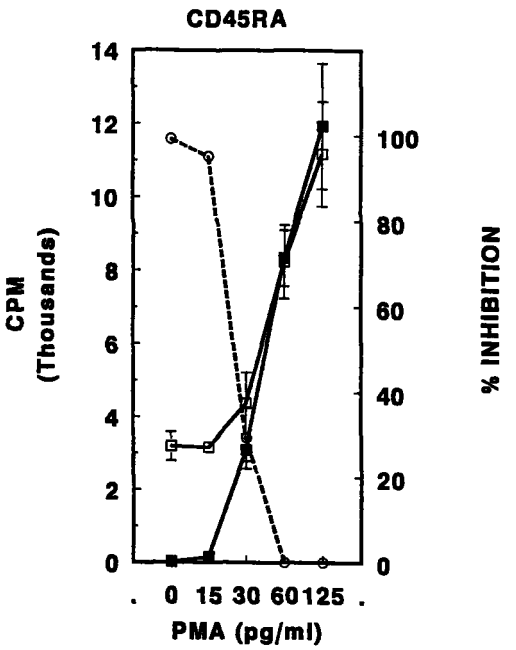

Figure 4. **PMA abrogates dexamethasone induced inhibition of naive CD4+ T cells.**

Naive CD4+ T cells were activated with the combination of anti-CD2 and anti-CD28, together with various concentrations of PMA, both in the absence (□——□) and in the presence (■——■) of 10^{-7} M DEX. [^{3}H]TdR incorporation (mean cpm ± SD) was measured after 4 days of culture. The dashed line shows the percentage inhibition by DEX. Representative titration curves out of three experiments with different donors are shown.

cells, and that these cells therefore also require the activation of PKC in order to become insensitive to DEX.

DISCUSSION

In the present study we show that naive $CD4^+$ T-cells are more sensitive to DEX than memory $CD4^+$ T-cells, but that this difference in sensitivity depends on the route of activation. DEX is thought to form a complex with a glucocorticoid receptor and this complex binds to the transcription factor AP-1 [19,20]. In turn AP-1 expression is an important intermediate of PKC induced T-cell activation [21]. Because AP-1 is involved in the transcription of the IL-2 gene, DEX inhibits IL-2 production [19,22] and as a consequence T cell proliferation.

Both naive and memory $CD4^+$ T-cells were sensitive to DEX when stimulated with anti-CD3, a route of activation which involves activation of PKC [16,18]. At suboptimal DEX concentrations, memory $CD4^+$ T-cells were less sensitive than naive $CD4^+$ T cells, which is in line with the fact that memory cells are more easily activated by anti-CD3 [5], possibly resulting in a stronger activation of PKC and higher levels of AP-1. In a previous study performed with unseparated $CD4^+$ T cells we showed that the alternative pathway of T cell activation through simultaneous triggering of CD2 and CD28 is largely insensitive to DEX [12], which would be consistent with the observation that this pathway is PKC-independent [16]. However, employing $CD4^+$ T cell subsets, we found in the CD2/CD28 pathway that only naive cells are higly sensitive to DEX. In an unseparated population of $CD4^+$ T cells, memory cells probably produce sufficient cytokines which allow naive $CD4^+$ T cells to escape from suppression by DEX. In the present study also exogenous IL-2 was capable to abrogate the suppressive effect of DEX (data not shown).

On the basis of the results obtained with naive cells it can be concluded that DEX-resistance is not an intrinsic property of the alternative pathway of $CD4^+$ T-cell-activation. As a matter of fact, we demonstrated that naive $CD4^+$ T-cells required both CD3-triggering and CD28-triggering to become insensitive to DEX. The effect of CD3-triggering in the induction of DEX-resistance is probably mediated through the activation of PKC and a subsequent increase of endogenous AP-1, since also PMA was capable of rendering the CD2/CD28 pathway resistant to DEX (Figure 4).

Memory cells stimulated with anti-CD2 and anti-CD28 may be more resistant to DEX for several reasons. First of all, it might be that memory CD4+ T-cells are relatively resistant in the CD2/CD28 pathway because these cells display an increased activity of PKC [23] which could be responsible for higher steady state levels of endogenous AP-1. Secondly, memory CD4+ T-cells may display a higher DEX resistance because of their increased capacity to produce both IL-2 and IL-4 [10]. Recently, Kam *et al.* showed that preincubation of T-cells with the combination of IL-2 and IL-4 reduces the affinity of the glucocorticoid receptor and T-cell response to GC [24]. Thirdly, because the CD28-pathway is not inhibited by PKC inhibitors like 1-alkyl-2-methyl glycerol (AMG) and staurosporin [16] DEX-resistance found when memory cells are triggered with CD28 may involve a mechanism different from PKC activation and AP-1 induction. Indeed it has been shown that activation via CD28 results into binding of members of the NF-κB family to a unique CD28RE in the IL-2 promoter [25,26], and possibly a different regulation of IL-2 gene transcription.
Altogether our data show, that naive cells are more susceptible to inhibition by DEX than memory cells. It is thus expected that the effects of GC *in vivo* will have a greater impact on primary immune responses than on secondary immune responses, which are dominated by the relative DEX-resistant memory CD4+ T-cell population. That indeed naive CD4+ T cells *in vivo* might be more sensitive to DEX, is illustrated by Chiapelli *et al.*, who showed an absolute and a relative decrease in the number of naive CD4+ T-cells after DEX administration to healthy human volunteers [27].

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CHAPTER 6

DEXAMETHASONE SENSITIVITY AND BCL-2 EXPRESSION OF LYMPHOCYTES IN MULTI-INFARCT DEMENTIA AND ALZHEIMER'S DISEASE

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ABSTRACT

Alzheimer's disease is accompanied by a disturbed hypothalamic-pituitary-adrenal axis which might have an effect on immune function. In the present study we investigated this by determining the sensitivity of peripheral blood mononuclear cells (PBMC) from patients with different forms of dementia with respect to dexamethasone (DEX). DEX-sensitivity of PBMC from multi-infarct dementia (MID) patients was higher than in patients with senile dementia of the Alzheimer type (SDAT) or controls, whereas PBMC from patients with clinical signs of both MID and SDAT (MIXED group) show an intermediate sensitivity. These differences could not be explained by quantitative differences in the expression of glucocorticoid receptors as measured by flowcytometry.
By contrast, the expression of bcl-2 was lower in PBMC from MID patients as compared to cells from SDAT patients or controls, whereas the MIXED group showed an intermediate expression; a low bcl-2 expression correlated with a high DEX-sensitivity. Finally, our observation that amyloid precursor protein (APP) was expressed in a lower density in PBMC of MID patients is in support for a role of both APP and bcl-2 in the pathogenesis of Alzheimers' disease and multi-infarct dementia.

INTRODUCTION

Several causes are thought to play a role in the development of dementia. Unlike in multi-infarct dementia (MID), the vascular system seems not to be etiologically involved in senile dementia of the Alzheimer type (SDAT). In this type of dementia it is assumed that alternative processing of amyloid precursor protein (APP) gives rise to the formation of β-amyloid (Hardy and Higgins, 1992) which is an important component of the so-called senile plaques. In addition to ß-amyloid an other neuropathological hallmark of SDAT is the presence of neurofibrillary tangles (Kidd, 1983; Glenner and Wong, 1984).
At present a definitive diagnosis SDAT can only be made post mortem since no biological markers are available, although progress is being made in this respect (Farlow et al., 1992; Van Nostrand et al., 1992). Such biological markers are of value to make a discrimination between MID and SDAT. Furthermore, such markers would be of importance to gain more insight into the etiology and

pathogenesis of these diseases.
Recent observations support the idea that characteristics of peripheral blood mononuclear cells (PBMC) may be useful in this regard. Patients with Alzheimer's disease (AD) are characterized by a disturbed hypothalamic-pituitary-adrenal axis, as apparent from the fact that these patients are less able to downregulate cortisol production after a dexamethasone (DEX) challenge (Parnetti et al., 1990; Balldin et al., 1983; Leake et al., 1990). Recently, we have demonstrated that such a diminished responsiveness to DEX can also be found with the use of PBMC (Nijhuis et al., 1994a).
In the present study we focussed on several markers on PBMC that are either shared with the central nervous system or that might play a role in the sensitivity of PBMC for DEX. We paid attention to APP, because this precursor protein of β-amyloid is also produced and expressed by PBMC, although its function is unknown (Monning et al., 1990). In addition to its role in the etiology of Alzheimer's disease, APP might be involved in MID since parts of APP have been found inhibitory for blood clotting factors IXa and XIa (Smith et al., 1990; Van Nostrand et al., 1990; Bush et al., 1990 ; Schmaier et al., 1993). Because glucocorticoids (GC) may exert their effect in part by the induction of apoptosis (Kabelitz et al., 1993; Cohen and Duke, 1992; Zubiaga et al., 1992), we evaluated the expression of bcl-2, a molecules that is involved in the protection against apoptosis (Korsmeyer, 1992), and as such may also be involved in the regulation of GC responsiveness.
These parameters were studied in a group of 43 consecutive patients entering a screening programme for cognitive impairment. Our data show that PBMC from patients with MID display a higher sensitivity to DEX and that the cells express lower levels of bcl-2 as compared to age-matched controls.

MATERIALS AND METHODS

Patients

Patients with senile dementia of the Alzheimer type (SDAT; n = 11; mean age 78 years, 5 male and 6 female), multi-infarct dementia (n = 7; mean age 81 years, 5 male and 2 female), or a combination thereof (n = 7; mean age 79 years, 5 male and 2 female) formed part of a group of 43 consecutive patients

with cognitive impairment, which entered a six week screening programme in a regional psychiatric hospital. Patients were diagnosed according to the DSM-III-R criteria and the criteria of the Dutch consensus meeting on the diagnosis of dementia (Dutch Consensus Development Conference: Diagnosis of the Dementia syndrome, 1988; Utrecht). In 18 patients miscellaneous causes for their cognitive impairment were established. The results from this group were excluded from the study. The mean age of the control group (n = 11; 5 male and 6 female) was 75 years.

Cell cultures

PBMC were obtained by Percoll (Biochrom KG, Berlin, Germany) density centrifugation (ρ = 1.077 g/cm^3) and cryopreserved in culture medium containing 20 % (v/v) Foetal Calf Serum (FCS, Seralab, Crawley Down, UK) and 10 % (v/v) DMSO, using a software-directed programme for the cryopreservation of lymphocytes as described elsewhere (Bom-van Noorloos et al., 1980). Per experiment, cell suspensions from an equal number of patients and controls were thawed by dropwise dilution (at least ten-fold) in ice-cold culture medium containing 20 % FCS. The preservation time of PBMC from all donors used in this study was less than one year. Viable cells ($\geq$ 80 %) were enumerated by trypan blue exclusion. In agreement with previous studies (Bom-van Noorloos et al., 1980), we found no changes in the phenotype of the cells after this procedure, with the exception of a slight decrease in the percentage of monocytes. All cultures were performed in Iscove's modified Dulbecco's medium (Biochrom KG) supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamin (2 mM) and β-mercaptoethanol (50 μM).
PBMC ($4x10^4$/well) were cultured in flat bottom microtiter plates (Costar, Cambridge, MA). Cells were stimulated with 0.5 μg/ml PHA (Murex, Dartford, UK) in the absence or in the presence of DEX (Sigma, St. Louis, MO). This PHA concentration, being optimal for proliferation of PBMC, but suboptimal for the induction of IL-2, allows a sensitive discrimination between individual patients and controls with respect to differences in DEX sensitivity of PBMC.
Cell proliferation was measured by adding 0.25 μCi methyl-tritium-thymidine ([^{3}H]TdR; specific activity 2 Ci/mmol; Radiochemical Centre, Amersham, UK) during the last 6 hours of culture. Cells were harvested onto glass fibre filter paper (Packard Instrument Company, Meriden, CT). Filters were counted using a

Matrix 96 β-counter (Packard Instrument Company). This procedure has an efficiency of about 20 % as compared to standard liquid scintillation counting.

IL-2 and IL-4 assays

To determine IL-2, cells were stimulated with an optimal concentration of PHA i.e. 10 μg/ml. For IL-4, cells were stimulated with the combination of anti-CD2 (CLB-T11.1/1 and CLB T11.2/1), each in a 1:2000 dilution of murine ascites and 2 μg/ml of purified antibody anti-CD28 (CLB-CD28/1). These antibodies were a generous gift of Dr. R.A.W. van Lier from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (Amsterdam, The Netherlands). All supernatants were harvested at day 3 and stored at -20 °C until assay.
IL-2 activity was assessed with the use of the CTLL-2 cell line. In the CTLL-2 bio-assay, five thousand cells were cultured in the presence of serially diluted supernatants. Human recombinant IL-2 (Cetus, Emeryville,CA) was used as a standard. During the last 4 hours of the 24 hour culture period cells were pulsed with 0.25 μCi [^{3}H]TdR. IL-4 levels in the supernatant were assessed using an ELISA technique as described elsewhere (Van der Pouw-Kraan, 1992) employing reagents kindly provided by Dr. van der Pouw-Kraan, CLB, Amsterdam, The Netherlands. Plates were read using a Biorad Microplate reader (Biorad, Richmond, CA). Human recombinant IL-4 was used as a standard (Genzyme, Cambridge, MA). The detection limit of the assay was 100 pg/ml.

Phenotypic analysis

For phenotypic analysis, $5x10^4$ cells were incubated with a saturating amount of FITC-, PE- or PercP- conjugated mAbs. Thereafter, cells were washed and analyzed on a FACScan (Becton & Dickinson, Mountain View, CA). Gates were set on the basis of forward and sideward scatter, including all viable leucocytes. Seven thousand gated events were analyzed for green and red fluorescence. To determine the expression of CD45RA or CD45RO within the $CD4^+$ T cell population, only viable cells stained with anti-CD4-PercP were included. The following reagents were used: 2H4-FITC (anti-CD45RA; Coulter, Hialeah, FL), and UCHL-1-FITC (anti-CD45RO; DAKO, Glostrup, Denmark). Anti-CD3-FITC,

anti-CD4-PE, anti-CD4-PercP, anti-CD8-PE and anti-CD14-FITC were purchased from Becton & Dickinson.

Expression of intracellular receptors

PBMC were fixed and permeabilized as described elsewhere (Aiello et al., 1992) with some modifications. Briefly, cells were washed twice with PBS to remove the FCS from the culture medium. Thereafter the cells were fixed (5 minutes, 4 °C) with 2 % paraformaldehyde in PBS. Subsequently, the cells were permeabilized by adding an equal volume of 0.4 % Triton X-100 for an additional 5 minutes. Finally, the cells were washed twice with PBS containing 0.5 % BSA. $5x10^5$ permeabilized PBMC were incubated with 1) 5 µg/ml mAb number 7, a mouse antibody crossreactive with the human glucocorticoid receptor (GCR ; Okret et al., 1984), kindly provided by Dr. A.C Wikström, Karolinska Institutet, Huddinge, Sweden , or 2) anti-bcl-2 (DAKO) or 3) 7.5 µg/ml anti-Alzheimer precursor protein A4 (Boehringer Mannheim GmbH, Germany). Staining occurred with Goat-anti-Mouse-FITC (CLB) as second step antibody. Double staining for $CD4^+$ T cells was performed with a CD4PercP conjugate (Becton & Dickinson) in 5 % (v/v) normal mouse serum. After each incubation step, cells were washed twice. Finally, 15,000 PBMC were analyzed on a FACScan (Becton & Dickinson). Irrelevant mouse IgG1 (for bcl-2 and APP) and IgG2a (for GCR) antibodies were used as isotype controls. Results for each individual patient or control are expressed as relative fluorescence intensity, i.e. mean fluorescence intensity found with the respective specific antibodies divided by the mean fluorescence intensity of the isotype control.

Statistics

Statistic analysis was performed using the two-tailed Mann-Whitney U-test. Differences were considered to be statistically significant at a confidence level of 95 % or higher ($p < 0.05$).

RESULTS

Increased sensitivity of T cell proliferation to dexamethasone in MID patients

As can be seen in Figure 1A, stimulation of PBMC with 0.5 μg/ml PHA resulted in a mean T cell proliferation that was comparable in SDAT patients and age-matched controls. Although there was a tendency towards a lower response in MID patients and patients with clinical signs of both MID and SDAT (further designated as MIXED) this appeared not significantly different. Since the peak of the proliferative response may vary from patient to patient between day 4 and 5 we compared data on both days. However, this did not influence the results.

When the sensitivity of PBMC for DEX was studied, a difference in sensitivity was found between the patients. As Figure 1B demonstrates, 10^{-7} M DEX caused 58 %, 41 % and 23 % inhibition of the PHA response in MID, MIXED, and SDAT, respectively. This was significantly higher (MID: $p < 0.005$ and MIXED: $p < 0.02$) than the inhibition of the responses found with PBMC from age-matched controls (9.1 % inhibition). Similar statistically significant results were obtained at day 4 or with 10^{-6} M DEX (data not shown).

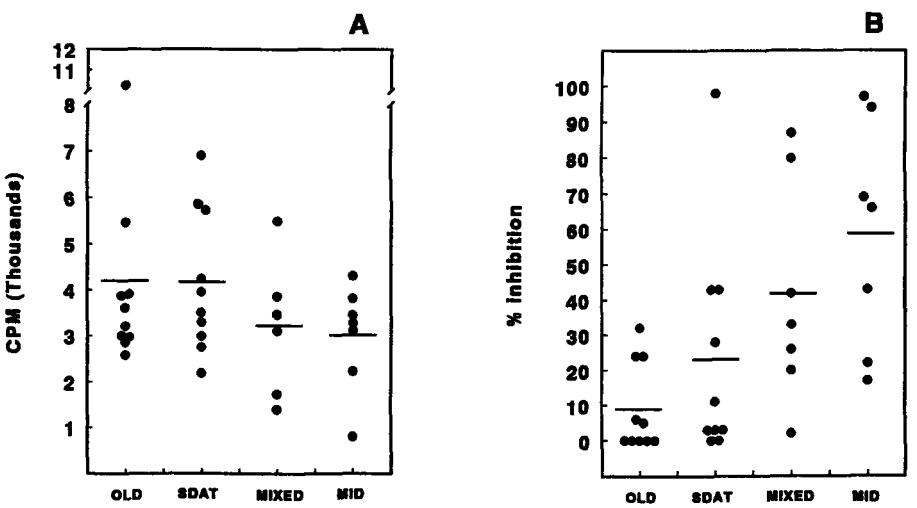

Figure 1. **Increased sensitivity of PBMC from MID patients to dexamethasone** PBMC were stimulated with 0.5 μg/ml PHA in the absence (A) and in the presence (B) of 10^{-7} M DEX. For each individual donor the response at day 5 is shown. MIXED refers to the patients with clinical signs of both MID and SDAT.

Increased sensitivity to dexamethasone in MID is not due to a different composition of the CD4$^+$ T cells or differences in the production of IL-2 and IL-4

Recently we demonstrated that memory CD4$^+$ T cells are less sensitive to DEX than naive CD4$^+$ T cells (Nijhuis et al., 1995). Moreover, we have shown that culture conditions which result into a high production of interleukins are less sensitive to DEX (Nijhuis et al., 1994b). Therefore, we investigated whether differences in DEX sensitivity of PBMC between patients with MID and controls were related to a different composition of the CD4$^+$ T cell subset or to a difference in the ability of PBMC to produce IL-2 and IL-4. As depicted in Table 1, the percentages of CD3$^+$, CD4$^+$ and CD8$^+$ T cells, within the mononuclear cell fraction, were comparable between the different groups. Moreover, no differences in the distribution of naive CD45RA$^+$ and memory CD45RO$^+$ cells within the CD4$^+$ T cell population were observed between patients and controls.

Table 1: Phenotype of T cells from controls and patients with different types of dementia

			% of PBMC*			% of CD4$^+$ T cells‡	
Subjects	mean# age	n	CD3	CD4	CD8	CD45RA	CD45RO
SDAT	78±7.8	10	45.7±11.7	28.4±9.2	19.1±11.5	49.9±14.0	53.9±16.1
MID	81±7.4	7	47.7±12.4	28.9±9.8	16.7± 8.8	41.9±14.3	59.6±13.2
MIXED	79±4.6	7	35.9±13.4	22.0± 11	15.7± 6.8	40.1±17.2	66.6±19.5
C'trols#	75±4.6	10	44.2± 8.8	30.7±7.1	16.1± 7.9	50.0±17.5	59.3±17.3

* Shown are the relative number of cells (mean ± SD) in a total PBMC population.
‡ The percentage of CD4$^+$ T cells expressing CD45RA or CD45RO was calculated after gating the CD4$^+$ (PercP-positive) cells.
\# mean age ± SD; C'trols = Controls

The inhibition of the synthesis of IL-2 and IL-4 is an important characteristic of DEX (Arya et al., 1984 ; Wu et al., 1991). IL-2 production was induced by PHA or by the combination of anti-CD2 and anti-CD28. Because PHA stimulation did not result into detectable IL-4 levels (data not shown), IL-4 production was studied only after stimulation with anti-CD2 and anti-CD28. As can be seen in

Figure 2, the mean IL-2 production for controls, SDAT, MID, and MIXED after PHA stimulation varied from 16 to 28 U/ml, and from 39 to 58 U/ml after simultaneous CD2 and CD28 perturbation. Despite the tendency towards a lower IL-2 production in the MID group, the difference with the controls failed to reach statistical significance ($p = 0.33$ and 0.63 for PHA and anti-CD2/CD28, respectively).

The mean IL-4 production in the different groups ranged from 500 to 700 pg/ml, without showing a significant difference. Therefore, it can not be concluded that a lower IL-2 or IL-4 production in the MID group was responsible for the stronger inhibition by DEX.

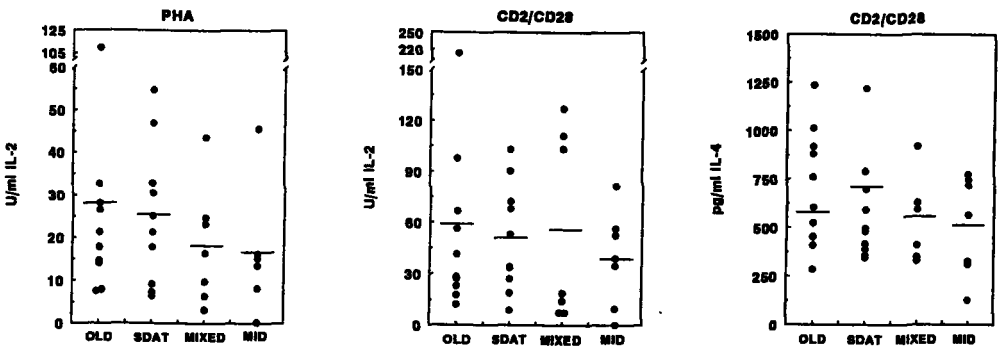

Figure 2. **Cytokine production by PBMC from patients with different types of dementia**
PBMC were stimulated with 10 μg/ml PHA (IL-2) or the combination of anti-CD2 and anti-CD28 (IL-2 and IL-4; see Material and Methods). For each individual donor the amount of interleukin in the supernatant at day 3 is shown. MIXED refers to the patients with clinical signs of both MID and SDAT.

Decreased expression of bcl-2 and APP, but not of the glucocorticoid receptor, in $CD4^+$ T cells from patients with MID

Recently, molecules have been identified that are thought to be involved in the complex process of apoptosis and cell survival. For instance β-amyloid, derived from APP, is a putative inducer of apoptosis in neuronal cells (Loo et al., 1993). Moreover, it has been suggested that DEX, through binding to its GCR, may be

involved in the induction of apoptosis in mature T cells (Zubiaga et al., 1992). Molecules like bcl-2 on the other hand, seem to play a crucial role in the protection of cells against apoptosis (Korsmeyer, 1992).

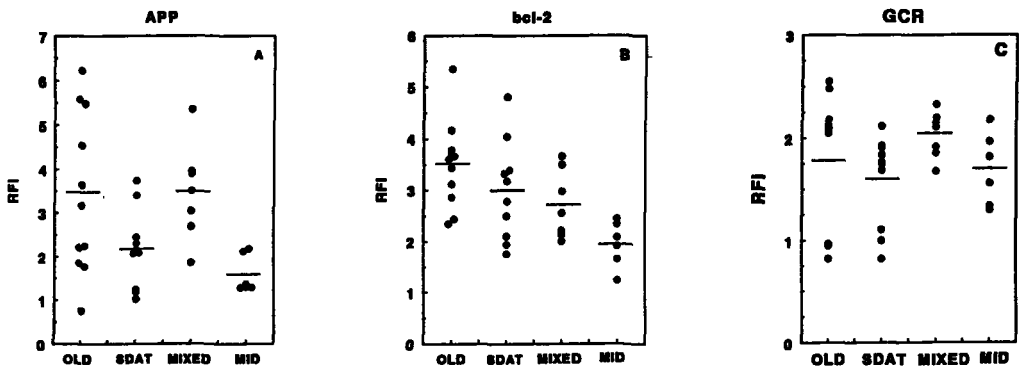

Figure 3. **PBMC from MID patients express lower levels of APP and bcl-2**
PBMC were fixed and permeabilized to detect the level of expression of APP, bcl-2 and GCR (see Material and Methods). The density is depicted as relative fluorescence intensity (RFI), i.e. the ratio between the mean fluorescence intensities found with the specific antibodies and an irrelevant isotype control antibody. MIXED refers to the patients with clinical signs of both MID and SDAT.

Therefore the observed differences in sensitivity of PBMC to DEX as shown in Fig. 1B might be due to differences in the expression of these molecules.
As can be seen in Figure 3A, the expression of APP on $CD4^+$ T cells from MID patients (mean RFI 1.6 ; $p < 0.05$), unlike that on $CD4^+$ T cells from SDAT patients (mean RFI 2.2 ; $p = 0.16$) or the MIXED group (mean RFI 3.5 ; $p = 0.87$), is lower than on the cells from age-matched controls (mean RFI 3.4). Moreover, as shown in Fig 3B, $CD4^+$ T cells from MID patients (mean RFI 2.0 ; $p < 0.005$), in contrast to $CD4^+$ T cells from SDAT (mean RFI 3.0) and MIXED patients (mean RFI 2.7), expressed lower levels of bcl-2 than $CD4^+$ T cells from controls (mean RFI 3.5). Bcl-2 expression in $CD4^+$ T cells from MID patients was also significantly lower than in SDAT patients (Fig 3B ; $p < 0.05$). Interestingly, $CD4^+$ T cells from patients in the MIXED group express bcl-2 at an intermediate level. By contrast, the GCR density was comparable between all groups tested (Figure 3C). These data therefore show that only $CD4^+$ T cells

from MID patients express lower levels of APP and bcl-2 than $CD4^+$ T cells from controls.

The extent of inhibition by DEX is negatively correlated with the level of bcl-2 expression

To evaluate whether the inhibition by DEX depends on the expression of the molecules discussed above, we investigated the correlation between the expression of bcl-2, APP and GCR and the extent of inhibition by DEX. This was done by including the data from all subjects tested (controls, SDAT, MID and MIXED; n =25). As shown in Figure 4, inhibition by DEX was negatively correlated with the expression of bcl-2 (Fig. 4B ; $p < 0.05$), i.e. a high bcl-2 expression is associated with DEX resistance. By contrast, no correlation was observed with the expression of GCR or APP (Fig. 4A and C). This suggests that a lower expression of bcl-2 contributes to the increased DEX sensitivity of PBMC from patients with MID as compared with controls, SDAT and MIXED patients.

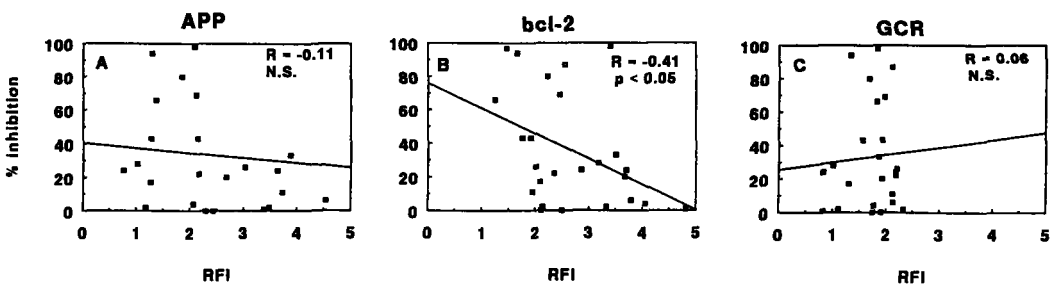

Figure 4. **Inhibition of proliferation by dexamethasone correlates with bcl-2 expression**
Inhibition of proliferation by DEX was plotted against the relative fluorescence intensities of bcl-2, APP and GCR, using data from Figure 1 and 3 from all four groups tested. Correlation coefficients and P values were obtained through linear regression.

DISCUSSION

In a previous study we demonstrated that PBMC from early onset AD patients display a diminished sensitivity to DEX in response to PHA (Nijhuis et al., 1994a). This confirmed *in vivo* studies that showed that cells from the pituitary gland in AD patients are also less sensitive to the action of DEX (Parnetti et al.,1990 ; Balldin et al., 1983; Leake et al., 1990).

The present study emphasizes the question, whether this phenomenon is restricted to PBMC from patients suffering from early onset AD or can be extended to PBMC from SDAT patients or patients with other forms of dementia. Our data show that PBMC from patients with MID or with symptoms of both AD and MID display and increased sensitivity to DEX as compared to age-matched controls, despite similar proliferative responses to PHA (Figure 1). The higher sensitivity for DEX, particular in MID, could not be explained by a difference in expression of the GCR between the several groups (Figure 3), although this does not exclude the possibility of an altered affinity of GCR in patients.

In contrast to our previous data obtained with early-onset AD patients, PBMC from patients with SDAT, did not show a different sensitivity to DEX as compared to controls. Perhaps this reflects a difference between the familial cases of AD (which reside more in the early-onset group) and sporadic cases, because SDAT tends to be non-familial (Van Duijn, 1992). Furthermore, the results may be influenced by the differences in diagnostic procedures used in these two studies, although both methods are widely accepted. Finally, we cannot exclude the possibility that the difference in mean age of the age-matched controls for both AD groups being 70 years in the early onset group and 75 years in this study of SDAT patients is explanatory, for instance when DEX-resistance develops during aging.

Our data show that PBMC from patients with MID and MIXED display an increased sensitivity to DEX as compared to age-matched controls, despite similar proliferative responses to PHA (Figure 1). Again no quantitative differences in GCR expression were found although a qualitative difference can not be excluded.

Daynes and Araneo (1989) showed for the murine system that DEX can enhance IL-4 production, while inhibiting IL-2 production, suggesting a differential effect of DEX on IL-2 and IL-4 producing cells. In the human system memory $CD4^+$ cells produce a variety of cytokines, including IL-4, whereas

naive $CD4^+$ T cells are restricted to the production of IL-2 (Salmon et al., 1989). We have previously shown that memory $CD4^+$ T cells display a lower sensitivity for DEX than naive $CD4^+$ T cells (Nijhuis et al. 1995). We therefore examined the possibility that differences in DEX sensitivity between the different groups of dementia as compared to controls, were due to a difference in the composition of the $CD4^+$ T cell compartment or the production of IL-2 and IL-4. However, no significant differences were found with respect to these parameters.

One of the mechanisms by which DEX exerts its effect on PBMC might be by the induction of apoptosis (Kabelitz et al., 1993; Cohen and Duke, 1992; Zubiaga et al., 1992). Recently, data have become available showing that several molecules are involved in the protection against apoptosis, e.g. bcl-2 (Korsmeyer, 1992) . Moreover, it has been suggested that β-amyloid the presence of which in senile plaques forms one of the neuropathological hallmarks of AD, plays a role in the apoptotic process (Loo et al., 1993).

In the present study we demonstrated that $CD4^+$ T cells from MID patients express less bcl-2 than cells from control donors. Because bcl-2 expression showed a negative correlation with the extent of inhibition of PHA induced proliferation by DEX (Figure 4), it is likely that the increased sensitivity of PBMC in MID to DEX was indeed due to a lower bcl-2 expression.

Altogether, this study indicates that the DEX sensitivity of PBMC is increased in MID patients and that this might be causally related with the level of expression of the bcl-2 protein. If these findings reflect a lower expression of bcl-2 on neuronal cells, this could be responsible for a higher susceptibility of these cells for apoptosis and as a consequence increased cell death. Our finding that the expression of APP in PBMC of MID patients is decreased is of interest because parts of APP are natural inhibitors of blood clotting factors IXa and XIa (Smith et al., 1990; Van Nostrand et al., 1990; Bush et al., 1990; Schmaier et al., 1993). A lower APP expression might therefore contribute to the development of MID through a lack of blood clotting inhibition.

Our studies have shown that PBMC may be useful tools to study the pathogenesis of dementia, with regard to structures shared with the CNS. Further studies on PBMC from patients that suffer from different types of dementia, with respect to the regulation of bcl-2 and APP, may provide information that contributes to a better understanding of the pathogenesis of the multi-infarct dementia and senile dementia of the Alzheimer type.

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CHAPTER 7

GENERAL DISCUSSION

GENERAL DISCUSSION

The objective of the study presented in this thesis was to approach Alzheimer's disease (AD) research from an immunological point of view. This was based on several considerations. Firstly, a causal relationship between the classical neuropathological hallmarks *i.e.* β-amyloid containing plaques as well as the neurofibrillary tangles and the development of AD, is still not firmly established. This is illustrated by the lack of discriminating biochemical diagnostic criteria and the use of consensus criteria with respect to the clinical presentation of the disease. Secondly, bidirectional interactions between the central nervous system (CNS) and the immune system have been extensively described since the early seventies.

Indeed, circumstantial evidence for impairment of the immune system in AD has been published over the years. Whether these are epiphenomena of the CNS pathology or representative of a general cell-biological defect that contributes to the pathogenesis of AD remains to be established. Eikelenboom *et al.* for instance suggest that the sequence of inflammatory events in the brain of Alzheimer patients may all take place within the CNS following brain injury or other pathogenic insults without involvement of cells from the immune system [1]. On the other hand, the identification of the Amyloid Precursor Protein (APP), of which β-amyloid is derived by processing, as a ubiquitous cell-membrane protein broadened the view on the pathogenesis of AD [2].

Observations concerning an impairment of the Hypothalamic Pituitary Adrenal (HPA) axis in AD led to the experiments presented in Chapter 2 [3-5]. The HPA axis is a clear example of how the CNS can influence the immune system, namely through the production and release of neuroendocrine hormones. One of these, *i.e.* ACTH induces the release of glucocortiocoids (GC) of which the immunosuppressive properties are well documented. In recent years however it is becoming increasingly clear that these hormones play an important role in maintaining or altering, the homeostatic balance of the immune system [6].

To examine 'HPA-mediated' changes in the immune system an experimental approach was followed in which peripheral blood mononuclear cells (PBMC) from early-onset AD patients were stimulated with phytohaemagglutinin (PHA) *in vitro* and exposed to the synthetic GC dexamethasone (DEX). It appeared that PBMC from early-onset AD patients were less sensitive to DEX than PBMC from age-matched controls (Chapter 2). This observation is in line with the observation that AD patients are low responders in the dexamethasone suppres-

sion test, and is therefore indicative of a general cell biological phenomenon. The lower sensitivity to DEX could not be explained by a lower expression of GCR as measured with a monoclonal antibody. The possibility that the GCR have a lower affinity in AD patients was not investigated. However, several studies indicate that stress related hormones are involved in the switch from a T_H1-like (IL-2 dominated) into a T_H2-like (IL-4 dominated) immune response [6]. Daynes and Araneo showed in mice that DEX enhances IL-4 production whereas it inhibits IL-2 production [7]. Moreover, it has been found that IL-4 may render cells insensitive to DEX [8]. The importance of giving priority to the study of the relation between DEX sensitivity and cytokine production became evident from studies in which we evaluated immunological changes during human ageing (Chapter 3).

Young and elderly human donors were selected according to the SENIEUR protocol to minimize the effect of underlying disease on the immune parameters under investigation, *i.e.* phenotype of the $CD4^+$ T-cell population, proliferation and interleukin production by PBMC. These studies showed an age related increase of $CD4^+$ memory T-cells, on the basis of the expression of surface membrane molecules that are generally accepted as being associated with T-cell memory, namely the presence of CD45R0, and the absence of CD45RA and CD27 [9]. This acquisition of a memory phenotype with age was accompanied by a significant increase of IL-4 production by PBMC from elderly donors.

Because of the age-related increase in IL-4 production and lack of information with regard to IL-4 production in AD patients, which could even be higher than in controls, we investigated the IL-2 and IL-4 production of PBMC from AD patients. Furthermore, we assessed the phenotype of $CD4^+$ T-cells. However, no differences between AD and controls were observed with respect to these parameters. Interestingly, the controls showed, as expected a significant negative correlation was found in controls between the amount of interleukins produced and the extent of inhibiton by DEX, whereas no such correlation was observed in early-onset AD (Chapter 2). These data suggested that in AD patients the decreased DEX-sensitivity was not due to a change in the composition and functional characteristics of the T-cells. This lack of correlation between DEX-sensitivity and immunological parameters in AD patients prompted us to study the mechanism of DEX mediated suppression in more detail in normal donors.

First, the suppressive effect of DEX was investigated in unseparated $CD4^+$ T-cells using different activation pathways (Chapter 4). It was demonstrated that

DEX inhibits both IL-2 and IL-4 production. The inhibitory effect on IL-2 is in accordance with the literature data [10]. Also the inhibitory effect on IL-4 production is a confirmation of recently published data by Wu et al. [11]. These data are however not in agreement with murine studies which showed that IL-2 production is inhibited by DEX, whereas IL-4 production is stimulated [7]. Differences in experimental approach might be responsible for the discrepancy: Daynes et al. used T-cell clones whereas the human studies employed fresh isolated $CD4^+$ T-cells.

Although no differential effects on IL-2 or IL-4 were found, it became evident that different activation pathways were not equally sensitive to DEX (Chapter 4). Activation routes that were protein kinase C (PKC) dependent were highly sensitive to DEX, which is in line with studies showing that DEX acts via interference with the transcription factor AP-1 [12,13]. It was therefore not unexpected to find that activation of $CD4^+$ T-cells via a PKC independent pathway, namely via CD2 and CD28 was largely insensitive to DEX. This suggests that the induction of IL-2 gene expression through CD2 and CD28 is less dependent on AP-1. Data that sustain this view have been published by Lu et al. who showed that for IL-2 gene expression induced by CD28 triggering AP-1 binding activity is not essential [14]. Alternatively, other factors than IL-2 are involved in the abrogation of DEX induced inhibition. It can be concluded that the sensitivity of $CD4^+$ T-cells to DEX depends on their activation stage and the activation pathways that are involved.

The observation that the activation state of $CD4^+$ T-cells determines their sensitivity to DEX was substantiated by our studies on naive and memory $CD4^+$ T-cells (Chapter 5). The rationale for this approach is the fact that naive and memory $CD4^+$ T-cells have different activation requirements [15-17], probably because of a higher expression level of several cell adhesion molecules. In agreement with the studies described for total $CD4^+$ T-cells, naive $CD4^+$ T-cells, having a relatively low activation state, could be rendered insensitive by accessory signals such as anti-CD28, or additional PKC activation. In support of the idea presented in this thesis that memory cells are more resistant to DEX as a consequence of a higher activation state is the observation of a higher PKC expression in memory $CD4^+$ T-cells [18]. Also of support is the finding that memory $CD4^+$ T-cells produce both IL-2 and IL-4, which synergistically reduce the glucocorticoid receptor (GCR) binding affinity in T-cells, and consequently the ability of GC to be inhibitory for proliferation [8]. Altogether, the data from Chapters 5 and 6 illustrate that the action of DEX on $CD4^+$ T-cells is dependent

on the pathway of activation as well as the type of $CD4^+$ T-cell under investigation.

The *in vivo* implications of these observations might be that GC have a greater impact on primary immune responses mediated by naive $CD4^+$ cells than on secondary immune responses which are dominated by memory T-cells. Moreover, GC might be involved in directing an immune response towards a T_H2 like response since IL-4 reduces GCR binding affinity and as a consequence the T-cell response to GC [8]. IL-4 producing cells, i.e. pre-existing T_H0 and T_H2 cells are therefore likely to have a growth advantage over T_H1 cells. Since IL-4 promotes T_H2 development from naive cells [19], a T_H2 like immune response would be indirectly favoured by GC. Consequently, disease states that are predominately T_H2-like might benefit to a lesser extent from immunosuppressive therapy by GC. Depending on the state of activation of the cells, aggravation of the disease state might even be possible. That this indeed might be the case is illustrated by Kam et al. who described a steroid resistant subset of asthmatic patients [8].

On the basis of these data concerning the mechanism of action of DEX it was expected that our early observations concerning a lower sensitivity of PBMC of AD patients (Chapter 2) could be explained by differences in composition of the $CD4^+$ T-cell population and cytokines produced. This was, however, not the case. Almawi et al. have demonstrated that IL-1, IL-6 and IFN-γ -cytokines that may derive from a variety of cells- can render PBMC resistant to the suppressive effects of DEX [20]. The possibility that PBMC from AD patients produce higher amounts of these factors, and are therefore more resistant to DEX, was not investigated. At least for IL-6 it has been found that there is no increase in plasma levels in AD [21].

Apart from the fact that GC inhibits IL-2 gene transcription, another possible effect of GC is the induction of apoptosis. Zubiaga *et al.* showed that DEX induced apoptosis occurs in mature T-cells, apart from its well-known apoptotic effect on thymocytes [22,23]. In relation to this thesis it was important to evaluate the expression of bcl-2. Bcl-2 is a cytoplasmic protein, of which the expression level correlates positively with cell survival [24]. Because the expression of bcl-2 might also determine effects mediated by GC, studies were performed to establish the role of bcl-2 in Alzheimer's disease.

In addition the observed differences in DEX sensitivity, as described in Chapter 2, could be related to a difference in the expression of CD27, a structure that has also been shown to play a role in the protection against apoptosis. These

studies were performed in a group of patients that comprised AD patients, multi-infarct dementia patients and those with symptoms of both AD and MID, in an attempt to segregate the different types of dementia with respect to the abovementioned markers (Chapter 6). An important discrepancy with the results described in Chapter 2 was that in this study, no differences in DEX sensitivity were observed between this group of AD patients and the controls. In Chapter 7, the possible reasons for this discrepancy were discussed extensively. The results obtained in this second study however, revealed several interesting new aspects concerning dementia and the relation to the immune system. The MID population appeared to be very distinct with respect to DEX sensitivity and bcl-2 expression: MID patients were very sensitive to the action of DEX and expressed significantly less bcl-2 protein than controls, or patients from the AD group. A significant correlation between the percentage of inhibition and the level of bcl-2 expression, suggested a causal relationship between these two findings. Interestingly, and supporting the differences between MID and controls as well as AD, the group consisting of patients with symptoms of both MID and AD expressed bcl-2 at an intermediate level.

Altogether, this thesis provides evidence for a role for GC in the regulation of the immune response with emphasis on the differential effects on distinct $CD4^+$ T-cell activation pathways and on different subsets of $CD4^+$ T-cells. Since GC concentrations are regulated by a neuroendocrine feedback system (the HPA axis), GC may act as an intermediate between the CNS and the immune system. Since in AD it has been shown that the HPA axis is disturbed, these data support the hypothesis that alterations in the neuroendocrine homeostatic balances might lead to altered properties of cells from the immune system and change the course of an immune response. In AD both the pituitary gland and PBMC are less sensitive to DEX, which points to a more general cell-biological phenomenon. Perhaps a diminished sensitivity of cells in the CNS to DEX reflects a relative loss of GC mediated physiological control of protein synthesis in neurons. Data with respect to GC control of protein synthesis in neurons that would support this possibility, are at present however limited [reviewed in 25]. In addition to an increased sensitivity to DEX in MID, PBMC from these patients display a decreased expression of bcl-2. It might therefore be that regulation of bcl-2 expression governs the responsiveness of lymphocytes, but also neurons to GC.

In conclusion, it has been demonstrated in this study that PBMC are a useful and easy accessible tool in AD and MID orientated research as it provides information concerning the interactions between the CNS and the immune

system. Moreover, general cell biological defects occurring in AD and MID may already be investigated during life. Further studies on PBMC may thus accelerate the development of diagnostic and therapeutic approaches to dementia.

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SUMMARY

This thesis deals with the regulatory properties of glucocorticoids (GC) on $CD4^+$ T cells and the possible implications thereof in disorders characterised by cognitive impairment, i.e. Alzheimer's disease (AD) and multi-infarct dementia. These items are dealt with in the context of age-related changes of the immune system.

Starting with a general introduction on the functioning of the immune system, in particular the $CD4^+$ subset thereof, the age-related changes in the immune system are briefly reviewed in Chapter 1. The second part of Chapter 1 describes the bidirectional interactions between the immune system and the central nervous system.

By means of the dexamethasone suppression test it has been demonstrated *in vivo*, that in AD patients the pituitary gland is less sensitive to the synthetic glucocorticoid dexamethasone (DEX). Chapter 2 shows that peripheral blood mononuclear cells (PBMC) from these patients, when cultured *in vitro* using PHA as mitogen, are also less sensitive to DEX. In the recent years an increasing body of evidence has become available showing that GC may play a regulatory role in the immune response by differential effects on T_H1 and T_H2 cells. Therefore it was evaluated whether the decreased sensitivity to DEX in AD patients was related to differences in production by PBMC of IL-2 (a T_{H1} type interleukin) and IL-4 (a T_H2 type interleukin) or the composition of the $CD4^+$ T cell subset i.e. $CD45RA^+$ (naive $CD4^+$ T cells, IL-2 producing) versus $CD45RO^+$ (memory $CD4^+$ T cells, IL-2 and IL-4 producing). However, no differences between patients and age-matched controls were observed. Unexpectedly, since inhibition of IL-2 production is one of the prime effects of DEX, in AD patients DEX senstivity was not correlated with IL-2 or IL-4 synthesis. Furthermore the level of glucocorticoid receptor (GCR) expression was also comparable between patients and controls. It is suggested that other interleukins than IL-2 and IL-4 or a different affinity of the GCR are explanatory for the observed differences.

The relevance of paying attention to DEX sensitivity and interleukin production is illustrated by data in Chapter 3 were age-related changes in phenotype and interleukin production of PBMC is described. The data show that the age related increase of the memory $CD4^+$ T cell population, based on the relative increase of CD45RO and decrease of CD27 expression, is accompanied with an increase in IL-4 production. In contrast to several other studies an age-related decrease in IL-2 production was not observed, probably as a result of using the SENIEUR

protocol to select young and aged donors, which minimizes the effect of underlying disease on immune parameters.

To obtain more insight in the immunoregulatory properties of DEX, studies were performed in normal donors. In Chapter 4, using unseparated $CD4^+$ T cells, it is shown that the immunosuppressive action of DEX is dependent on the activation state of $CD4^+$ T cells as well as the activation pathway that is used. PKC dependent activation pathways were highly sensitive to DEX but could be rendered insensitive by additional PKC stimulation through PMA, a direct PKC activator. CD28 mediated activation of $CD4^+$ T cells appeared to be largely resistant to the action of DEX. These observations are compatible with the mechanism of action of DEX. It primarily interferes with the transcription factor AP-1, which is an important factor in PKC induced T cell activation. Furthermore, AP-1 expression does not seem to be essential in CD28 mediated $CD4^+$ T cel activation. Interestingly, at supraoptimal PKC stimulation DEX exhibited a stimulatory effect on $CD4^+$ T cell proliferation. When $CD4^+$ T cells are separated in a naive ($CD45RA^+$) and memory ($CD45RO^+$) $CD4^+$ T cell subset it appears that these subsets display a different sensitivity to DEX (Chapter 5). Naive $CD4^+$ T cells are far more sensitive to DEX mediated inhibition than memory $CD4^+$ T cells. This was true for PKC mediated T cell activation (via immobilized anti-CD3) as well as CD28 mediated T cell activation (via combined perturbation of CD2 and CD28). When both activation routes were combined (via CD3 and CD28 triggering or PMA and CD28 triggering), the effects of DEX were largely overcome. The data from Chapter 4 and 5 illustrate that at least two mechanisms contribute to DEX resistance, namely the magnitude of PKC activation of $CD4^+$ T cells and the relative contribution of CD28 triggering to activation of $CD4^+$ T cells. Probably in memory $CD4^+$ T cells these two mechanisms are more effectively triggered than in naive cells.

The results from Chapters 4 and 5 did not contribute to a further explanation of the observed decreased sensitivity to DEX in AD patients. Therefore, it was studied whether molecules that may protect against the action of DEX were differentially expressed. In this respect the expression of the cytoplasmic protein bcl-2, which protects the cell against apoptosis, is of importance. In an attempt to segregate AD from multi-infarct dementia (MID) these two different types of dementia were compared (Chapter 6). It appeared that PBMC from MID patients were very sensitive to DEX mediated suppression and displayed a lower bcl-2 expression. A significant correlation between the percentage of inhibition and expression of bcl-2 suggested a causal relation. Moreover a group of patients

with clinical symptoms of both AD and MID displayed intermediate levels of bcl-2 expression.

The data presented in this thesis are discussed in Chapter 7. Since GC act as an intermediate between the CNS and the immune system, disruption of the neuroendocrine homeostatic balance may lead to altered properties of different elements of the immune system. It is hypothesised that the altered properties of the PBMC in dementia disorders (Chapters 2 and 6) may reflect comparable alterations in neurons: a decreased DEX sensitivity in neurons could result into a relative loss of GC mediated control of protein synthesis, and thus play a role in neuropathology.

SAMENVATTING

Dit proefschrift behandelt de regulerende eigenschappen van glucocorticoiden (GC) met betrekking tot $CD4^+$ T cellen en de mogelijke implicaties daarvan op het immuunsystem in ziekten, die worden gekenmerkt door cognitieve stoornissen (Ziekte van Alzheimer en muli-infarct dementie). Deze onderwerpen worden behandeld in de context van leeftijds gerelateerde veranderingen in het immuunsysteem.

Na een algemene inleiding over het functioneren van het immuunsysteem, met name de $CD4^+$ T cellen, wordt er een kort overzicht gegeven van de leeftijds gerelateerde veranderingen in het immuunsysteem. Het tweede gedeelte van hoofdstuk 1 beschrijft de interacties tussen het immuunsysteem en het centraal zenuwstelsel.

In vivo studies, waarbij gebruikt gemaakt is van de dexamethason suppressie test, hebben laten zien dat in AD patiënten de hypofyse minder gevoelig is voor het synthetische glucocorticoid dexamethason (DEX). Hoofdstuk 2 laat zien dat perifeer bloed mononucleaire cellen (PBMC) van deze patiënten, indien *in vitro* gestimuleerd met PHA, ook minder gevoelig zijn voor DEX. In de afgelopen jaren zijn er meer en meer gegevens beschikbaar gekomen, die wijzen op een regulatoire rol van GC in de immuunrespons en mogelijk een differentiëel effect op T_H1 en T_H2 cellen. Daarom werd onderzocht of de verminderde gevoeligheid voor DEX in patiënten met AD gerelateerd was aan verschillen in produktie door PBMC van IL-2 (een T_H1 type interleukine) en IL-4 (a T_H2 type interleukine). Echter, er werden geen verschillen tussen patiënten en leeftijdscontroles gevonden. Onverwacht, omdat remming van IL-2 productie een van de belangrijkste kenmerken van DEX gemedieerde immuunsuppressie is, was er geen verband tussen de DEX gevoeligheid en produktie van deze interleukinen in patiënten met AD. Bovendien, de mate van glucocorticoid receptor (GCR) expressie was ook vergelijkbaar tussen patiënten en controles. Mogelijk vormen de betrokkenheid van andere interleukinen en /of een verminderde affiniteit van de GCR een verklaring voor de gevonden verschillen.

Het belang om aandacht te schenken aan DEX gevoeligheid in relatie tot interleukine produktie wordt onderstreept door gegevens uit hoofdstuk 3, waar leeftijdsgerelateerde veranderingen in fenotype en interleukine produktie van PBMC worden beschreven. De experimenten laten zien dat de leeftijds-gerelateerde toename van de memory $CD4^+$ T populatie, gebaseerd op de relatieve toename van CD45R0 en afname van CD27 expressie, vergezeld gaat

van een toename in IL-4 produktie. In tegenstelling tot enkele andere studies werd er geen leeftijdsgerelateerde afname in IL-2 produktie gezien. Dit wordt waarschijnlijk veroorzaakt door het gebruik van het SENIEUR protocol voor de selectie van jonge en oude donoren, welke het effect van onderliggende ziekten op de immuun parameters minimaliseert.
Om meer inzicht te verkrijgen in de immuunregulerende eigenschappen van DEX werden studies uitgevoerd met PBMC van gezonde jonge donoren. In hoofdstuk 4, gebruik makend van ongescheiden $CD4^+$ T cellen, wordt getoond dat de immuunsuppressieve werking van DEX zowel afhankelijk is van de activatie-toestand van $CD4^+$ T cellen als van de route van activatie. PKC afhankelijke activatieroutes bleken zeer gevoelig voor DEX maar konden ongevoelig gemaakt worden door extra PKC stimulatie met PMA, een directe PKC activator. CD28 afhankelijke $CD4^+$ T celactivatie bleek grotendeels resistent voor de werking van DEX. Deze bevindingen zijn in overeenstemming met het werkingsmechanisme van DEX. Het interfereert primair met de nucleaire transcriptiefactor AP-1, welke een belangrijke factor is in PKC geinduceerde T cell activatie. Bovendien lijkt AP-1 expressie niet van wezenlijk belang voor CD28 gemediëerde $CD4^+$ T cell activatie. Als $CD4^+$ T cellen worden gescheiden in naieve ($CD45RA^+$) en memory ($CD45RO^+$) $CD4^+$ T cellen, blijkt dat deze twee subsets een verschillende gevoeligheid voor DEX hebben (Hoofdstuk 5). Naieve $CD4^+$ T cellen zijn veel gevoeliger for DEX dan memory $CD4^+$ T cellen. Dit geldt zowel voor PKC gemediëerde T cel activatie (via geïmmobiliseerd anti-CD3) als CD28 gemediëerde T cell activatie (via gecombineerde stimulatie van CD2 en CD28). Als de twee activatie routes werden gecombineerd (via CD3 en CD28 stimulatie dan wel PMA en CD28 stimulatie), dan worden de effecten van DEX grotendeels teniet worden gedaan. De experimenten uit hoofdstukken 4 en 5 illustreren dat ten minste twee mechanismen bijdragen aan DEX resistentie, namelijk de mate van PKC activatie en de relatieve bijdrage van CD28 stimulatie aan de activatie van $CD4^+$ T cellen. Waarschijnlijk worden beide activatie routes in memory $CD4^+$ T cellen effectiever aangesproken dan in naieve $CD4^+$ T cellen.
De resultaten uit hoofdstukken 4 en 5 boden geen verdere verklaring voor de eerder gevonden verminderde gevoeligheid voor DEX van PBMC van patiënten met AD. Daarom werd de expressie van moleculen, welke de cel zouden kunnen beschermen tegen de werking van DEX, bestudeerd. Hierin speelt de expressie van het cytoplasmatische eiwit bcl-2, dat de cel beschermt tegen apoptosis, een belangrijke rol. In een poging tot differentiatie werden PBMC van patiënten met

dementie van verschillende etilogie bestudeerd. PBMC van patiënten met MID bleken verhoogd gevoelig voor DEX gemediëerde suppressie. PBMC van deze patiëntengroep brachten ook het eiwit bcl-2 lager tot expressie. Een significante correlatie tussen de percentages remming door DEX en het niveau van bcl-2 expressie suggereerde een causaal verband. Bovendien, de patiëntengroep met klinische symptomen van AD zowel als MID bracht bcl-2 op een intermediair niveau tot expressie.

De experimenten beschreven in dit proefschrift worden geëvalueerd in hoofstuk 7. Omdat GC werkzaam zijn als een intermediair tussen het CZS en het immuunsysteem, leidt een verstoring van de neuroendocriene homeostatische balans mogelijk tot veranderde eigenschappen van de verschillende elementen van het immuunsystem. Tot slot wordt de hypothese geformuleerd dat veranderde eigenschappen van PBMC in ziekten gepaard gaande met dementie, wellicht vergelijkbare veranderingen in cellen van het CZS reflecteren: een verminderde gevoeligheid voor DEX in neuronen zou kunnen betekenen dat er een afname van GC gemediëerde regulatie van eiwit synthese optreedt, hetgeen mogelijk een rol speelt in het ontstaan van neuropathologie.

Abbreviations

ACTH	Adrenocorticotropic hormone
AD	Alzheimer's disease
AMG	1-Alkyl-2-methyl-glycerol
APC	Antigen presenting cell
APP	Amyloid precursor protein
CD	Cluster of differentiation
CNS	Central nervous system
cpm	counts per minute
CsA	Cyclosporine A
DAG	Diacylglycerol
DEX	Dexamethasone
DMSO	Dimethylsulfoxide
FACS	Fluorescence activated cell sorter
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
GC	Glucocorticoids
GCR	Glucocorticoid receptor
GGFP	Glial growth promoting factor
GMF	Glial maturation factor
GSF	Glial stimulating factor
GTP	Guanosinetriphosphate
HPA	Hypothalamic pituitary adrenal
IFN	Interferon
IL	Interleukin
IL-2R	Interleukin-2 receptor
IP-3	Inositoltriphosphate
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
MID	Multi infarct dementia
MS	Multiple sclerosis
NK	Natural killer
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PE	Phycoerythrin
PercP	Peridinin-chlorophyll-protein
PHA	Phytohaemagglutinin
PIP-2	Phosphatidylinositolbiphosphate
PKC	Protein kinase C
PMA	Phorbol-12-myristate-13-acetate
PLC	Phospholipase C
PTK	Protein tyrosine kinase
RFI	Relative fluorescence intensity
SD	Standard deviation
SDAT	Senile dementia of the Alzheimer type
TCR	T cell receptor
T_H	T helper
U	Unit
VIP	Vasoactive intestinal peptide

CURRICULUM VITAE

De schrijver van dit proefschrift werd op 19 juni 1960 geboren te Den Haag. In Castricum werd op 23 juni 1978 het VWO diploma in ontvangst genomen op het Bonhoeffer College.

In september van dat jaar werd de studie Scheikunde begonnen aan de Universiteit van Amsterdam, alwaar op 24 juni 1981 het kandidaatsexamen behaald werd. Later dat jaar werd, na drie keer te zijn uitgeloot, alsnog de studie Geneeskunde aangevangen aan de Universiteit van Amsterdam. Tijdens deze studie was hij van 1984 tot 1987 kandidaatsassitent bij de vakgroep Biochemie van dezelfde Universiteit. Het doktoraal-, en artsexamen werden respectievelijk afgelegd op 4 juli 1986 en 22 februari 1989.

Van juni tot en met december 1989 was hij werkzaam als doktoraalassistent bij het Centraal Laboratorium voor de Bloedtransfusiedienst in Amsterdam onder begeleiding van Dr. R.A.W. van Lier.

Van 1 januari 1990 tot en met 31 december 1993 was hij verbonden als wetenschappelijk medewerker aan de afdeling Immunologie van het Instituut voor Experimentele Gerontologie-TNO in Rijswijk (hoofd: Dr. J. Rozing) wat later fuseerde met een tweetal andere TNO instituten tot het Instituut voor Verouderings- en Vaatziekten Onderzoek, gevestigd te Leiden (algemeen directeur: Prof. dr. D. Knook). Onder supervisie van Dr. L. Nagelkerken werd dit proefschrift bewerkt.

Vanaf 1 februari 1994 is hij in opleiding tot patholoog aan de Universiteit van Amsterdam (hoofd: Prof. dr. J.J. Weening). In het kader daarvan is hij vanaf 1 april 1995 gedurende twee jaar werkzaam in het Streeklaboratorium voor de Pathologie en Microbiologie te Enschede (opleider: Dr. J. van der Stadt).

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