Neuroimmune and molecular aspects of antidepressants

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Neuroimmune and molecular aspects of antidepressants

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Chapter 1

General introduction

1. MAJOR DEPRESSION

1.1. General

Everyone now and then feels unhappy or sad, or experiences feelings of depressed mood. However, this everyday sadness does not resemble the clinical features of major depression. The latter is characterized by persistent depressed mood, lack of enjoyment or inability to experience pleasure (anhedonia), reduced energy, pessimistic thoughts, feelings of worthlessness and inappropriate guilt, anxiety and slowness (psychomotor retardation). In addition, the patient may display vegetative symptoms such as reduced appetite, weight loss and sleep disturbances. Depressed patients are often not able to work or to perform normal daily activities. Furthermore, a great deal of patients suffers from suicidal ideation and a number of them actually commit suicide. Thus, major depression is a highly disabling and life-threatening disease that affects both the patient and the persons in his/her environment.

According to the Global Burden of Disease study, conducted by the World Health Organization, the World Bank and Harvard University in the early 1990's, major depression is the leading cause of disability world-wide, and second to ischaemic heart disease in the Western world. The study predicted that by the year 2020 unipolar major depression is the major cause of years lost due to disability throughout the developing regions of the world (Murray and Lopez, 1996). In addition, mental illnesses in general outrank all other causes of disability in the United States, Canada and Western Europe (Iglehart, 2004).

The life-time prevalence of major depression is estimated as high as 16.1%, and affects twice as many women as men (Doris et al., 1999). The "burden" of this disease is further highlighted by its chronic course. A Dutch study pointed out that newly originated episodes of major depression had a median duration of 3 months. Twenty percent of the patients did not recover within 24 months (Spijker et al., 2002).

Despite the development of new antidepressants, about 30% of patients do not respond to the first treatment. Eventually, about 10 % of patients stay refractory to antidepressant treatment. Although the side effect profile of the second generation antidepressants has markedly improved, they still display uncomfortable side effects. Moreover, there is a delay in the onset of clinical improvement of depressive symptoms after starting antidepressant therapy.

The above indicates that the search for better antidepressants, with an improved side effect profile, a faster onset of action, and which are useful in treatment resistant patients, remains open. The major obstacles for designing and developing better antidepressants is the lack of reliable animal models of

depression, and the fact that the exact pathophysiology of major depression has not been resolved.

Several hypothesis concerning the aetiopathogenesis of major depression have been formulated, but so far, no conclusive evidence for one of them has emerged. A summary of the most important biological disturbances that could be relevant for the pathophysiology of major depression is given below.

1.2. Biological findings in major depression

The first biological systems proposed to underlie major depression were the monoamines. This hypothesis was primarily based on the fact that the first discovered antidepressants (mono-amine oxidase inhibitors and tricyclic antidepressants) increased brain concentrations of monoamines, especially those of serotonin and noradrenaline. Moreover, all parts of the limbic system, which is implicated in the control of emotions, mood, anxiety and cognition, are strongly innervated and regulated by both the serotonergic and noradrenergic system (Ressler and Nemeroff, 2000).

There is no conclusive direct evidence for a deficiency in either serotonin or noradrenaline in depressed patients. However some findings strongly suggest a role of serotonin in major depression (Maes and Meltzer, 1995; Smith and Cowen, 1997), namely: 1) the availability of L-tryptophan (the precursor of serotonin) to the brain is lower in depressed patients, and 2) remitted patients treated with antidepressants that primarily target the serotonergic system show a relapse of depressive symptoms after taking a tryptophan-free diet. Further, evidence from challenge studies indicate that 5HT₂-receptors may be upregulated (i.e. supersensitive), whereas 5-HT_{1A}-receptors may be downregulated (in number or density) in depressed patients. Moreover, treatment with antidepressants may restore these abnormalities in 5-HT-receptor function (Smith and Cowen, 1997).

Evidence for a role of noradrenaline in major depression comes from the observation that chemical depletion of catecholamines may induce depressive-like symptoms, which can be reversed by several antidepressants that target the noradrenergic system (Anand and Charney, 1997). Although some studies suggest that both α_2 -adrenergic and β -adrenergic receptors may be down-regulated in depressed patients, these findings are still a matter of debate (Schatzberg and Schildkraut, 1995; Anand and Charney, 1997). However, down-regulation of both receptors is the most consistent effect of antidepressant treatment in clinical and pre-clinical studies (Anand and Charney, 1997).

Despite the useful information that emerged from the intensive research on the role of monoaminergic neurotransmitter systems in major depression, it is of limited value in elucidating the exact pathophysiology of the disease. Major

depression arises from the complex interaction of multiple susceptibility genes and environmental factors. Moreover, although most antidepressants exert their initial effect by increasing intrasynaptic monoamine concentrations, their clinical antidepressant effects occur only after chronic treatment (2-8 weeks). This indicates that downstream adaptational mechanisms are necessary for the therapeutic effect.

One of the most consistent biological findings in major depression is the hyperactivity of the hypothalamic-pituitary-adrenal (HPA)-axis. This neuro-endocrine system regulates physiological responses to stress by controlling the release of corticosteroids. Hypothalamic secretion of corticotropin releasing hormone (CRH) stimulates the pituitary to release adrenocorticotrophic hormone (ACTH) which is released in the blood stream. ACTH triggers the secretion of glucocorticoids (i.e. cortisol in humans) by the adrenal cortex. Cortisol ultimately inhibits its own secretion through a negative feedback mechanism by binding with glucocorticoid receptors (GRs) in the hypothalamus and pituitary, thereby inhibiting the release of CRH and ACTH respectively. Hyperactivity of the HPA-axis in major depression is characterized by: 1) increased concentrations of cortisol in plasma, urine, saliva and cerebrospinal fluid of depressed patients, 2) an enhanced cortisol response to ACTH, and 3) increased concentrations of CRH in cerebrospinal fluid. Dysfunction of the glucocorticoid receptor in the hypothalamus has been implicated in HPA-axis hyperactivity in major depression. This is supported by the notion that administration of the synthetic glucocorticoid dexamethasone fails to suppress cortisol secretion in depressed patients. Some studies suggest that antidepressants enhance glucocorticoid receptor function and may thus restore this "glucocorticoid resistance" (Pariante and Miller, 2001).

It has long been recognized that major depression shows a high co-morbidity with other physical diseases such as cardiovascular disease, auto-immune disorders (e.g. rheumatoid arthritis, multiple sclerosis) and chronic infectious diseases (Irwin, 2002). Therefore, the status of immune function in depressed patients has been subject of intense investigation. Changes in several aspects of the immune system have been described. Early reports described that major depression is associated with a decrease in Natural Killer Cell activity and a lowered proliferative response of lymphocytes to mitogen stimulation (Herbert and Cohen, 1993; Zorrilla et al., 2001). Depressed patients also had lower numbers of circulating lymphocytes. Although these findings point to a state of immunosuppression in depressed patients, other immune variables show an activation of certain aspects of the immune system. Indeed, it is now clear that major depression is accompanied by an activation of the inflammatory response system (Maes, 1999). Evidence for this includes: 1) increased numbers of circulating monocytes and neutrophils; 2) increased numbers of circulating

activated lymphocytes; 3) the presence of an acute phase response, with positive acute phase proteins (such as haptoglobin) being upregulated (for review see Maes, 1999; Zorrilla et al., 2001). Furthermore, several studies found that major depressed patients had higher levels of pro-inflammatory cytokines in their serum or plasma. The *ex-vivo* production of these molecules is also enhanced. Pro-inflammatory cytokines have dramatic effects on brain function and neuro-endocrine homeostasis, and profoundly affect behaviour in animals and humans. It has therefore been proposed that pro-inflammatory cytokines play an important role in the pathophysiology of major depression. This will be dealt with in detail below, but first an introduction to the nature and physiology of the cytokine network is presented.

2. CYTOKINES AND MAJOR DEPRESSION

2.1. General features of cytokines

Cytokines are small (15 – 44 kD) glycoproteins, primarily secreted by immune cells, but also endothelial, epithelial, and neuronal cells can produce them. They interact with a specific membrane-bound receptor on target cells. Given the large number of known cytokines (probably many more to be discovered) and the diverse functions attributed to each of them, classification of cytokines is still obscure. Nevertheless, when examining systemic activity of the immune system in depressed patients, differentiation in 2 major groups is justified, namely pro-inflammatory and anti-inflammatory cytokines. Pro-inflammatory cytokines are mainly produced by activated immune cells and stimulate others, and, thereby, enhance inflammatory reactions. Anti-inflammatory cytokines on the other hand, tend to inhibit activated cells and temper inflammatory responses.

Monocytes and macrophages, activated by the recognition of pathogens, produce a diverse array of pro-inflammatory cytokines such as interleukin-1 (IL-1), tumor necrosis factor- α (TNF- α), IL-6 and IL-12. IL-1 and TNF- α promote inflammatory reactions, i.e. they stimulate other monocytes/macrophages and neutrophils to migrate to the site of infection and to phagocytosis. IL-6 activates lymphocytes and induces B-cells to differentiate to antibody producing plasma cells. After the initial activation, monocytes/macrophages also produce anti-inflammatory cytokines or proteins such as IL-10 and IL-1 Receptor antagonist (IL-1RA). IL-10 is a major de-activator of activated monocytes/macrophages, lymphocytes and neutrophils, and directly inhibits pro-inflammatory cytokine production. IL-1RA binds the IL-1 receptor and blocks the action of IL-1. Thus, the major monocytic pro-

inflammatory cytokines are IL-1, TNF- α and IL-6 and are as such indicative for an activation of the monocytic arm of the immune system.

Activated T-lymphocytes can be divided in two major groups according to the cytokines they produce, namely T-helper-1 (Th1) and Th2-cells. Th1-cells mainly produce interferon- γ (IFN- γ), IL-2 and TNF- β ; Th2-cells produce IL-4, IL-5, and IL-10. These cytokines have differential functions, i.e. Th1 cytokines stimulate cell-mediated immunity (CMI) whereas Th2 cytokines promote humoral immunity. CMI is characterized by the activation of monocytes/macrophages, neutrophils, cytotoxic T-cells (Tc-cells) and the stimulation of B-cells to produce opsonizing antibodies (and thus facilitating phagocytic activity). For example, IFN- γ (produced by Th1-cells) stimulates macrophages to phagocytosis; IL-2 further stimulates Th1-cells and induces Tc-cells to cytotoxic activity. On the other hand, IL-4 and IL-5 from Th2-cells mainly stimulate B-cells to produce non-opsonizing antibodies like IgE, and activate eosinophils, basophils and mast cells, which release several allergic mediators. In addition, Th1 and Th2 responses are mutually exclusive, i.e. IFN- γ inhibits Th2-cells, whereas IL-4 and IL-10 inhibit Th1-cells. This results in a polarization of the phenotype of the immune response. This phenotype is determined by the kind of microbial antigen and the micro-environment at the time of immune activation. For more information on Th1/Th2-responses and the accompanying cytokines, see Abbas et al. (1996).

It is clear that the concepts pro/anti-inflammatory and Th1/Th2-cytokines are more or less overlapping. Pro-inflammatory cytokines induce the production of Th1 cytokines, which have by themselves pro-inflammatory capacities; Th2 cytokines are anti-inflammatory and inhibit Th1 and/or pro-inflammatory cytokine production. One exception in this respect is IL-6. It has many pro-inflammatory properties, but is, besides monocytes/macrophages, produced by Th2-cells. Nevertheless, IL-6 is an important activator of the inflammatory response system. A major aspect of IRS is the acute phase response, which is predominantly induced by IL-6.

As mentioned before, cytokines act through specific membrane bound receptors. Some of these receptors can be released in a soluble form in the circulation. In most cases, they are shed from the cell membrane of activated leukocytes. For example, the soluble IL-2 receptor (sIL-2R) is shed from activated T-cells. As such, increased levels of sIL-2R indicates ongoing T-cell activation. Soluble cytokine receptors usually act as antagonists, since they prevent the respective cytokine from binding with its receptor on the target cell. Again, IL-6 is an exception to this rule. The IL-6/sIL-6R complex in serum can bind to gp130, a membrane-bound molecule that mediates IL-6 signaling. Therefore, sIL-6R can enhance the biological activity of circulating IL-6.

2.2. Neuroimmunology: cytokine-brain interaction

Although cytokines have long been implicated in the regulation of the immune response, it is now clear that they play (together with other aspects of the immune system) a central role in homeostasis of the whole body. Nowadays, the immune system is regarded as a sense organ that, through the release of cytokines, communicates the status of peripheral infection and injury to the brain. As such, cytokines perform important regulatory functions, not only on the immune system, but for example also on brain function, endocrine and neuro-endocrine regulation, cardiovascular functioning and the reproductive system.

Concerning the central nervous system, all brain cells (both glial and neuronal cells) express various cytokine receptors and are capable of producing cytokines (Vitkovic et al., 2000; Szelenyi, 2001). There is a specific bi-directional communication between the immune system and the brain. Cytokines produced by peripheral immune cells affect brain neurotransmission and neurotransmitter turn-over (Linthorst and Reul, 1998). Various pro-inflammatory cytokines also increase HPA-axis activity at all levels (Dunn, 2000).

Several possible pathways by which peripherally produced cytokines influence brain function have been described (Connor and Leonard, 1998; Maier and Watkins, 1998). First, although the size of the cytokines prevents diffusion through the blood-brain-barrier (BBB), passive transport across the BBB is possible at locations where a functional BBB is absent (i.e. the circumventricular organs). This mechanism could be particularly relevant when high concentrations of circulating cytokines are involved (e.g. in the case of cytokine therapy). In addition, active transport mechanism for some cytokines have been described, but their exact physiological role remains elusive. Second, circulating cytokines can induce cytokine production within the brain by interacting with receptors on vascular endothelial cells, that respond with *de novo* production of cytokines or other inflammatory intermediates, such as prostaglandin E₂ (PGE₂), that can enter the brain. In this way, cytokine production can spread through the brain parenchyma. Third, it has been shown that i) cytokines can trigger the afferent fibres of the vagus nerve; ii) stimulation of the vagus nerve induces cytokine production in several brain areas; iii) sectioning of the vagus nerve abrogates behavioural and neurochemical responses to peripheral administration of cytokines or endotoxin (for review see Maier et al., 1998). Thus, the effects of peripheral cytokines on the central nervous system are, at least in part, mediated by afferent neuronal pathways.

On the other hand, peripheral immune responses are controlled by the central nervous system. Immune cells express a broad array of neurotransmitter receptors and transporters (Gordon and Barnes, 2003). Lymphoid organs and

the spleen (which are important centers for immune regulation) are strongly innervated by efferent nerves of the sympathetic nervous system. These nerve terminals release noradrenaline and neuropeptides that strongly influence the nature of the ongoing immune response. For example, noradrenaline produces a shift in the Th1/Th2-balance, by inhibiting Th1- and promoting Th2-responses (Elenkov et al., 2000). Recently, it has been reported that the efferent vagus nerve, through the release of acetylcholine, influences the release of cytokines by activated macrophages (Pavlov et al., 2003; Wang et al., 2003).

2.3. The cytokine hypothesis of major depression

The notion that pro-inflammatory cytokines may underlie psychiatric disorders, especially major depression, comes from both human studies and animal experiments. As mentioned before, there is evidence that depressed patients suffer from an ongoing inflammatory reaction. One aspect of this is that higher concentrations of circulating pro-inflammatory cytokines such as IL-6, IL-1 β and TNF- α are found in depressed patients (Maes et al., 1997; Lanquillon et al., 2000; Mikova et al., 2001; Owen et al., 2001). Also *ex-vivo* production of these cytokines is increased (Seidel et al., 1995, 1996). Whether these pro-inflammatory cytokines contribute to the pathophysiology of major depression has long been (and still is) a matter of debate, but strong evidence favours this hypothesis. Indeed, administration of pro-inflammatory cytokines to humans (e.g. in the treatment of persistent viral infections or malignancies) induces depressive symptoms and even clinical major depression in previously psychiatrically healthy patients (Capuron et al., 2000). Also a moderate activation of the cytokine system induces cognitive and mood disturbances in healthy volunteers, even in the absence of physiological signs of sickness (Reichenberg et al., 2001). Many diseases that are associated with a hypersecretion of pro-inflammatory cytokines (e.g. cancer, chronic infections, atherosclerosis, auto-immune disorders) have a high co-morbidity with major depression (Pollak and Yirmiya, 2002). In animals, pro-inflammatory cytokines provoke "sickness-behaviour", a syndrome that resembles many clinical features of major depression, such as anhedonia, psychomotor retardation, reduced social exploration, decreased appetite, anorexia and sleep disturbances (Dantzer, 2001). Moreover, antidepressants and anti-inflammatory cytokines (such as IL-10 and IL-1RA) efficiently attenuate or completely prevent these cytokine-induced depressive symptoms (Dantzer, 2001; Musselman et al., 2001).

Several mechanisms that may underlie cytokine-induced depression have been proposed. First, virtually all pro-inflammatory cytokines are known activators of the HPA-axis (Dunn, 2000; Haddad et al., 2002), resulting in adrenal

hypersecretion of corticosteroids. As mentioned above, hyperactivity of the HPA-axis is implicated in the aetiology of major depression. On the other hand, corticosteroids are known to inhibit cytokine production and temper several facets of immune activation. Thus, sustained increases in circulating corticosteroids should reduce cytokine secretion. However, it has recently been shown that pro-inflammatory cytokines decrease glucocorticoid receptor function, and may induce glucocorticoid resistance (Pariante et al., 1999; Pariante and Miller, 2001). Thus, pro-inflammatory cytokines disturb the negative feed-back mechanism of glucocorticoids. This results in a sustained HPA-axis hyperactivity and a continuous secretion of pro-inflammatory cytokines.

Secondly, cytokines have dramatic effects on brain neurotransmission and neurotransmitter turn-over. Regarding this subject, most studies report that acute administration of cytokines or inflammatory stimuli (i.e. LPS) increase serotonin and noradrenaline secretion and turn-over, thereby facilitating serotonergic and noradrenergic neurotransmission (Linthorst and Reul, 1998; Dunn et al., 1999). Although these data seem less compatible with the monoamine hypothesis of depression, it has been proposed that chronic administration will result in mono-amine depletion and, hence, in a disturbance of monoaminergic neurotransmission (Pollak and Yirmiya, 2002). Another, indirect mechanism of cytokine effects on serotonergic neurotransmission, implicates the enzyme indoleamine 2,3 dioxygenase (IDO), which catalyses the breakdown of tryptophan through the kynurenine pathway. Pro-inflammatory cytokines (especially IFN- γ , TNF- α) up-regulate both the transcription and activity of IDO, leading to depletion of circulating tryptophan, and thus to decreased serotonin synthesis. In addition, it has been proposed that (neuro-)toxic metabolites of kynurenine contribute to neurodegeneration, hippocampal atrophy and neuropsychiatric disorders (for review see Wichers and Maes, 2004).

Although further investigations are required, the cytokine hypothesis of major depression offers an alternative explanation for the multi-factorial pathophysiology of the disease. It states that a moderate, but chronic hypersecretion of pro-inflammatory cytokines induces alterations in both neurological and neuro-endocrine networks, resulting in depressed mood and in several depression-related vegetative and somatic disturbances.

3. ANTIDEPRESSANTS

3.1. Different classes of antidepressants

Although the nature of major depression has been described for many centuries (starting by the Greek philosopher Hippocrates), an efficient and biological directed treatment for the disease was only introduced in 1938, when patients were treated with electroconvulsive therapy. Pharmacological treatment became available when antidepressants were serendipitously discovered in the 1950's. The first antidepressants were clearly effective in reducing the depressive symptomatology, but displayed a broad range of (sometimes dangerous) side effects. With the introduction of fluoxetine (Prozac[®]), a number of other so-called "second generation" antidepressants became available. The major advantage of these drugs over the first generation antidepressants was their better side effect profile. However, as mentioned before, clinical efficiency has only slightly improved. This despite the fact that psychiatrists now have of a number of antidepressants, with different proposed mechanisms of action, at their disposal.

Classification of antidepressants is based on their primary mechanism of action, except for the group of tricyclic antidepressants (TCAs), which are classified according their chemical structure. Table 1 presents the major classes of antidepressants with the most important substances of each class and their primary mechanism of action. Most antidepressants increase the availability of neurotransmitters (usually serotonin and/or noradrenaline) in the synaptic cleft, either by blocking the reuptake of the neurotransmitter in the presynaptic neuron, or by inhibiting the enzymes responsible for the neurotransmitter breakdown in the synaptic cleft. Selective Serotonin Reuptake Inhibitors (SSRIs) block the reuptake of serotonin, while Selective Noradrenaline Reuptake Inhibitors (NRIs) inhibit that of noradrenaline. Antidepressants that block the reuptake of both serotonin and noradrenaline belong to the Serotonin-Noradrenaline Reuptake Inhibitors (SNRIs). TCAs also block the reuptake of both neurotransmitters, but the ratio between serotonin and noradrenaline reuptake inhibition differs for each. For example, desipramine primarily blocks noradrenaline reuptake, whereas clomipramine selectively inhibits that of serotonin. Apart from reuptake inhibition, TCAs also interact with various receptor types, an effect that is associated with side effects. Another way of increasing neurotransmitter concentration in the synaptic cleft is by inhibition of their breakdown by the enzyme Monoamine Oxidase (MAO). Inhibitors of MAO (MAOIs) were, together with the TCAs, the first antidepressants in clinical practice, but they are no longer used due to dangerous side effect. This was partly because MAOIs interacted in an irreversible manner with the

enzyme. There is however currently one antidepressant available (i.e. moclobemide) that is a reversible inhibitor of MAO-A (RIMA) and displays fewer side effects than the MAOIs.

The above-described antidepressants are by far the most used, but recently a group of atypical antidepressants has emerged. Their primary mechanism of action is not limited to increases in synaptic concentrations of serotonin or noradrenaline, and may in fact be completely unrelated to these increases. For example, nefazodone and trazodone, which is sometimes described as a heterocyclic antidepressant according to its chemical structure, are antagonists of the 5-HT_{2A}-receptor. Mirtazapine blocks α_2 -adrenergic receptors, and thereby increases the activity of noradrenergic (and partly serotonergic) neurons. Some atypical antidepressants do not seem to have a primary effect on neurotransmitters. Rolipram, for instance, is an inhibitor of phosphodiesterase type 4 (PDE4), an enzyme that catalyses the breakdown of the second messenger cyclic Adenosine Monophosphate (cAMP). Although the clinical efficacy of rolipram proved to be equal to that of TCAs, it is not on the market any more due to severe side effects. However, several pharmaceutical companies are still trying to develop new PDE4-inhibitors as potential antidepressants. A remarkable substance that recently came available as an antidepressant on the European market is tianeptine. It appears to have the opposite effect as SSRIs, namely it enhances the reuptake of serotonin and thereby decreases serotonin availability in the synaptic cleft.

From the above, it is clear that substances with different biochemical and pharmacological effects can display antidepressant properties. The viewpoint that the working mechanism of antidepressants is to increase neurotransmitter availability has therefore recently been questioned. It is more likely that the primary effects of SSRIs, TCAs, NRIs and SNRIs on reuptake inhibition of either serotonin or noradrenaline is not directly related to their antidepressant effect. Rather, it appears that these effects induce adaptational changes in neuronal functioning which eventually translate into antidepressant effects. This phenomenon would also explain the fact that no currently available antidepressant can significantly reduce the delay in therapeutic effect. Indeed, all antidepressants have to be taken for several weeks before clinical improvement emerges. The biochemical and pharmacological effects on the other hand, are seen only a few minutes to hours after administration of the antidepressant. There is now more and more evidence that antidepressant effects are associated with (molecular) neurobiological changes, such as increases in synaptic plasticity and/or neurogenesis, and changes in the activity of intracellular signalling cascades (Manji et al, 2003).

Table 1
Classes of antidepressants with different biochemical effects

Class	Antidepressants	Primary biochemical effect
Tricyclic antidepressants (TCAs)	imipramine, desipramine, amitriptyline, nortriptyline, clomipramine	Reuptake inhibition of serotonin, and/or noradrenaline, interaction with various receptor types
Selective Serotonin Reuptake Inhibitors (SSRIs)	fluoxetine, paroxetine, citalopram, sertraline, fluvoxamine	Selective inhibition of the reuptake of serotonin
Selective Noradrenaline Reuptake Inhibitors (NRIs)	reboxetine, mianserine	Selective inhibition of the reuptake of noradrenaline
Serotonin and Noradrenaline Reuptake Inhibitors (SNRIs)	venlafaxine, duloxetine	Inhibition of the reuptake of both serotonin and noradrenaline
Monoamine Oxidase Inhibitors (MAOIs)	phenelzine, tranylcipromine	Blockade of monamine breakdown
Reversible Inhibitors of MAO-A	moclobemide	Reversible inhibition of the breakdown of monoamines
Atypical antidepressants	nefazodone, trazodone	Antagonism of 5-HT ₂ -receptors
	mirtazapine	Antagonism of α_2 -adrenergic receptors
	rolipram	Inhibition of PDE4
	tianeptine	Enhancement of serotonin reuptake

3.2. Antidepressants and cytokines

As stated previously, the working mechanism(s) of antidepressants are generally thought to be mediated by their effects on central neurotransmission. Since the emerging evidence of the possible role of cytokines in major depression, the influence of antidepressants on cytokine production has been subject of several studies. Indeed, the cytokine-hypothesis of major depression implies that antidepressants should interfere in cytokine production. Several studies that addressed this issue are reviewed in Chapter 2. The overall conclusion is that antidepressants have anti-inflammatory effects *in-vitro* and in several animal models of depression. Thus, antidepressants seem to inhibit pro-inflammatory cytokines, and enhance the production of IL-10. The effect of antidepressant treatment on cytokine production in depressed patients has also been examined, but the results are inconclusive. Several methodological issues may underlie this inconsistency (see Chapter 2).

4. THE cAMP/PKA-PATHWAY

4.1. General

Cells are dynamic entities that respond to physiological alterations in their immediate environment and to molecules secreted by neighbouring or distant cells. These chemical messages interact with specific receptors at the cell membrane, which subsequently generate an intracellular signal.

Cyclic adenosine monophosphate (cAMP) is one of the best characterized second messenger molecules. It is produced by the enzyme adenylate cyclase that is coupled to certain G-protein coupled receptors (GPCRs). Activation of such receptors leads to rapid stimulation of adenylate cyclase and subsequent cAMP-production. Receptors that are linked to adenylate cyclase include receptors for hormones, neurotransmitters, purines and prostaglandines. Among the cAMP-coupled neurotransmitter receptors are serotonergic 5-HT₄, 5-HT₅ and 5-HT₇ receptors, β_2 -adrenergic receptors and histaminergic H₂-receptors. The binding of a ligand with these receptors causes conformational changes and reorganization of receptor associated G-protein subunits. This results in activation of the membrane-bound adenylate cyclase and a subsequent rise in intracellular cAMP-concentrations. The major effect of cAMP is the activation of protein kinase A (PKA, see Figure 1). Inactive PKA consists of two catalytic subunits (PKA-c) that are held together by two regulatory subunits (PKA-r). Cyclic AMP binds with the regulatory subunits causing the release of the catalytic units. The active PKA-subunits are then able to activate other kinases, enzymes and transcription factors by phosphorylation. One of the best known targets of PKA is the cAMP responsive element binding protein (CREB), a transcription factor that binds to a cAMP responsive element (CRE) in the DNA, which is found in the promotor region of many genes. Binding of CREB to the CRE can have either stimulatory or inhibitory effects on gene transcription.

Intracellular homeostasis of cAMP is predominantly controlled by phosphodiesterase type 4 (PDE4) that hydrolyses cAMP to AMP. PDEs are a group of enzymes that are responsible for the breakdown of cyclic nucleotides such as cAMP and cyclic guanosine monophosphate (cGMP). They differ in the specificity and affinity for the respective cyclic nucleotides. PDE4 is highly specific for the breakdown of cAMP. Inhibition of PDE4 consequently results in the rapid and sustained accumulation of intracellular cAMP.

Thus, modulation of the cAMP/PKA-pathway can be achieved by:

- stimulation of receptors that are positively coupled to adenylate cyclase (by natural ligands or artificial agonists)
- direct stimulation or inhibition of adenylate cyclase
- inhibition of PDE4
- stimulation or inhibition of PKA

These strategies have been employed to study the role of the cAMP/PKA-pathway on the production of cytokines by immunocompetent cells.

Figure 1
Schematic presentation of intracellular cAMP/PKA-signalling.

4.2. Cyclic-AMP/PKA and cytokine production

Cytokine production is controlled by many different processes. As mentioned previously, cytokine production is induced by immunological activation of immune cells (e.g. by antigens) and/or by cytokines secreted from neighbouring cells. The binding of a cytokine with its receptor activates receptor associated protein kinases (usually tyrosine kinases), which in turn activate other kinases

and transcription factors. In this way, the cells respond rapidly to cytokine stimulation, resulting in certain immunological activities, such as induction or enhanced production of other cytokines. Stimulation of the cAMP/PKA-cascade does not directly induce cytokine production, but this signalling pathway strongly modulates cytokine expression induced by other stimuli such as antigens, lipopolysaccharides and mitogens (for review see Zidek, 1999). The influence of cAMP-signalling on cytokine production has been examined on several cell types, mainly T-lymphocytes, monocytes, macrophages, lymphoid or myeloid cell lines and peripheral blood mononuclear cells (PBMCs). The latter is the purified fraction of lymphocytes and monocytes from whole blood. Also microglia (the macrophage-like cells in the brain) have been used to study the role of cAMP/PKA in cytokine production. In most studies, modulation of the cAMP/PKA-activity was achieved by agonists of adenylate cyclase coupled receptors (β_2 -adrenergic receptors, H_2 -histaminergic receptors and EP_2 -prostaglandin receptors), PDE4-inhibition or stimulation with cAMP-analogues such as dibutyryl-cAMP. Chapter 6 provides a summary of the most important findings on this subject. In general, stimulation of the cAMP/PKA-pathway decreases the stimulated production of IFN- γ , TNF- α and IL-12, while it enhances that of the IL-10. Thus, increased cAMP/PKA-activity provokes anti-inflammatory effects *in-vitro*. The effects of cAMP/PKA-signalling on other cytokines is not yet clear and presumably depends on the cell type, stimulus and micro-environment.

5. GENERAL HYPOTHESIS

If pro-inflammatory cytokines play a role in the pathophysiology of major depression, then antidepressants should interfere with production and/or action of these cytokines. Therefore, the effects of antidepressants on the production of various cytokines in either human whole blood or in primary rat brain cells are explored. Further, if antidepressants of different classes decrease pro-inflammatory and/or increase anti-inflammatory cytokine production, then this effect resembles that of substances that up-regulate the activity of the cAMP/PKA-pathway. Therefore, it is hypothesized that: a) antidepressants enhance cAMP/PKA-signalling either by increasing intracellular cAMP-levels or by augmenting PKA-activity; b) this antidepressant-induced increase in cAMP/PKA-activity changes the pattern of cytokine production.

6. CHAPTER OVERVIEW

This introductory chapter provides a concise background on the field of “psychoneuroimmunology”, specifically in relation to the pathophysiology of major depression. It contains an introduction to the biology of major depression, the immune system and cytokines, brain-cytokine interactions, the different classes of antidepressants, and the cAMP/PKA signalling cascade.

In Chapter 2, an overview of the current knowledge about the influence of antidepressants on cytokine production is presented. Human studies, animal models of depression and *in-vitro* culture models, have all been used to examine the effects of antidepressants on the immune system in general and on cytokines in particular. The respective literature is critically reviewed with respect to methodology and the type of model used.

The reliable measurement of cytokines is a crucial aspect of most experiments presented throughout this thesis. Handling and storage of samples is an important aspect of pre-analytical variability. The study in Chapter 3 provides evidence that long-term storage of samples has no influence on the correct measurement of cytokines.

Stimulated human whole blood is a reproducible and robust method to study the influence of chemical therapeutics on cytokine production. Although this model has been used previously to study the effects of some antidepressants (Maes et al., 1999), the effects of different classes of antidepressants are further explored in Chapter 4. One of the possible mechanisms by which antidepressants can influence cytokine production is by their serotonergic action, i.e. either by interacting with serotonergic receptors on peripheral immune cells, or by blocking serotonin reuptake and thereby increasing extracellular serotonin concentrations. Therefore, the direct effects of serotonin, serotonergic receptor agonists and antagonists, and the effect of serotonin depletion are examined in Chapter 5, using the same whole blood stimulation model. Another possible mediator is the cAMP/PKA-pathway. Although there are numerous reports about the role of cAMP and PKA on cytokine production, none of them have employed stimulated whole blood. In addition, most of these studies only examine a single aspect of cAMP/PKA signalling (mainly enhancing or inhibiting PKA-activity, or blocking PDE4), or they present data about a limited number of cytokines. The study in Chapter 6 examines the influence of cAMP/PKA-signalling on stimulated cytokine production (in human whole blood) by modulating the cascade at all levels, i.e. at the level of cAMP-formation by adenylate cyclase, at the level of cAMP-degradation by PDE4, and at the level of PKA-activity.

To test the hypothesis that the effects of antidepressants on cytokine production are mediated by an increase in cAMP/PKA-activity, whole blood was

stimulated in the presence or absence of inhibitors of adenylate cyclase (SQ22536) or PKA (Rp-8-Br-cAMPS). If the hypothesis is correct, then these inhibitors should reverse the effects of the antidepressants on cytokine production. The data of these experiments are presented in Chapter 7. Further, the effect of antidepressants on intracellular cAMP-concentrations in human peripheral blood mononuclear cells is explored in Chapter 8.

As explained elsewhere in this chapter, it is likely that the effects of peripheral cytokines on brain function are mediated by cytokines produced within the central nervous system. However, the effects of antidepressants on cytokine production by brain cells has never been examined before. Chapter 9 presents data on the production of cytokines by re-aggregating whole brain cell cultures ("spheroid" cultures), and the influence of the antidepressant imipramine hereon.

Finally, the results of the experiments are critically reviewed in Chapter 10. Directions for future research are discussed.

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Chapter 2

Effects of antidepressants on the production of cytokines

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ABSTRACT

There is now evidence that major depression is associated with an upregulation of the inflammatory response system (IRS). One of the major factors in this IRS activation is the hyperproduction of pro-inflammatory cytokines. Recently, a number of studies examined whether there is a causative role of these inflammatory mediators in the aetiology of major depression. Studies with animal models and cytokine immune therapy in humans suggest that pro-inflammatory cytokines induce depressive symptomatology. Moreover, these depressive symptoms can be effectively reversed by antidepressant treatment. Thus, it may be suggested that antidepressants suppress pro-inflammatory cytokine production and/or action, resulting in improvement of depressive symptoms. The influence of antidepressants on cytokine production has been examined in culture systems *in-vitro*, and in animal models of depression - in which cytokine production is induced by endotoxin administration. Results suggest that antidepressants of several classes decrease the production of pro-inflammatory cytokines like interferon- γ and tumor necrosis factor- α , and increase that of interleukin-10, an anti-inflammatory cytokine. Further, the effect of antidepressive treatment on cytokine secretion and on plasma levels of cytokines in depressed patients has been studied. Unfortunately, different approaches to examine cytokine production and different techniques to measure cytokines in plasma are used in these studies. Despite this, current data point to a normalization of cytokine plasma levels and cytokine production after antidepressant treatment. It is clear, however, that more research is warranted and we strongly argue the need for higher standardization in the methodology used to examine the cytokine network in depressed patients.

1. INTRODUCTION

In the past decade, it has become clear that there is a strong interaction between the peripheral immune system and the central nervous system (CNS). Immune activation influences brain functioning and the CNS modulates the immune response. Moreover, immune activation can lead to profound alterations in psychological status (e.g. mood and behaviour). Therefore, alterations of the immune system have been intensively investigated in psychiatric disorders like major depression, dysthymia and schizophrenia (for review see: Maes, 1999; Kronfol and Remick, 2000; Muller et al., 2000).

The most important link whereby the immune system influences the brain is established by the cytokine network. Initially, cytokines were discovered and described as proteins that were produced by immunocompetent cells to stimulate or inhibit the function of other immune cells. Now it is clear that these molecules do much more than solely participating in the regulation of the immune response and that various cell types, including cells of the CNS (astrocytes, microglia and even neurons) can produce cytokines. However, classification of cytokines is still based on their role in immune homeostasis. Two classification systems can be distinguished. One of them denotes cytokines as pro-inflammatory or anti-inflammatory, whereas the other uses the terms T-helper1(Th1)-like or Th2-like cytokines. These terms are not exactly the same, although there is an overlap. Pro-inflammatory cytokines promote inflammatory reactions and, as such, they tend to stimulate or activate immunocompetent cells. In contrast, anti-inflammatory cytokines inhibit inflammation and deactivate immune cells. The most important pro-inflammatory cytokines are interleukin-1 (IL-1), IL-6, IL-8, tumor necrosis factor (TNF) and interferon- γ (IFN- γ). The prototypical anti-inflammatory cytokine is IL-10, which is a major deactivator of monocytes, macrophages and lymphocytes. Interleukin-1 receptor antagonist (IL-1RA) is another anti-inflammatory protein that specifically antagonizes the activities of IL-1. The distinction between Th1- and Th2-like cytokines is based on the respective lymphocyte subset that secretes them. T-helper 1 (Th1) lymphocytes produce IL-2, IFN- γ and TNF- β , whereas Th2 lymphocytes mainly produce IL-4, IL-5 and IL-10 (Abbas et al., 1996).

Cytokines act through specific membrane bound receptors. Binding of a cytokine with its receptor induces activation of several intracellular pathways, eventually leading to changes in gene expression. Interestingly, some cytokine receptors can be cleaved from the plasma membrane by proteases. Furthermore, alternatively spliced mRNA, coding only the extracellular part of receptors, has been described. These "soluble receptors" enter the circulation in concentrations that often exceed that of their ligand. Soluble cytokine receptors usually act as antagonists because they compete with the membrane bound receptor for

binding. One important exception is the soluble IL-6 Receptor (sIL-6R), since the IL-6/sIL-6R-complex in serum can bind with gp130 (the final signal transduction molecule for IL-6) on the cell membrane, thereby augmenting the biological activity of IL-6 (Peters et al., 1998).

The pro-inflammatory cytokines have profound effects on CNS and endocrine functioning. They increase central monoamine metabolism and are potent activators of the hypothalamic-pituitary-adrenal axis (HPA-axis). Both systems are disturbed in major depression.

Much attention has focused on the role of cytokines in the aetiology and pathophysiology of mood disorders, especially major depression. A complete overview of these studies is far beyond the scope of this review. The topic has been excellently reviewed in recent publications (Connor and Leonard, 1998; Dantzer et al., 1999; Licinio and Wong, 1999; Maes, 1999).

If pro-inflammatory cytokines play a causative role in major depressive disorders, then antidepressants should downregulate these cytokines or interfere with their actions. Accumulating evidence has been published that antidepressants modulate cytokine production. This paper reviews studies concerning the influence of antidepressants on cytokines in depressed patients and experimental animals, and on *in-vitro* cytokine production.

2. INFLUENCE OF ANTIDEPRESSANT TREATMENT ON CYTOKINES IN DEPRESSED PATIENTS

2.1. Methods to study cytokine production in humans

One approach to study the influence of antidepressants on cytokine production in humans is to look at differences in cytokine patterns before and after antidepressant therapy. Indeed, several investigators addressed this issue. However, the methods employed to investigate changes in the cytokine homeostasis differ greatly among the reported studies. Circulating cytokine levels are very low in healthy subjects and in patients with psychiatric disorders, and, hence, are very difficult to detect in serum or plasma of these subjects. Therefore, the production of cytokines is often examined *ex-vivo*, e.g. in the supernatant of cultured white blood cells or cultured whole blood. In both cases, cytokine production is induced by the addition of lipopolysaccharides (LPS) and/or mitogens, like phytohaemagglutinin (PHA) or Concanavalin A (Con A). In the first case, leukocytes are purified from blood to obtain a mixture of predominantly lymphocytes and monocytes, usually referred to as Peripheral Blood Mononuclear Cells (PBMCs). In the latter case, whole blood from the patient (sometimes diluted in culture medium) is directly cultured, without

purification of specific cell types. Thus, besides lymphocytes and monocytes, all other cell types capable of producing cytokines (including neutrophils, granulocytes and platelets) are present in the culture. It has been shown that diluted, stimulated whole blood is a good and reliable method to study cytokine production *ex-vivo* or *in-vitro*. Compared to PBMC-cultures, whole blood cultures reflect better the *in-vivo* conditions and are more reproducible in terms of cytokine production (De Groote et al., 1992, 1993; Zangerle et al., 1992; Yaqoob et al., 1999). First, natural cell-to-cell interactions are preserved in whole blood cultures, including endogenous immunoregulatory mediators present in the plasma. Second, purification of white blood cells modifies the lymphocyte/monocyte ratio, leading to reduced monocyte concentrations (De Groote et al., 1992). Since some cytokines are secreted by one cell type, whereas others can be produced by several cell types, and since the production of cytokines is influenced by other cytokines, it is better to measure cytokines in cultures with a preserved lymphocyte/monocyte ratio.

Nowadays, cytokines are mostly determined by means of enzyme-linked immunosorbent assays (ELISA) or radio-immunoassays (RIA). These methods use specific mono- or polyclonal antibodies to detect the respective cytokine in biological fluids or culture medium. Earlier, cytokines were measured using certain cell-lines that displayed a dose-dependent biological response to the cytokine. This response could be proliferation, growth inhibition or the secretion of certain proteins. However, these bio-assays only measure biological active cytokines. This biological activity can be influenced by the presence of soluble receptors or by degradation of the cytokine in the sample (through the action of endogenous proteases or by freezing and thawing of the samples). Moreover, cell lines might not solely respond to one cytokine, but also to other proteins (including cytokines), hormones or other factors present in the sample, leading to non-specific responses. This phenomenon can be checked using neutralizing antibodies added to the sample (in which case the biological activity of the cytokine is blocked). Overall, bio-assays have the advantage of measuring biological active cytokines, but immuno-assays perform much better in terms of sensitivity, specificity, reliability and reproducibility. It should also be noted that there are now numerous commercial immuno-assays (e.g. ELISAs) available. Over the last years they have been significantly improved in terms of sensitivity. Moreover, specific assays for cytokine detection in serum or plasma have been developed. We strongly recommend to use these ultra-sensitive ELISAs when measuring cytokine levels in serum or plasma.

All methods mentioned above have been employed by investigators to study cytokine patterns in psychiatric patients. It is therefore difficult to interpret and to compare divergent (and sometimes conflicting) results obtained by different laboratories. One should always keep in mind the differences between the

methods used: e.g. cytokine levels in plasma versus *ex-vivo* cytokine production - stimulated PBMCs versus whole blood cultures - cytokine measurement by immunoassays versus bio-assays.

2.2. Ex-vivo cytokine production in depressed patients before and after pharmacotherapy

Table 1 gives an overview of studies that examined *ex-vivo* cytokine production before and after antidepressant treatment. In an early study, Weizman et al. reported that the stimulated production of IL-1 β by PBMCs was significantly enhanced after a 4 week treatment with clomipramine compared with that before the start of the treatment (Weizman et al., 1994). In this study, stimulated production of IL-1 β and IL-2 in major depressed patients before treatment was significantly lower compared to matched healthy controls. It should be noted that indomethacin was added to these cultures to inhibit prostaglandin formation, which affects cytokine production. However, indomethacin may change cytokine patterns as well. So it is difficult to compare this study with later reports that examined cytokine production without indomethacin. Recently, it was found that the production of IL-1 β in stimulated whole blood is not different in patients with melancholic and non-melancholic depression and healthy controls, although higher monocyte counts were found in patients with non-melancholic depression (Rothermundt et al., 2001b). Antidepressive treatment did not affect the production of IL-1 β . It is noteworthy that the aim of this study was to investigate differences in immune parameters between melancholic and non-melancholic major depressed patients. The majority of these patients were already on different types of antidepressants at baseline. Thus, conclusions regarding the influence of antidepressant treatment on IL-1 β production are not reliable. Anisman et al. found increased production of IL-1 β in dysthymic patients, which persisted despite successful antidepressant treatment (Anisman et al., 1999).

Seidel et al. (1995) examined the stimulated production of IL-1 β , IL-2, IL-6, IL-10, sIL-2R and IFN- γ in whole blood of healthy controls and major depressed patients before and after therapy. The production of IFN- γ and sIL-2R was significantly higher in patients as compared to controls. After six weeks of treatment, IFN- γ , sIL-2R, IL-2 and IL-10 production was significantly reduced compared to pre-treatment values (Seidel et al., 1995, 1996). Unfortunately, the type of treatment was not specified in these papers. Recently, Rothermundt et al. (2001a) reported that the stimulated production of IFN- γ , IL-2 and IL-10 was significantly lower in patients suffering from melancholic depression compared to healthy controls, but not in non-melancholic depressed patients. The

production of these cytokines normalized after two weeks of treatment, but patients were not free of antidepressants at the start of the study.

Others reported no effect of four and twelve weeks of moclobemide treatment on the stimulated production of TNF- α in PBMCs of major depressed patients (Landmann et al., 1997). It is noteworthy that the TNF- α production did not differ between patients before therapy and healthy controls. In contrast, Lanquillon and colleagues reported an increase in the unstimulated TNF- α secretion in whole blood from depressed patients (Lanquillon et al., 2000). Moreover, this secretion was significantly reduced in patients who responded to a six-week treatment with amitryptiline. TNF- α secretion in non-responders decreased not significantly. Interestingly, in the same study, it was shown that the secretion of IL-6 was significantly normalized in both responders and non-responders, where responders had significantly lower and non-responders significantly higher IL-6 levels before treatment-start as compared to healthy controls (Lanquillon et al., 2000).

Table 1

Summary of studies that examined *ex-vivo* cytokine production in depressed patients before and after antidepressant treatment.

Study	Antidepressant	Cytokines examined	Outcome
Weizman et al., 1994	Clomipramine	IL-1 β	Increase
Rothermundt et al., 2001b	Not specified	IL-1 β	No effect
Anisman et al., 1999	Sertraline	IL-1 β	No effect
Seidel et al., 1995, 1996	Not specified	IFN- γ	Decrease
		sIL-2R	Decrease
		IL-2	Decrease
		IL-10	Decrease
Rothermundt et al., 2001a	Not specified	IFN- γ	Increase
		IL-2	Increase
		IL-10	Increase
Landmann et al., 1997	Moclobemide	TNF- α	No effect
Lanquillon et al., 2000	Amitryptilyne	TNF- α	Decrease
		IL-6	Increase in treatment responders Decrease in treatment non-responders

2.3. Cytokine levels in serum or plasma

Other investigators examined plasma levels of cytokines in major depressed patients before and after antidepressant therapy (see Table 2). Maes et al. reported that subchronic (five weeks) treatment with fluoxetine or various tricyclic antidepressants (TCAs) had no effect on increased IL-6, sIL-6R and sIL-2R levels (Maes et al., 1995). Additionally, there was no influence of antidepressant treatment on serum IL-6 and IL-1RA levels, which were increased in major depressed patients, including treatment-resistant depressed patients (Maes et al., 1997). The plasma level of IFN- γ was reported not to be different between healthy controls and depressed patients, and treatment with moclobemide for up to three months did not affect the IFN- γ concentration (Landmann et al., 1997). In contrast, two other studies found that antidepressant treatment significantly lowered the initially increased serum IL-6 concentrations in depressed patients (Sluzewska et al., 1995; Frommberger et al., 1997). Sluzewska et al. (1995) reported that only 6 out of 22 patients had increased IL-6 levels. Treatment with fluoxetine for eight weeks reduced IL-6 levels in these patients to those of healthy controls and patients with non-elevated IL-6 levels. This may indicate that circulating levels of pro-inflammatory cytokines are not increased in all depressed patients, and that increased cytokine production may be related to certain subtypes of depressive disorders and/or the state of the patient. However, in patients where increased cytokine levels are apparent, antidepressant treatment may normalize this increase. Finally, in a recent study it was found that treatment with TCAs significantly increased the plasma level of the TNF-receptor type II (Hinze-Selch et al., 2000). This effect was not seen after treatment with paroxetine. The authors suggest that activation of the TNF- α system might precede the induction of weight gain by TCAs. However, it is well known that soluble TNF-receptors may antagonize the effects of TNF- α , indicating that TCAs may display anti-inflammatory effects regarding the TNF- α system.

It is clear that divergent and contradictory results regarding the influence of antidepressant treatment on cytokine production are reported. Discrepancies in study results may be related to differences in methodology, the heterogeneity of the study population and the patient status regarding the use of antidepressants. Obviously, more studies are needed to fully clarify the influence of antidepressant treatment on cytokines in depressed patients.

Table 2

Summary of studies that examined serum or plasma cytokine levels in depressed patients before and after antidepressant treatment.

Study	Antidepressant	Cytokines examined	Outcome
Maes et al., 1995	Flouxetine/TCAs	IL-6	No effect
		sIL-2R	No effect
		sIL-6R	No effect
Maes et al., 1997	Fluoxetine/Trazodone	IL-6	No effect
		IL-1RA	No effect
Landmann et al., 1997	Moclobemide	IFN- γ	No effect
Frommberger et al., 1997	Various types	IL-6	Decrease
Sluzewska et al., 1995	Fluoxetine	IL-6	Decrease
Hinze-Selch et al., 2000	TCAs	sTNF-RII	Increase
	Paroxetine	sTNF-RII	No effect

3. EFFECTS OF ANTIDEPRESSANTS ON CYTOKINE PRODUCTION *IN-VITRO*

The effects of antidepressants have also been examined in several *in-vitro* culture systems. Xia et al. (1996) reported on the effects of imipramine, clomipramine and citalopram on cytokine secretion in isolated monocytes and T-lymphocytes. They found that a 24 hour pre-incubation with all antidepressants examined, significantly decreased the stimulated production of IL-1 β , IL-6 and TNF- α by monocytes. The same effect was seen on the production of IL-2 and IFN- γ from stimulated T-lymphocytes. In the latter case, the inhibition was more pronounced with imipramine and clomipramine in comparison to citalopram. Maes and co-workers examined the effects of antidepressants on cytokine production in stimulated, diluted whole blood cultures. In these studies, several classes of antidepressants were compared in view of their effect on the IFN- γ /IL-10 production ratio. IFN- γ is predominantly produced by regulatory Th1-lymphocytes and promotes the production of other pro-inflammatory cytokines. On the contrary, IL-10 is the major anti-inflammatory cytokine and downregulates pro-inflammatory cytokine production. Therefore, the balance between IFN- γ and IL-10 production is critical in determining the pro- or anti-inflammatory capacities of the culture supernatant (Katsikis et al., 1995).

The authors showed that clomipramine reduces the secretion of IFN- γ (Maes et al., 1999a), and that imipramine enhances the production of IL-10 (Kubera et al., 2001a). Both TCAs significantly reduced the IFN- γ /IL-10 production ratio. Sertraline and fluoxetine significantly reduced IFN- γ and increased IL-10 production. Hence, both SSRIs significantly decreased the IFN- γ /IL-10 ratio (Maes et al., 1999a; Kubera et al., 2001a). Venlafaxine, a serotonin noradrenaline reuptake inhibitor (SNRI), significantly enhanced the production of IL-10 (Kubera et al., 2001a), whereas the heterocyclic antidepressant (HCA) trazodone decreased the secretion of IFN- γ (Maes et al., 1999a). Most important, both antidepressants reduced the IFN- γ /IL-10 ratio. In summary, TCAs, SSRIs, SNRIs and HCAs have negative immunoregulatory effects by decreasing the production of IFN- γ relative to that of IL-10. Another study examined the influence of moclobemide on cytokine production in whole blood cultures (Lin et al., 2000). It was found that moclobemide enhanced the stimulated production of IL-10, but did not affect that of IFN- γ . Interestingly, moclobemide suppressed the spontaneous (unstimulated) release of the pro-inflammatory cytokines TNF- α and IL-8. These data indicate that moclobemide may have anti-inflammatory capacities, by inhibiting pro- and stimulating anti-inflammatory cytokine production.

The effect of the mood stabilizer lithium on *in-vitro* cytokine production was subject of several studies. Early studies reported that lithium increases the production of IL-2 in PBMCs and T-cells (Wilson et al., 1989; Wu and Yang, 1991; Kucharz et al., 1993), and stimulated monocytic TNF- α secretion (Kleinerman et al., 1989). A recent study showed that lithium reduced the spontaneous and the LPS-induced IL-8 production by monocytes (Merendino et al., 2000). Maes et al. reported that in stimulated whole blood, lithium increased the production of the pro-inflammatory cytokines TNF- α and IL-8, but not IL-6 and IFN- γ (Maes et al., 1999b). Also the production of the anti-inflammatory cytokines IL-10 and IL-1RA was significantly enhanced in lithium treated cultures. However, lithium did not influence the IFN- γ /IL-10 production ratio (Maes et al., 1999b). In a recent study, lithium produced a shift in the Th1/Th2-balance by enhancing IL-4 and IL-10 production and decreasing that of IL-2 and IFN- γ in stimulated whole blood (Rapaport and Manji, 2001). To summarize, lithium may influence both pro- and anti-inflammatory cytokine production. It has been suggested that lithium can restore mild immune activation and disequilibria in cytokine production (Sluzewska et al., 1997; Maes et al., 1999b; Rapaport et al., 1999).

Overall, it is concluded that antidepressants of different classes have, at least *in-vitro*, negative immunoregulatory effects in terms of cytokine secretion.

4. ANTIDEPRESSANTS AND CYTOKINES IN ANIMAL MODELS OF DEPRESSION

Several animal models have been developed to study the pathophysiology of major depression. Commonly studied models of depression are submission to chronic mild stress, lesion of the olfactory bulb and injection of lipopolysaccharides (LPS). These models have been evaluated on the basis of the induction of depressive like symptoms also seen in major depressed patients, like anhedonia, reduced social activity, sleep disturbances, alterations in food consumption, and on the basis of the reversal of these symptoms by antidepressant treatment. Only recently, few studies have been undertaken to assess immune parameters and cytokine secretion in these animals.

Administration of lipopolysaccharides to animals induces an array of symptoms commonly referred to as sickness behaviour (Dantzer et al., 1998b). Reduced food intake, weight loss, decreased social and locomotor activity, and anhedonia (mostly measured as a decrease in the consumption and preference for saccharine solution) are part of the behavioural symptoms (Yirmiya, 1996). The symptoms of sickness behaviour can be effectively attenuated by pretreatment with antidepressants (Yirmiya, 1996; Shen et al., 1999; Yirmiya et al., 2001). It is thought that the LPS-induced production of pro-inflammatory cytokines forms the basis for this phenomenon (Dantzer et al., 1998a). Therefore, the effect of antidepressant treatment on cytokine production in endotoxaemic animals has been investigated. Shen et al. reported that chronic treatment with the TCA desipramine inhibits the LPS-induced TNF- α production and potentiates the secretion of IL-10 in response to LPS administration (Shen et al., 1999). The desipramine pretreatment also attenuated the LPS-induced behavioural response. However, neither the SSRI paroxetine, nor the SNRI venlafaxine had any effect on the behavioural response or on the secretion of TNF- α and IL-10. In contrast, chronic treatment with imipramine or fluoxetine had no effect on the LPS-induced splenic TNF- α and IL-1 β mRNA expression, although fluoxetine was effective in attenuating some of the effects on behaviour and also on the LPS-induced activation of the hypothalamo-pituitary-adrenal axis (HPA-axis) (Yirmiya et al., 2001). In a recent study, it was found that the atypical antidepressant tianeptine effectively reduced symptoms of sickness behaviour induced by peripheral administration of either LPS or IL-1 β (Castanon et al., 2001). This effect was not seen when LPS or IL-1 β was administered by intracerebroventricular injection, suggesting that tianeptine attenuated peripheral rather than central cytokine actions on behaviour. Additionally, only chronic, but not acute, tianeptine pretreatment attenuated the LPS-induced sickness-behaviour.

Connor et al. (2000) examined the production of IL-1 β and TNF- α after an LPS-challenge in olfactory bulbectomized rats. This animal model of depression exhibits many behavioural, neurochemical, endocrine and immune alterations that are qualitatively observed in clinically depressed patients. Furthermore, chronic antidepressant treatment is able to reverse these effects (Kelly et al., 1997). Olfactory bulbectomized rats displayed decreased IL-1 β and TNF- α levels after LPS injection compared to sham-operated control rats. Chronic pretreatment with desipramine decreased the production of IL-1 β and TNF- α in control rats and further reduced the production of these cytokines in olfactory bulbectomized rats (Connor et al., 2000).

It has been shown that chronic imipramine treatment in animals subjected to chronic mild stress (CMS) effectively reverses the reduced sucrose intake (a measure for anhedonia) in the stressed animals (Kubera et al., 1995). This reversal is accompanied by a reduced proliferation of splenocytes in response to mitogen, only in the stressed animals. Moreover, in these stressed animals (and not in control animals), the production of IL-1 and IL-2 by splenocytes was significantly reduced after chronic imipramine administration (Kubera et al., 1996). In another study, it was shown that chronic desipramine treatment significantly increases the capacity of splenocytes to produce IL-10, from both control and CMS-subjected animals. The capacity of splenocytes to produce IL-2, IL-4 and IFN- γ was unaffected (Kubera et al., 2001b). In addition, acute administration of desipramine in mice, caused an increase in the stimulated production of IL-10 from splenocytes, an effect that was completely reversed by an acute stressor (Kubera et al., 1998). In the same splenocytes, the production of IFN- γ , IL-2 and IL-4 were unaltered following desipramine treatment. The secretion of IFN- γ however, was severely inhibited by the acute stressor, regardless of desipramine treatment. Recent studies from Kubera et al., showed that prolonged (28 day) administration of the TCAs amitriptyline and desipramine and the SSRIs fluoxetine and citalopram, increases the stimulated production of IL-10 by splenocytes from normal (not subjected to chronic mild stress) mice (Kubera et al., 2000a, c).

It is concluded that the production of the pro-inflammatory cytokines TNF- α and IL-1 β is reduced after chronic (pre-)treatment with TCAs like imipramine and desipramine. In addition, both antidepressants seem to upregulate the production of IL-10. Hence, especially TCAs have anti-inflammatory effects *in-vivo* and could contribute to successful antidepressant therapy in depressed patients that display hypersecretion of cytokines (Shen et al., 1999). The influence of SSRIs on cytokine production in these animal models remains unclear.

5. EFFECTS OF ANTIDEPRESSANTS ON CYTOKINES IN THE BRAIN

Only few studies report on the effects of antidepressants on cytokine expression in the brain. Suzuki et al. found that imipramine, fluvoxamine and maprotiline increase mRNA levels of IL-1 β and IL-1RA in several regions of the rat brain (Suzuki et al., 1996). The expression of IL-1RA mRNA was much more increased than that of IL-1 β , suggesting that chronic exposure to the antidepressants induces an IL-1 antagonistic activity in the brain. Interestingly, the maximal increase in IL-1RA mRNA was observed after 28 days of treatment, which parallels that of the clinical onset of antidepressants. However, the effects of the antidepressants was not specific, since also several neuroleptics were found to increase the mRNA of these cytokines (Suzuki et al., 1996).

It has been shown that some cytokines, including TNF- α , may play a physiological role in neurotransmission (Vitkovic et al., 2000). Thus, low levels of TNF- α are found within neurons of the hippocampus, locus coeruleus (LC) and hypothalamus of normal rats (Ignatowski and Spengler, 1994). Acute desipramine administration induces an accumulation of TNF- α mRNA in the LC and increases TNF- α protein levels in the hippocampus (Ignatowski and Spengler, 1994). Chronic desipramine administration also increases total hippocampal TNF- α content but decreases neuronal associated TNF- α in hippocampus and LC (Ignatowski et al., 1997), although no differences were found in the accumulation of TNF- α mRNA (Nickola et al., 2001). Whether this points to an increased release or degradation of TNF- α protein is not clear, just as is the role of this phenomenon in antidepressant drug action. It is suggested that TCAs as well as SSRIs may alter the regulatory effect of TNF- α on noradrenaline release in conjunction with α_2 -adrenergic receptors (Nickola et al., 2001).

6. POSSIBLE MECHANISMS OF ANTIDEPRESSANT ALTERED CYTOKINE PRODUCTION

Only few studies have addressed the issue of how antidepressants may modulate cytokine secretion. Nevertheless, several possible mechanisms can be suggested from the present data. It should be noted that one must distinguish the different approaches used to examine the effects of antidepressants on cytokine secretion. Thus, the mechanism underlying the effects of antidepressants on *ex-vivo* cytokine production by lymphocytes may be different from that underlying the decrease in plasma levels of pro-inflammatory cytokines in depressed patients.

Moreover, different mechanisms may be involved in the modulation of cytokine production in humans versus laboratory animals.

A first possible mechanism may be related to the effect of antidepressants on the serotonergic system. The presence of receptors for serotonin on immune cells has been demonstrated, both in humans and in animals (Aune et al., 1993, 1994; Stefulj et al., 2000). Further, serotonin may influence cytokine production. Arzt et al. showed that serotonin inhibits the stimulated production of IFN- γ by human PBMCs and of TNF- α in human monocytes (Arzt et al., 1988, 1991). In a recent study it was shown that serotonin, in supra-physiological concentrations (1.5 and 15 $\mu\text{g/ml}$), decreased the IFN- γ production in stimulated whole blood (Kubera et al., 2000b). These findings are similar to those found with antidepressant drugs. There are, however, numerous reports which show immune-enhancing effects of serotonin. This discrepancy may be explained by the following. Experiments with para-chlorophenylalanine (PCPA: an inhibitor of serotonin synthesis that depletes intracellular serotonin) indicate that endogenous serotonin is necessary for optimal immune function. PCPA inhibits T-cell proliferation and activation (Young et al., 1993; Aune et al., 1994; Young and Matthews, 1995), effects that can be reversed by the addition of serotonin to the culture medium. Kubera et al. (2000b) found that PCPA inhibits the production of both IFN- γ and IL-10. Further, low doses of serotonin (in the range of physiological concentrations) promote the production of IFN- γ and IL-2 (Hellstrand et al., 1993; Young et al., 1993). Taken together, these data suggest that intracellular serotonin and low concentrations extracellular serotonin are necessary for optimal production of cytokines and normal immune functions, whereas higher concentrations of serotonin (e.g. supra-physiological) are immunosuppressive and inhibit cytokine production. Lymphocytes and monocytes are able to synthesize and secrete serotonin upon stimulation with mitogens (Finocchiaro et al., 1988; Aune et al., 1994). Therefore, in *in-vitro* studies with immune cells, stimulation with LPS or mitogens results in increased concentrations of extracellular serotonin. Antidepressants (especially SSRIs) that inhibit the reuptake of serotonin may further increase serotonin concentrations in culture medium (Ferjan and Erjavec, 1996). This may result in high extracellular serotonin concentrations and the subsequent inhibition of cytokine production. As such, this may explain the inhibitory effect of antidepressants on cytokine secretion when added *in-vitro*. In Section 2, it was mentioned that treatment with antidepressants might normalize elevated cytokine levels in depressed patients. It has been shown that prolonged pharmacotherapy can result in a depletion of serotonin from platelets and blood cells, and in a decrease of serotonin plasma levels (Karage et al., 1994; Fekkes et al., 1997; Alvarez et al., 1999). Therefore, since physiological serotonin levels are indispensable for cytokine production, it is suggested that

reduction of these concentrations and/or depletion of intracellular serotonin might result in a down-regulation of cytokine production and in the subsequent decrease of cytokine plasma levels.

A second possible mechanism of antidepressant altered cytokine production revolves around the capacity of antidepressants to increase intracellular concentrations of cyclic adenosine monophosphate (cAMP). Indeed, it has been shown that antidepressants of several classes may increase cAMP levels in immune cells. Xia et al. (1996) found that a 24h. pre-incubation with imipramine, clomipramine or citalopram enhances cAMP concentrations after stimulation in both monocytes and lymphocytes. Others found that fluoxetine at pharmacological concentrations increases cAMP formation in T-lymphocytes when optimal stimulation conditions were employed (Edgar et al., 1999). cAMP is formed from ATP by the enzyme adenylate cyclase. The activity of this enzyme is modulated by several G-protein coupled receptors. For example, β_2 -adrenergic receptors are positively coupled to adenylate cyclase and stimulate cAMP formation after ligand binding. Some serotonergic receptors (5-HT-receptors) are also positively coupled to adenylate cyclase (5HT-4, 5HT-6 and 5HT-7 receptors), whereas others are negatively coupled and will downregulate adenylate cyclase activity (5HT-1A receptor). It is thought that cAMP activates Protein Kinase A (PKA), a kinase that in turn stimulates other downstream enzymes, including transcription factors like cAMP responsive element binding protein (CREB). Activation of the cAMP/PKA cascade results in altered cytokine expression (for review see Zidek, 1999). It is generally believed that pharmacological augmentation of cAMP down-regulates the expression of pro-inflammatory cytokines like IFN- γ and TNF- α (Benbernou et al., 1997; Eigler et al., 1998; Brideau et al., 1999) and increases that of IL-10 (Platzer et al., 1995, 1999; Benbernou et al., 1997; Eigler et al., 1998). Thus, antidepressant induced increases in cAMP levels, may result in a decreased production of IFN- γ and in an enhanced IL-10 secretion. However, we should mention that in our laboratory, we were not able to confirm that antidepressants (imipramine and paroxetine) increased cAMP levels in human PBMCs after stimulation with LPS and PHA (Kenis et al., 2003). Moreover, in stimulated whole blood, the antidepressant induced decrease in IFN- γ and TNF- α production, could only partially be reversed by pre-incubation with antagonists of adenylate cyclase (which reduces cAMP formation) or PKA (see Chapter 7). In this respect, it should be noted that the influence of fluoxetine on cAMP levels is dependent on the conditions of stimulation (Edgar et al., 1999).

7. CONCLUSION

Accumulating evidence suggests that major depression is associated with an upregulation of pro-inflammatory cytokines. It remains elusive whether these cytokines play a causative role in major depressive disorders, although the majority of data point to this direction. Therefore, it is warranted to examine the influence of antidepressants on cytokine production, since a causative relationship implies an inhibitory effect of antidepressants on pro-inflammatory cytokines. Reports from studies comparing cytokine levels and/or *ex-vivo* cytokine production before and after pharmacotherapy in depressed patients, present divergent and sometimes conflicting results. Nevertheless, we carefully conclude that antidepressant treatment may normalize cytokine production in depressed patients. As mentioned before, differences in methodology and heterogeneity of the study population may underlie these discrepancies. The need to use more standardized and immunological relevant methods in the investigation of cytokine homeostasis in psychiatric patients is put forward. When examining *ex-vivo* cytokine production, the use of diluted, stimulated whole blood cultures is preferred. Circulating cytokine levels in serum or plasma should be determined with specific, sensitive and well-standardized immuno-assays (e.g. ELISA). Further, it is possible that not all patients suffering from major depression display a hypersecretion of cytokines. It may therefore be warranted to differentiate patients into individuals with elevated cytokine levels and those who have not. The examination of cytokine alterations during treatment and the comparison of different antidepressants in this effect, may give more insight into both the pathophysiology of major depression and the mechanism of action of antidepressant drugs. Concerning *in-vitro* studies, it is clear that antidepressants of different classes have negative immunoregulatory effects. These data indicate that antidepressants may downregulate pro- and upregulate anti-inflammatory cytokines. However, it should be noted that they reflect acute effects on cytokine production. In view of the delayed clinical onset of antidepressants, one should keep in mind that only studies dealing with chronic exposure to antidepressants may be relevant. Yet, it is conceivable that acute effects on cytokine production, which are maintained during prolonged treatment, is reflected in improved depressive symptomatology only after the biological processes related to the behavioural effects of cytokines are restored. The use of animal models may be a suitable tool to examine the influence of antidepressants on cytokine production. Chronic treatment with TCAs seems to inhibit pro-inflammatory cytokine secretion and enhances the production of IL-10. Whether the same is true for other antidepressants remains to be determined. Animal models may also serve

as an efficient tool to study cytokine expression in the brain and the influence of antidepressants on it. Until now, no studies have really dealt with this issue. More research is obviously needed to establish the exact influence of antidepressants on cytokine production and the mechanisms underlying these effects. Undoubtedly, clarification of these questions will provide more insight into the pathophysiology of major depression and could contribute to the search for more efficient antidepressant drugs.

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Chapter 3

Stability of interleukin-6, soluble interleukin-6 receptor, interleukin-10 and CC16 in human serum

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ABSTRACT

The stability of interleukin 6 (IL-6), its soluble receptor (sIL-6R), IL-10 and CC16 or uteroglobin (an endogenous cytokine inhibitor) in human serum was examined using an accelerated stability testing protocol according the Arrhenius equation. Further, the effect of time delay between blood sampling and sample processing, clotting temperature and repeated freeze-thaw cycles on serum levels of these proteins were determined. Paired serum samples were stored at 4°C, 20°C, 30°C and 40 °C for 1 to 21 days. We found that IL-6 and CC16 concentrations did not change at 4°C, 20°C and 30°C. IL-6 concentrations significantly declined after 11 days at 40°C. The concentrations of sIL-6R and IL-10 did not change at 4°C but significantly decreased at 20°C (after 21 and 14 days respectively), 30°C and 40°C (after 1 day at both temperatures for both cytokines). Arrhenius-plots indicated that sIL-6R and IL-10 are stable for at least several years at -20°C and -70°C, respectively. Since their relative stability, no Arrhenius-plot could be calculated for IL-6 and CC16. The concentrations of the proteins examined were not significantly altered by repeated freeze-thaw cycles, nor by extended clotting times at 4°C or 20°C. We conclude that serum samples for the determination of IL-6, sIL-6R and CC16 can be stored at -20°C for several years, but for IL-10 determinations, storage at -70°C is recommended.

INTRODUCTION

During the past decade, cytokines and other inflammatory mediators have been extensively investigated in various diseases, including autoimmune diseases, psychiatric disorders (major depression, schizophrenia, anxiety and eating disorders) (Lin et al., 1998; Maes et al., 1999) and neurodegenerative diseases (e.g. Alzheimer's disease) (Bonaccorso et al., 1998). To study the role of cytokines in these human diseases, samples of biological fluid (serum, plasma, cerebrospinal fluid, synovial fluid, etc.) are collected, processed and concentrations of the respective cytokines determined by immuno-assays, e.g. radio immuno-assays (RIA's) or enzyme immuno-assays (EIA). However, two types of bias can interfere with study results, i.e. the biological and pre-analytical variability. Biological variability comprises all fluctuations in analyte concentrations due to natural conditions, such as seasonal and diurnal variability, age, gender, the menstrual cycle and other conditions that change the analyte concentration *in-vivo* (Fraser, 1986; Maes et al., 1995). Pre-analytical variability can be described as the variation in the analyte concentrations due to pre-analytical conditions such as blood sampling, storage etc. The present study focuses on the pre-analytical variability as a possible source of error in studies concerning cytokines or other serum proteins.

When collecting serum or plasma for cytokine determinations, attention should be paid to the processing of the samples, since it is known that anti-coagulants and time before centrifugation may influence cytokine concentrations (Thavasu et al., 1992; De Jongh et al., 1997; Flower et al., 2000). Cytokines have short half lives *in-vivo* and may be further degraded *ex-vivo*. On the other hand, *ex-vivo* induction of cytokines or other inflammatory mediators during blood processing can occur (Riches et al., 1992; Thavasu et al., 1992). This is especially important when blood samples are collected and later transported to the laboratory for processing (centrifugation, separation of plasma or serum and storage). Moreover, in longitudinal studies, samples are collected over time, stored at -20°C or -70°C , and then simultaneously analysed in order to minimize inter-assay variability. However, it has remained unknown whether inflammatory mediators like cytokines are degraded during long-term storage or denatured by the freezing and thawing process. Thus, it is of great importance to know how long samples can be stored before measurement of the respective protein by immuno-assay is affected.

Therefore, we examined the stability of several inflammatory mediators using an accelerated stability testing protocol. This procedure is based on the Arrhenius equation, which describes a linear relationship between the logarithm of the reaction rate constant (e.g. the degradation rate) and the inverse of the absolute temperature (Kirkwood, 1977; Anderson and Scott, 1991; Ruiz et al.,

1996). The degradation rate constant k at different temperatures is determined. The logarithm of k is plotted versus the inverse of the absolute temperature, which gives the so called Arrhenius-plot. Using the equation of the best-fit line, one can calculate the degradation rate constant at every desirable temperature.

The specific aims of this study were 1) to examine the stability of interleukin 6 (IL-6), soluble IL-6 Receptor (sIL-6R), IL-10 and Clara Cell Protein 16 (CC16, an endogenous cytokine inhibitor) in serum using an accelerated stability testing protocol according to Arrhenius; 2) to examine the influence of clotting time (e.g. the time delay between blood sampling and sample processing) on protein concentration measurement by ELISA; and 3) to examine the effects of repeated freeze-thaw cycles on the measured protein concentration.

METHODS

Samples

Since serum concentrations of IL-6 and IL-10 are very low in healthy humans, the stability of these cytokines was examined in serum of five post-surgical patients admitted on an Intensive Care Unit. The stability of sIL-6R and CC16 was investigated in serum of the same patients and in that of three healthy volunteers. Blood of the patients was drawn from the first stopcock of an arterial line system (Pressure Monitoring Kit, Baxter BV, Utrecht, The Netherlands) after initial elimination of 10 ml of blood. Then blood was collected in three 10 ml and eight 5 ml vacuumed serum tubes (SST Vacutainer® Hemoguard, Becton Dickinson, Meylan, France). Blood from the healthy volunteers was drawn using Vacutainer Systems (Becton Dickinson, Meylan, France). The collected blood was then processed as described below.

Accelerated stability testing protocol

The blood in the three 10 ml serum tubes was allowed to clot for 30 minutes at room temperature and subsequently centrifuged for 15 minutes at 1600xg. The serum from each subject was pooled in a sterile tube (50 ml, Falcon®, Becton Dickinson) and subsequently fractionated in sterile 1.5 ml eppendorf tubes, 0.3 ml per tube. Then, the tubes were stored at the different stress temperatures. Eleven tubes were put at each stress temperature: 4°C, 20°C, 30°C and 40°C. Three tubes were immediately frozen at -20°C and served as controls.

For up to 21 days, at regular time points, one tube from each temperature was frozen at -20°C. At the end, all samples were kept at -20°C until analysis.

Influence of clotting time

To check the influence of the time delay between blood sampling and centrifugation, and the influence of clotting temperature, serum tubes were processed as follows.

Immediately after blood collection, four tubes per subject were put at 4°C and four were kept at room temperature. After 30 minutes, one tube at each temperature was taken and centrifuged for 15 minutes at 1600xg. Serum was immediately collected and frozen at -20°C. The same procedure was repeated after 24, 48 and 96 hours. Samples were kept frozen until cytokine analysis.

Cytokine analysis

Cytokines were determined by enzyme linked immunosorbent assays (ELISA) from Eurogenetics (Tessenderlo, Belgium). Analysis was performed according manufacturer instructions. As stated by the manufacturer, the inter- and intra-assay variability was lower than 10% for all assays used.

Calculations and statistics

We assumed that the concentration of the proteins in the samples that were kept frozen during the 21-day stress period remained constant. The concentrations of the proteins in the stressed samples were compared to these control samples and the percentage recovery was calculated. After checking for normality using the Kolmogorov-Smirnov test, student t-test was used to compare the recovery after each time point (at each temperature) with the control samples. Further, the recovery was plotted versus days of heat stress and the slope of the best-fit line was determined. For IL-10 and sIL-6R, only the first three time points were used to calculate the best fit of the 40°C line because the concentration of both cytokines decreased to less than 20% after five days.

Then, the degradation rate was plotted versus the inverse of the absolute temperature times 1000. The equation of the best-fit line was used to calculate the degradation constant at -20°C and -70°C.

The kinetics of protein denaturation are comparable with that of a first order reaction (Kirkwood, 1977; Ruiz et al., 1996), which means that the degradation rate is proportional to the concentration of the respective analyte.

The equation is:

$$-d[C]/dt = k [C]$$

or $\log ([C]/[C]_0) = - kt/2.303$

where $[C]_0$ is the initial concentration and $[C]$ is the concentration after time t . We additionally calculated the time after which 90% of the respective cytokine can be recovered, by substituting $[C]/[C]_0 = 0,90$ in the last equation.

RESULTS

Stability

The stability in serum of sIL-6R and CC16 was investigated in 5 patients and 3 healthy controls, while the stability of IL-6 and IL-10 in serum was investigated in patients only. The concentration of sterile samples of the subjects stored during 21 days at 4°C, 20°C, 30°C and 40°C were compared to triplicate samples of the same person frozen at -20°C. The mean concentrations of the serum proteins in patients and controls of the frozen samples are summarized in Table 1. The data depicted in Figure 1(A) show that the IL-6 concentration in the serum samples stored at 4°C, 20°C and 30°C remained similar to the concentration in the frozen samples over 21 days. The relative stability of IL-6 in serum at these three temperatures yielded to few data to calculate an Arrhenius-plot. The samples stored at 40°C showed a decline down to 88% from the concentration measured starting from day 11, via the significant lower recovery of 66% at day 18 ($t=-4.00$, $p<0.05$) down to 59% of the concentration of the frozen samples at day 21.

The data in Figure 2(A) show the concentration of sIL-6R in serum at the different temperatures. The sIL-6R concentration in serum samples stored at 4°C remained similar to that in the frozen samples over the whole duration of the experiment. A significant, apparently linear, decline in the mean concentration of sIL-6R in serum stored at 20°C was observed down to 87% of the concentration of the frozen samples after 21 days ($t=-2.30$, $p<0.05$). After one day of storage at 30°C the concentration declined down to 91% ($t=-1.93$, $p<0.1$) of the frozen samples, down to the significant lower recovery of 85% at day 2 ($t=-3.08$, $p<0.01$). The concentration of sIL-6R in serum stored at this temperature of 30°C further declined gradually over time down to 63% at day 21 ($t=-7.45$, $p<0.0001$). The concentration of sIL-6R in the serum samples stored at 40°C showed a 50% decline after one day ($t=12.00$, $p<0.0001$) and declined to undetectable concentrations at day 12. The values of the degradation rate constant at each temperature are shown in Table 2. After calculation of the Arrhenius-plot, the graph and formula shown in Figure 2(B) were obtained for sIL-6R. Using this equation, we determined the k -value at -20°C and calculated the storage period after which 90% of sIL-6R can still be recovered at this

temperature. The projected k -value is 6.398×10^{-6} and the predicted storage time is thus approximately 45 years at -20°C .

Table 1

Serum concentrations of the examined inflammatory markers.

Patient	n	IL-6 Mean (SD)	sIL-6R Mean (SD)	IL-10 Mean (SD)	CC16 Mean (SD)
HV-1	2	5.9 (0.0)	131.6 (6.4)	1.7 (0.1)	26.4 (0.8)
HV-2	3	0.7 (0.6)	207.0 (40.4)	1.2 (0.5)	61.4 (8.7)
HV-3	3	2.9 (1.2)	204.0 (19.9)	2.0 (1.3)	22.3 (1.8)
P1	3	187.6 (8.4)	184.1 (30.3)	26.5 (1.3)	22.4 (1.5)
P2	3	50.7 (2.4)	131.4 (10.4)	12.1 (3.1)	37.2 (43.2)
P3	3	33.3 (2.3)	245.9 (22.2)	13.7 (2.1)	73.1 (43.9)
P4	3	2617.5 (77.7)	79.9 (9.8)	121.8 (9.2)	16.6 (4.4)
P5	2	141.3 (11.7)	212.5 (5.5)	38.8 (5.7)	26.8 (20.1)

Concentrations of the examined proteins in the control samples (samples that were immediately stored at -20°C , and not subjected to heat stress). HV = Healthy Volunteer; P = Patient.

Table 2

k -values for sIL-6R and IL-10.

Temperature ($^\circ\text{C}$)	sIL-6R	IL-10
4	0.0802	-0.0715
20	0.8017	2.9556
30	1.4755	4.3875
40	32.191	11.901

Percentage recovery was plotted versus days of heat stress at each temperature. Values of the degradation rate constant k for sIL-6R and IL-10 at these different stress temperatures were determined as the slope of the best fit line.

Figure 1

Recovery of (A) IL-6 and (B) CC16 versus days of heat stress. Sample aliquots were stored for up to 21 days at 4°C (◇), 20°C (■), 30°C (○) or 40°C (X). The recovery was calculated as percentage of control samples (e.g. sample aliquots that were immediately stored at -20°C). One point represents the mean recovery in aliquots of serum samples from five patients for IL-6, and from five patients and three healthy volunteers for CC16.

Figure 2

(A) Recovery of sIL-6R versus days of heat stress. Sample aliquots were stored for up to 21 days at 4°C (◇), 20°C (■), 30°C (○) or 40°C (X). The recovery was calculated as percentage of control samples (e.g. sample aliquots that were immediately stored at -20°C). One point represents the mean recovery in aliquots of serum samples from five patients and three healthy controls.

(B) Arrhenius-plot for sIL-6R. The logarithm of the degradation rate constant k is plotted versus $1000 \cdot (1/T)$ (where T = temperature, expressed as °K).

The equation of the best-fit line is: $\log(k) = -5.7863 \cdot 1/T + 17.677$.

Figure 3
 (A) Recovery of IL-10 versus days of heat stress. Sample aliquots were stored for up to 21 days at 4°C (◊), 20°C (■), 30°C (○) or 40°C (X). The recovery was calculated as percentage of control samples (e.g. sample aliquots that were immediately stored at -20°C). One point represents the mean recovery in aliquots of serum samples from five patients.
 (B) Arrhenius-plot for IL-10. The logarithm of the degradation rate constant k is plotted versus $1000 \cdot (1/T)$ (where T = temperature, expressed as °K).
 The equation of the best-fit line is: $\log(k) = -2.7597 \cdot 1/T + 7.844$.

The concentrations of IL-10 in serum samples at the different temperatures during 21 days are shown in Figure 3(A). No significant decline in IL-10 concentrations in serum stored at 4°C was observed during the period of the experiment. The samples stored at 20°C showed a decline in IL-10 concentration to 63% from on day 14 ($t=-3.54$, $p<0.001$) and to 35% after 21 days. The IL-10 concentrations in the samples stored at 30°C declined from day 7 on ($t=-4.40$, $p<0.0001$). The serum concentration of IL-10 in the samples at 40°C was already decreased to 70% ($t=-2.91$, $p<0.01$) after one day of storage. Further decrease during the following days resulted in levels indistinguishable from background at day 14. The k -values for the degradation at these temperatures are shown in Table 2. Calculation of the Arrhenius-plot yielded the graph and formula demonstrated in Figure 3(B) for IL-10. The projected k -values at -20°C and -70°C are 8.63×10^{-4} and 1.776×10^{-6} respectively. The corresponding storage times at which 90% of the initial IL-10 concentration can be recovered are 122 days at -20°C and approximately 162 years at -70°C. The concentration of CC16 in the serum samples remained comparable to the frozen samples at all temperatures as shown in Figure 1(B). Therefore, no Arrhenius-plot could be calculated.

Clotting time

The data depicted in Table 3 show that the serum concentration of IL-6, sIL-6R, IL-10 and CC16 are not significantly different, at room temperature, between 30 minutes clotting time and a 4-day clotting, when stored at room temperature. In addition, no differences in concentration of the proteins are observed when clotting at room temperature was compared with clotting at 4°C for up to four days.

Influence of freeze-thaw cycles

The data summarized in Table 4 show that no significant decline in the concentrations of IL-6, sIL-6R, IL-10 or CC16 is observed after 2, 3 and 4 times repeated freeze-thaw cycles.

Table 3

Influence of clotting time and temperature on recovery of IL-6, sIL-6R, IL-10 and CC16 in serum.

	Temperature (°C)	% recovery (S.D.) after 4 day clotting
IL-6 (n=5)	4	96.9 (18.5)
	20	84.1 (10.8)
sIL-6R (n=8)	4	97.6 (17.9)
	20	103.1 (13.1)
IL-10 (n=5)	4	106.2 (35.6)
	20	109.9 (63.3)
CC16 (n=8)	4	105.0 (13.8)
	20	106.9 (14.5)

% recovery of the cytokines after 4 days clotting at 4°C and 20°C. Concentrations were compared with a standardized clotting time of 30 minutes and % recovery calculated. This experiment was performed on samples of patients only for IL-6 and IL-10, and on samples of patients and controls for sIL-6R and CC16.

Table 4

Influence of freeze-thaw cycles on recovery of IL-6, sIL-6R, IL-10 and CC16 in serum.

	% recovery (S.D.) after 4 freeze-thaw cycles
IL-6 (n=3)	100.9 (9.6)
sIL-6R (n=3)	117.4 (2.9)
IL-10 (n=3)	76.5 (37.9)
CC16 (n=3)	102.5 (2.0)

% recovery of the cytokines after 4 freeze-thaw cycles. Concentrations were compared with samples that were frozen only once.

DISCUSSION

To the best of our knowledge, this study is the first to report on the stability of several inflammatory markers in human serum, examined by means of an accelerated stability testing protocol. This method allows predicting stability for

longer storage periods at any temperature. We found that the recovery of IL-6 and CC16 did not decrease for up to 21 days at 4°C, 20°C and 30°C, and that CC16 concentrations even did not change at 40°C, whereas IL-6 concentrations decreased only after 21 days at this temperature. Soluble IL-6R and IL-10 were less stable, e.g. both protein concentrations did not change at 4°C but the recovery decreased gradually at 20°C and 30°C. The predicted storage time after which 90% of the initial concentration can be recovered is 45 years for sIL-6R at -20°C and 122 days and 162 years for IL-10 at -20°C and -70°C, respectively.

The only report to our knowledge that describes an accelerated stability testing protocol with cytokines, is that of Ruiz et al. (Ruiz et al., 1996). These authors examined the stability of tumor necrosis factor α (TNF- α) in cerebrospinal fluid (CSF) samples, and found it to be stable for at least 5 years at -70°C. They specifically used a pathological CSF-pool (with high TNF- α concentration) diluted with a normal CSF-pool to retain any degrading enzymes, and added sodium azide as preservative. In our study, undiluted serum samples were used for the test protocol and care was taken that to keep all samples sterile during the stress period.

In our study, the IL-6 concentrations did not significantly decline at 4°C, 20°C and 30°C. Therefore, it was not possible to calculate the degradation rate constant at those temperatures, and, hence, no Arrhenius-plot or projected stability time could be calculated. We conclude that IL-6 is very stable and that samples for IL-6 determination can be stored frozen for very long periods before recovery is significantly decreased. These results do not corroborate those from Spong et al., who found that IL-6 concentrations in frozen amniotic fluid samples decreased over time (Spong et al., 1998). A panel of amniotic fluid samples was measured two times with a one-year interval. The median IL-6 concentration from the second determination was significantly (20%) lower compared to those of one year earlier. However, several factors may explain the discrepancy between these and our findings. First, the higher protein concentration of serum may stabilize the IL-6 molecules. Moreover, high concentrations of sIL-6R are present in serum as compared to amniotic fluid (Opsjon et al., 1995). These molecules may bind IL-6 and protect it from proteolytic or thermal degradation. Second, detection of proteins in ELISA-systems is dependent on the binding of the antibodies with their respective epitopes on the protein. In some cases, these epitopes are conformational structures, which may change due to denaturation or proteolytic cleavage. The antibody then no longer binds its epitope and, hence, recovery in the ELISA decreases. We assume that the antibodies in our ELISA are able to detect partly denatured IL-6. In the study of Spong et al., a decrease in IL-6 concentrations was observed between two independent measurements (Spong et al., 1998). The

authors do not specify whether the same batch of ELISA was used; thus, interassay variation might be an interfering factor. To overcome this problem, we used an accelerated stability testing, so that all samples could be tested in one run with the same batch of reagents.

Another major finding of this study was that the concentrations of IL-6, sIL-6R, IL-10 and CC16 did not change when incubated at 4°C or 20°C for up to 4 days before centrifugation. De Jongh et al. (1997) also reported no change in IL-6 or sIL-6R concentrations when blood was incubated at 4°C for 24h before centrifugation (De Jongh et al., 1997). Others showed that IL-6 and IL-1 plasma levels were unchanged when stored for up to 3 hours at 37°C before centrifugation (Riches et al., 1992). Some studies, however, have shown a decrease in IL-6 recovery when blood samples were left unseparated for 24 hours at both 4°C and room temperature (Thavasu et al., 1992), and even after 4 hours at room temperature (Flower et al., 2000). Discrepancies between these studies are difficult to explain. In the study of Thavasu et al., IL-6 spiked blood samples were used (Thavasu et al., 1992). White blood cells or thrombocytes may take up the spiked IL-6, whereas *in-vivo* generated IL-6 may represent equilibrium between IL-6 production and uptake. Further, comparison between studies might be difficult due to differences in study design and/or immunoassays (e.g. radio- versus enzyme immunoassays, the antibodies used, etc.).

Finally, we showed that up to four repeated freeze-thaw cycles did not influence the recovery of IL-6, sIL-6R, IL-10 and CC16. This confirms the findings of other studies which demonstrated the resistance of IL-6, TNF- α , IL-1 α , IL-1 β , interferon- γ and other serum proteins (Leptin, Apolipoprotein E) to repeated freeze-thaw cycles (Thavasu et al., 1992; Flower et al., 2000; Schiele et al., 2000).

It is concluded that serum samples for the determination of IL-6, sIL-6R and CC16 can be stored at -20°C for several years, but for the determination of IL-10, serum samples should be stored at -70°C when longer storage periods (longer than three months) are required. Further, neither a time delay between blood sampling and serum separation, nor repeated freeze-thaw cycles have a negative influence on the recovery of these proteins. Thus, we showed that practical aspects of longitudinal and clinical studies (such as long term storage, extended clotting times and repeated freeze-thawing) do not influence the measurement of the proteins examined, including cytokines.

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Chapter 4

Anti-inflammatory effects of antidepressants through suppression of the interferon- γ /interleukin-10 production ratio

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ABSTRACT

There is some evidence that major depression and, in particular, treatment resistant depression (TRD) is accompanied by activation of the inflammatory response system and that pro-inflammatory cytokines may play a role in the aetiology of depression. This study was carried out to examine the effects of antidepressive agents, i.e. imipramine, venlafaxine, L-5-hydroxytryptophan (L-5-HTP) and fluoxetine on the production of interferon- γ (IFN- γ), a pro-inflammatory cytokine, and IL-10, a negative immunoregulatory cytokine.

Diluted whole blood of fluoxetine-treated TRD patients (mean age = 50.6 ± 3.9 years) and age-matched normal controls (mean age = 51.6 ± 1.7 years) and younger normal volunteers (mean age = 35.4 ± 9.6 years) was stimulated with phytohaemagglutinin (PHA, 1 $\mu\text{g/ml}$) and lipopolysaccharide (LPS, 5 $\mu\text{g/ml}$) for 48 hours with and without incubation with the antidepressants at 10^{-6} M and 10^{-5} M. IFN- γ and IL-10 were quantified by means of ELISA. The ratio of IFN- γ to IL-10 production by immunocytes was computed because this ratio is of critical importance in determining the capacity of immunocytes to activate or inhibit monocyte and T-lymphocytic functions.

All four antidepressive drugs significantly increased the production of IL-10. Fluoxetine significantly decreased the production of IFN- γ . All four antidepressants significantly reduced the IFN- γ /IL-10 ratio. There were no significant differences in the antidepressant-induced changes in IFN- γ or IL-10 between younger and older normal volunteers and TRD patients.

Tricyclic antidepressants, selective serotonin reuptake inhibitors, serotonin-noradrenaline reuptake inhibitors as well as the immediate precursor of serotonin have a common, negative immunoregulatory effect by suppressing the IFN- γ /IL-10 production ratio. It is suggested that the therapeutic efficacy of antidepressants may be related to their negative immunoregulatory effects.

INTRODUCTION

Recently, it has been demonstrated that major depression is related to activation of the inflammatory response system (IRS) (Maes, 1999). The evidence includes, amongst other things, an enhanced production of pro-inflammatory cytokines, such as interleukin-1 β (IL-1 β), IL-6 and interferon- γ (IFN- γ) (Seidel et al., 1995; Maes, 1999). An increased production of IL-1 and IL-2 has been observed in culture supernatant of mitogen-stimulated splenocytes in the mild, chronic stress rat and mice models of depression (Kubera et al., 1996, 1998a). It has been suggested that the increased production of pro-inflammatory cytokines may play a role in the aetiology of depression (Yirmiya, 1996; Maes, 1999). Indeed, IL-1, IL-6 and IFNs given to experimental animals and humans may produce behavioural alterations and symptoms similar to those observed in major depression, such as anhedonia, anorexia, weight loss, social withdrawal, psychomotor retardation, anergy, irritability and sleep disturbances (Maes, 1999).

If pro-inflammatory cytokines are involved in the aetiology of depression one would expect that antidepressive agents have negative immunoregulatory effects. Prolonged treatment with antidepressants suppresses the acute phase response in major depressed patients (Maes et al., 1997), and in the olfactory bulbectomized and chronic mild stress models of depression in rats (Sluzewska et al., 1994; Song and Leonard, 1994). A single as well as repeated injection of desipramine to naive mice increases the capacity of splenocytes to produce the negative immunoregulatory cytokine, IL-10 (Kubera et al., 1998b). *Ex-vivo* studies show that antidepressants inhibit the secretion of IL-1 β , IL-2, tumor necrosis factor- α (TNF- α), IFN- γ and inhibit the proliferative activity of T-cells and the cytotoxic activity of natural killer cells (Xia et al., 1996; Maes, 1999). Co-incubation of whole blood of normal humans with clomipramine, a tricyclic antidepressant (TCA) or sertraline, a selective serotonin reuptake inhibitor (SSRI), decreases IFN- γ production and increases IL-10 production (Maes et al., 1999b). IFN- γ is a T-helper 1 (Th1)-like, pro-inflammatory cytokine produced by activated T lymphocytes and natural killer cells, which boosts the production of IL-1 and IL-6 (Cavaillon, 1996). Interleukin-10 is produced by Th2-like lymphocytes, B-lymphocytes and monocytes, and has important anti-inflammatory and immunosuppressive properties through suppression of IFN- γ and other pro-inflammatory cytokines (Cavaillon, 1996). Therefore, the ratio of IFN- γ to IL-10 produced by immunocytes is of critical importance in determining their capacity to activate or inhibit monocytic and T-lymphocytic functions (Katsikis et al., 1995).

The aim of the present study is to examine the effects of antidepressive agents, i.e. imipramine (TCA), fluoxetine (SSRI), venlafaxine (serotonin-noradrenaline

reuptake inhibitor, SNRI) and the immediate precursor of serotonin, L-5-hydroxytryptophan (L-5-HTP) on the production of IL-10 and IFN- γ (and their ratio) by diluted whole blood of normal volunteers and patients with treatment resistant depression.

METHODS

Subjects

Blood samples for the determination of cytokine secretion were collected from 7 patients with TRD (mean age = 50.6 ± 3.9 years; male/female ratio: 2/5) and 7 age-sex-matched normal controls (i.e. > 45 years; mean age = 51.6 ± 1.7 years; male/female ratio: 2/5) and 12 younger normal volunteers (i.e. < 45 years; mean age = 35.4 ± 9.6 years; male/female ratio: 5/7). All subjects gave written informed consent after the study design was fully explained. The depressed inpatients were included if they met the DSM-IV diagnostic criteria for major depression. Diagnoses were made with the Structured Clinical Interview for DSM-III-R, Patient Version (SCID) (Spitzer et al., 1990). The depressed patients were resistant to one treatment trial with fluoxetine, 20-60 mg daily, administered daily for 6 weeks. They were taking fluoxetine when blood was sampled. The Hamilton Rating Scale for Depression (HDRS) score was used to measure severity of illness (Hamilton, 1960). The mean HDRS score in the depressed patients was $25.1 (\pm 3.2)$. The normal volunteers had a negative history of past and present axis-I psychiatric illnesses. None was a regular drinker and none had ever been taking antidepressant or antipsychotic agents. Normal volunteers and depressed subjects were free of any chronic medical illness. All subjects were free of acute infections or allergic reactions as well as drugs known to modify the immune or endocrine functions for at least one month before blood sampling. The subjects abstained from caffeine, nicotine and alcohol for at least 12 hr before blood sampling.

Whole blood stimulation

After an overnight fast, blood for the assays of IFN- γ and IL-10 was taken at 9 a.m. (± 30 minutes). The effects of antidepressants on cytokine secretion were studied by stimulating 1/10 diluted whole blood with PHA (1 $\mu\text{g}/\text{ml}$; Murex Diagnostics Ltd, Dartford, England) and LPS (5 $\mu\text{g}/\text{ml}$; *E. coli* 026:B6; lyophilized and sterilized by gamma-irradiation; Sigma, Belgium). 1.8 ml of RPMI-1640 medium with L-glutamine (Gibco BRL), 100 IU/mL penicillin (Sigma) and 100 $\mu\text{g}/\text{mL}$ streptomycin (Sigma) with PHA + LPS were placed into 24 well cell culture plates (Falcon 3047, Becton Dickinson). Diluted

whole blood stimulated with PHA + LPS offers the most appropriate and reproducible culture condition in order to measure cytokines, such as IFN- γ (De Groote et al., 1992; Maes et al., 1999b). Diluted whole blood cultures reflect the *in-vivo* immune cellular and humoral interactions and may be employed to examine the effects of drugs on cytokine secretion (De Groote et al., 1992; Maes et al., 1999b). The production of cytokines, such as IFN- γ , IL-6 and IL-10, under these culture conditions is related to *in-vivo* markers of cell-mediated immunity, such as serum concentrations of acute phase reactants and number of peripheral blood mononuclear cells, e.g. activated T-cells (De Groote et al., 1992; Maes et al., 1999a). The antidepressants were dissolved in sterile water, whereas sterile water alone served as the corresponding control. Twenty microliter of each antidepressant solution was added to the wells and gently mixed with the medium. A total of 0.2 ml of whole blood from each of 26 subjects were cultured with two concentrations, i.e. 10^{-5} M and 10^{-6} M, of imipramine hydrochloride (Ciba Geigy, Basel, Switzerland), venlafaxine hydrochloride (Wyeth, USA), L-5-hydroxytryptophan (Acros Organics) or fluoxetine hydrochloride (Eli-Lilly, Belgium). Viability of cells was checked by ethidium-bromide-dye exclusion. The samples were incubated for 24 hours in a humidified atmosphere at 37°C, 5% CO₂. Supernatants were taken off carefully under sterile conditions, divided into eppendorf tubes, and frozen immediately at -75°C. The drug concentrations employed here were chosen on the basis of literature on the therapeutic plasma concentrations of these agents. Thus, the 10^{-6} M concentrations employed here are in therapeutic range of plasma concentrations obtained during clinical treatment, whereas the higher concentrations correspond to the concentrations usually employed by other researchers in *ex-vivo* experiments which aim to examine the effect of drugs on isolated immunocytes. However, blood cells of patients are exposed to the antidepressants for longer periods of time and the intracellular loading of the drugs during *in-vivo* conditions may be much higher than the serum concentrations obtained during treatment with these drugs. IFN- γ and IL-10 were quantified by means of ELISA methods (Eurogenetics, Tessenderlo, Belgium) based on appropriate and validated sets of monoclonal antibodies as described by us previously (Maes et al., 1999b). All assays were carried out in one run on one and the same day. The intra-assay CV values for both analytes were less than 8%.

Statistics

Repeated measure design analysis of variance (RM design ANOVAs) was used to examine the i) within-subject variability with the effects of antidepressant drugs as temporal condition, i.e. positive control, and each of the four different

drugs at two concentrations; and ii) between-subject variability with the three study groups (younger controls, elderly controls and depressed patients) and gender as factors. Results of RM design ANOVAs were corrected for sphericity. Differences among treatment means were ascertained by means of the Dunn test. Tests on simple effects were employed to clarify main effects or significant interactions; a simple effect is defined as the effect of one variable at one level of the other variable. The IFN- γ /IL-10 ratio was computed as: z transformed IFN- γ - z transformed IL-10 (Maes et al., 1999b). IFN- γ and IL-10 and their ratio were processed in Box-Cox transformation.

RESULTS

Figure 1 shows that there were no significant effects of imipramine ($F=0.2$, $df=2/46$, $p=0.8$), venlafaxine ($F=2.7$, $df=2/40$, $p=0.08$) or L-5-HTP ($F=2.1$, $df=2/36$, $p=0.1$) on IFN- γ production (all results of RM design ANOVAs). There is a significant effect of fluoxetine on IFN- γ production ($F=14.8$, $df=2/42$, $p=0.00007$). IFN- γ production is significantly lowered by 10^{-6} M ($t=2.75$, $p=0.008$) and 10^{-5} M ($t=5.44$, $p=0.00003$) fluoxetine. IFN- γ production is significantly lower ($t=2.69$, $p=0.009$) after 10^{-5} M than after 10^{-6} M fluoxetine. There are no significant drug X group interactions. There are significant differences in IFN- γ between the three study groups ($F=7.4$, $df=2/23$, $p=0.003$). Analyses of simple effects show that there are significant inter-group differences in IFN- γ production in the positive control ($F=6.7$, $df=2/69$, $p=0.003$) and the imipramine 10^{-6} M ($F=7.0$, $df=2/69$, $p=0.002$) and 10^{-5} M ($F=6.4$, $df=2/69$, $p=0.003$) conditions. Dunn tests show significantly lower IFN- γ production in elderly volunteers than in younger volunteers ($t=3.79$, $p=0.001$) and depressed patients ($t=2.79$, $p=0.01$). There are no significant gender differences in IFN- γ ($F=0.3$, $df=1/20$, $p=0.6$) and no significant drug X gender or drug X gender X group interactions.

Figure 2 shows that imipramine ($F=28.3$, $df=2/44$, $p<10^{-4}$), venlafaxine ($F=18.1$, $df=1/30$, $p=0.0004$), L-5-HTP ($F=5.5$, $df=2/44$, $p=0.008$) and fluoxetine ($F=12.6$, $df=2/42$, $p=0.0001$) have significant enhancing effects on IL-10. Dunn tests show that i) IL-10 is significantly higher in the 10^{-5} M imipramine condition than in the control ($t=7.13$, $p<10^{-4}$) and 10^{-6} M ($t=5.64$, $p<10^{-4}$) conditions; ii) IL-10 is significantly higher in the 10^{-6} M ($t=3.32$, $p=0.002$) and 10^{-5} M ($t=6.00$, $p<10^{-4}$) venlafaxine condition than in the normal condition, and significantly higher in the 10^{-5} M than in the 10^{-6} M venlafaxine condition ($t=2.68$, $p=0.009$); iii) IL-10 is significantly higher in 10^{-5} M L-5-HTP condition than in the control condition ($t=3.12$, $p=0.003$); and iv) IL-10 is significantly higher in the fluoxetine 10^{-5} M condition than in the control ($t=5.03$, $p<10^{-4}$) and 10^{-6} M ($t=2.50$, $p=0.012$) fluoxetine conditions. There are no significant interactions between drug and

groups for any of the four antidepressants. There are no significant differences in IL-10 production between the three study groups. There are no significant gender differences in IL-10 ($F=1.7$, $df=1/20$, $p=0.2$) and no significant drug X gender or drug X gender X group interactions.

Figure 1

Effects of antidepressants on the stimulated production of IFN- γ (expressed in U/mL). Younger normal volunteers (●), older normal volunteers (○), patients with treatment resistant major depression (▼).

* Significantly different from the control condition.

Figure 2
Effects of antidepressants on the stimulated production of IL-10 (expressed in pg/ml). Younger normal volunteers (●), older normal volunteers (○), patients with treatment resistant major depression (▼).
* Significantly different from the control condition.

Figure 3

Effects of antidepressants on the IFN- γ /IL-10 production ratio. Younger normal volunteers (●), older normal volunteers (○), patients with treatment resistant major depression (▼).

* Significantly different from the control condition.

Figure 3 shows that imipramine ($F=8.33$, $df=1/32$, $p=0.006$), venlafaxine ($F=8.7$, $df=2/45$, $p=0.0009$), L-5-HTP ($F=5.8$, $df=2/35$, $p=0.007$) and fluoxetine ($F=27.5$, $df=2/38$, $p<10^{-4}$) significantly suppress the IFN- γ /IL-10 ratio. Dunn tests show i) a significantly lower IFN- γ /IL-10 ratio in the 10^{-5} M imipramine ($t=4.06$, $p=0.0004$) than in the control condition; ii) a lower ratio following venlafaxine 10^{-5} M ($t=3.88$, $p=0.0006$) and 10^{-6} M ($t=3.24$, $p=0.002$) than in control condition; iii) a lower ratio in the L-5-HTP 10^{-5} M condition than in the L-5-HTP 10^{-6} M and the control conditions; and iv) a lower IFN- γ /IL-10 ratio in the fluoxetine 10^{-6} M ($t=3.76$, $p=0.0007$) and 10^{-5} M ($t=7.41$, $p<10^{-4}$) conditions than in the control condition; and a lower ratio in the fluoxetine 10^{-5} M than in the 10^{-6} M condition ($t=3.65$, $p=0.0009$). There were no significant differences in the IFN- γ /IL-10 ratio between the study groups.

DISCUSSION

The main findings of this study are that imipramine, venlafaxine, L-5-HTP and fluoxetine significantly enhance IL-10 production and significantly suppress the IFN- γ /IL-10 ratio. These results extend those of (Xia et al., 1996) who found a trend toward a significant suppression of IFN- γ secretion and a significant inhibition of IL-1 β , IL-2 and TNF- α secretion by activated T-cells or monocytes pre-incubated with TCAs or SSRIs. Sertraline, clomipramine and trazodone, a heterocyclic antidepressant (HCA), significantly reduced the IFN- γ /IL-10 ratio (Maes et al., 1999b). The results of the above studies suggest that various types of antidepressive drugs, including TCAs, SSRIs, SNRIs, HCAs and L-5-HTP may have negative immunoregulatory effects. The effect of fluoxetine on the IFN- γ /IL-10 ratio in the patient group may be a large one. This effect may represent a priming effect by the oral dose on the cells prior to exposure to the *ex-vivo* dose. However, without a group of depressed untreated patients, it cannot be concluded whether the results of the patient group represent effects of depression or fluoxetine orally or both. Moreover, the results of the present study are laboratory (*ex-vivo*) observations only. Therefore, there is no evidence whether the findings are of clinical relevance. Nevertheless, prolonged treatment of depressed patients with antidepressants, including fluoxetine, is accompanied by a normalization of the initially increased production of IL-6, IFN- γ and positive acute phase proteins, suggesting that antidepressants have *in-vivo* anti-inflammatory effects (Seidel et al., 1995; Sluzewska et al., 1995; Maes et al., 1997; Maes, 1999). However, many drugs are known to alter cytokine production and in many cases these effects have no relationship to the primary function of these drugs. Systemic injection of LPS, which is a potent activator of the immune system, induced a depressive behavioural pattern in fluid-deprived rats, while chronic treatment with imipramine

reduces or blocks the suppressive effects of LPS on food consumption, body weight, social interaction and activity in the open-field test (Yirmiya, 1996). Bluthé et al. (1999) found that IL-10 antagonizes behavioural defects, such as depressed social behaviour, induced by LPS.

The mechanisms by which antidepressants modulate immune functions have remained elusive. Nahas et al. (1979) suggest that the immunological effects of TCAs are correlated with their liposolubility. Audus and Gordon (1982) hypothesized that TCAs may modulate immunocyte functions by binding to TCA binding sites. It is, however, unlikely that the immunological effects of these drugs are mediated through these binding sites, because TCAs are active *in-vitro* in the 10^{-4} - 10^{-6} M range, whereas the binding constant for this receptor is 4×10^{-8} M. The existence of receptors for noradrenaline and serotonin on human immunocytes provides another potential mechanism for the immune activity of antidepressants (Kubera et al., 2000). Stimulation of α_1 -, α_2 -adrenergic as well as 5HT1A or 5HT4 receptors on immunocytes by noradrenaline or serotonin increases the intracellular levels of cyclic Adenosine Monophosphate (cAMP) (Kubera et al., 2000). The latter plays a critical role in the production of IL-10 and IFN- γ . Thus, cAMP-elevating agents inhibit IFN- γ mRNA expression and the intracellular levels of IFN- γ , and increase the expression of IL-10 mRNA and the intracellular levels of IL-10 (Platzer et al., 1995; Benbernou et al., 1997). Xia et al. (1996) reported that TCAs as well as SSRIs significantly elevate intracellular cAMP-concentrations in T-lymphocytes and monocytes. Therefore, it is safe to hypothesize that the decrease in the IFN- γ /IL-10 ratio in immunocytes co-incubated with antidepressants results from increased cAMP-levels.

Finally, we found that the production of IFN- γ is significantly lower in the older than the younger volunteers and that the production of IFN- γ is significantly higher in fluoxetine-treated TRD patients than in age-matched normal volunteers. It is well known that ageing is associated with a decreased IFN- γ production (Candore et al., 1993). The higher IFN- γ production in depressed patients treated with fluoxetine may be explained by previous findings that IFN- γ production is significantly increased in depressed patients and that IRS activation is more pronounced in patients with TRD despite antidepressive treatments (Maes, 1999).

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Chapter 5

Effects of serotonin and serotonergic agonists and antagonists on the production of interferon- γ and interleukin-10

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ABSTRACT

Serotonin (5-HT) is a neurotransmitter and an immune modulator. *In-vitro*, antidepressants with a serotonergic mode of action have, at concentrations within the therapeutic range, negative immunoregulatory effects, i.e. they increase the production rate of interleukin-10 (IL-10), a negative immunoregulatory cytokine. We have hypothesized that part of these effects may be explained by the serotonergic activities of antidepressants on immunocytes. This study was carried out to examine the effects of 5-HT, p-chlorophenylalanine (PCPA), a 5-HT depleting agent, flesinoxan (a 5-HT_{1A} agonist), m-chlorophenylpiperazine (mCPP; a 5-HT_{2A/2C} agonist), and ritanserin (a 5-HT_{2A/2C} antagonist) on the production rate of interferon- γ (IFN- γ), a pro-inflammatory cytokine, and IL-10 by whole blood stimulated with polyclonal activators. The IFN- γ /IL-10 production ratio was computed, since this ratio reflects the pro- versus anti-inflammatory capacity of cultured whole blood. We found that 1) 5-HT, 150 ng/mL, 1.5 μ g/mL and 15 μ g/mL, significantly decreased the IFN- γ /IL-10 ratio; 2) PCPA (5 μ M) significantly suppressed the production of IFN- γ and IL-10; 3) flesinoxan (15 ng/mL; 1.5 μ g/mL) had no significant effects on the production of the above cytokines; and 4) mCPP (2.7 μ g/mL) and ritanserin (5.0 μ g/mL) suppressed the IFN- γ /IL-10 ratio. It is concluded that intracellular 5-HT may be necessary for an optimal synthesis of IFN- γ and IL-10, and that extracellular 5-HT concentrations at or above serum values may suppress the production of the pro-inflammatory cytokine IFN- γ . The negative immunoregulatory effects of antidepressive drugs are probably not related to their serotonergic activities.

INTRODUCTION

Serotonin (5-hydroxytryptamine, 5-HT) is a neurotransmitter, a vasoactive amine released by platelets at sites of inflammation and an immune modulator (Roszman et al., 1985; Mossner and Lesch, 1998; Kubera and Maes, 2000). In humans, 5-HT is produced, outside the central nervous system, by enterochromaffin cells and it is stored in circulating platelets and to a lesser extent in monocytes and lymphocytes (Essmann, 1978). 5-HT is released from platelets and lymphocytes/monocytes following stimulation, by platelet activating factors or lectins/interferon- γ (IFN- γ), respectively (Finocchiaro et al., 1988). Moreover, it has been demonstrated that lymphoid organs are innervated by serotonergic neurons and that 5-HT may influence the proliferation and differentiation of immunocompetent cells (Cohen, 1985; Felten et al., 1991). Pharmacological and molecular analyses have confirmed the presence of 5HT1A and 5HT2A/C receptors on activated human immunocytes and specific 5-HT transporter sites on macrophages/lymphocytes (Aune et al., 1994).

The effect of 5-HT on cell-mediated immunity and on the inflammatory response system (IRS) is still a matter of controversy. Some studies show that 5-HT has immunosuppressive effects. Devoino et al. (1968) reported that substances, which have the property to increase 5-HT concentrations, inhibit immunogenesis and antibody production in rodents. 5-HT may suppress delayed type hypersensitivity and transplantation immunity (Devoino and Morozova, 1988). Bonnet et al. (1984) found that 5-HT suppresses murine lymphocytic responses to phytohaemagglutinin (PHA) and/or allogeneic cells. Para-chlorophenylalanine (PCPA), an inhibitor of 5-HT synthesis, enhances the production of antibodies (Jackson et al., 1985). Other immune functions, however, may be stimulated by 5-HT. For example, cutaneous injection of 5-HT can initiate a delayed-type hypersensitivity reaction through local recruitment and activation of CD4⁺ T-helper cells (Ptak et al., 1991). Low doses (100 ng/ml) of exogenously added 5-HT stimulate T cell proliferation in response to interleukin-2 (IL-2) (Young et al., 1993; Young and Matthews, 1995). Enhancement of murine T-cell blastogenesis by 5-HT has been shown to occur through the 5-HT2 receptor (Young et al., 1993), whereas activation of human T-cells may occur through 5-HT1A receptors (Aune et al., 1994). Jahnova (1994) showed immuno-activating effects of 5-HT on the production of antibodies. (Boranic et al., 1987), on the other hand, reported that 5-HT as well as PCPA suppressed antibody production.

The effects of 5-HT on the production of cytokines by human immunocytes was the subject of a few studies. Arzt et al. (1991) showed that 5-HT inhibits, in a concentration-dependent way, the LPS-induced production of tumor necrosis

factor by human macrophages. This effect was blocked by the 5-HT₂ receptor antagonist, ketanserin. In humans, 5-HT promotes IFN- γ production by NK cells probably through a 5HT_{1A} receptor mediated mechanism (Hellstrand et al., 1993).

There is now evidence that major depression is accompanied by disorders in the peripheral and central turnover of 5-HT (Maes and Meltzer, 1995). There is also evidence that the production rate of pro-inflammatory cytokines, such as IL-1, IL-6 and IFN- γ is increased in patients with major depression (Seidel et al., 1995; Sluzewska et al., 1995; Maes, 1997; Connor and Leonard, 1998). It is hypothesized that the increased production of the above cytokines may play a role in the aetiology of depression (Maes et al., 1995; Yirmiya, 1996; Connor and Leonard, 1998). Indeed, IL-1, IL-6 and IFN- γ given to experimental animals and humans may produce depressive symptoms or full blown major depression (Bluthe et al., 1992; Yirmiya, 1996; Maes, 1997).

It is thought that antidepressants, such as selective 5-HT reuptake inhibitors (SSRIs), 5-HT-noradrenaline reuptake inhibitors (SNRIs) and some tricyclic antidepressants (TCAs) exert their antidepressant activity through modulation of the brain serotonergic system (Maes and Meltzer, 1995). Antidepressants have also significant negative immunoregulatory activities. In depressed patients, prolonged treatment with SSRIs normalizes the initially increased production of IL-6, IFN- γ and positive acute phase proteins (Seidel et al., 1995; Sluzewska et al., 1995; Maes et al., 1997). *In-vitro*, co-incubation of human whole blood with SSRIs (fluoxetine, sertraline), an SNRI (venlafaxine), TCAs (imipramine, clomipramine), a reversible MAO-A inhibitor (RIMA; moclobemide), and L-5-hydroxytryptophan (L-5-HTP), the immediate precursor of 5-HT, significantly increased the production of IL-10 and/or decreased that of IFN- γ (Maes et al., 1999; Lin et al., 2000; Kubera et al., 2001). The results suggest that antidepressants with a serotonergic mode of action have, at concentrations within the therapeutical range, negative immunoregulatory effects. The exact mechanisms by which antidepressive drugs affect cytokine production have remained elusive. It may be hypothesized that part of these effects may be explained by the serotonergic activities of antidepressants (Maes and Meltzer, 1995), such as depletion of intracellular 5-HT stores, increased extracellular 5-HT, 5-HT_{2A/2C} receptor blockade or 5-HT_{1A} receptor stimulation. However, to the best of our knowledge, the effects of serotonergic agonists and antagonists on the production of IFN- γ and IL-10 have not been studied as yet.

The specific aims of the present study were to examine the effects of 5-HT, PCPA (a 5-HT depleting agent), flesinoxan (a 5-HT_{1A} agonist), mCPP (a 5-HT_{2A/2C} agonist), and ritanserin (a 5-HT_{2A/2C} antagonist) on the production rate of IFN- γ and IL-10 by whole blood stimulated with polyclonal activators. The results of the present study provide a critical test to elucidate whether

serotonergic mechanisms modulate the production of IFN- γ and IL-10; and whether the effects of antidepressants on these cytokines are mediated by serotonergic mechanisms.

METHODS

Subjects

Blood samples for the assay of IL-10 and IFN- γ were collected from 26 subjects, i.e. 19 healthy volunteers, divided into two subgroups according to age (< 45 years versus $\geq$ 45 years) and seven major depressed patients who suffered from treatment resistant depression (TRD). The mean ages and male/female ratio of these subgroups were: 12 younger volunteers: mean age = 35.4 ± 9.6 years, male/female ratio: 5/7; 7 older volunteers, mean age = 51.6 ± 1.7 years, male/female ratio: 2/5; and TRD patients: mean age = 50.6 ± 3.9 years; male/female ratio: 2/5. The normal volunteers had a negative past and present history of axis-I psychiatric illnesses. None was a regular drinker and none had ever been taking antidepressant or antipsychotic drugs. All subjects, i.e. normal volunteers and depressed patients, were free of medical illness. All subjects were free of acute infections, inflammatory or allergic reactions for at least two weeks prior to the study. They were free of drugs known to modify the immune or endocrine functions for at least one month before blood was sampled. All subjects abstained from caffeine, nicotine and alcohol for at least 12 hr before blood was sampled. The depressed patients were admitted to the University Department of Psychiatry, AZ Stuivenberg, Antwerp, Belgium. Patients fulfilled with the DSM-IV diagnostic criteria for major depression (APA, 1994). We used the Structured Clinical Interview for DSM-III-R, Patient Version to make the diagnosis (Spitzer et al., 1990). The depressed patients were resistant to treatment with fluoxetine, 20-60 mg daily, administered daily for at least 6 weeks. Patients were still taking fluoxetine when blood was sampled. The mean Hamilton Depression Rating Scale score (Hamilton, 1960) in the depressed patients was $25.1 (\pm 3.2)$. All subjects gave written informed consent after the study design was fully explained.

Whole blood stimulation

After an overnight fast, blood for the assays of IFN- γ and IL-10 was taken at 9 a.m. (± 30 minutes). The effects of the serotonergic agents on these cytokines were examined by stimulating 1/10 diluted whole blood with PHA (1 μ g/ml; Murex Diagnostics Ltd, Dartford, England) and LPS (5 μ g/ml; E.Coli 026; lyophilized and sterilized by gamma-irradiation; Sigma, Belgium). Diluted

whole blood stimulated with PHA + LPS offers the most appropriate and reproducible culture condition in order to measure cytokine production (De Groote et al., 1992, 1993; Zangerle et al., 1992). Diluted whole blood cultures reflect the *in-vivo* immune cellular and humoral interactions and may be employed to examine the effects of drugs on cytokine secretion (De Groote et al., 1992, 1993; Zangerle et al., 1992; Maes et al., 1999). 1.8 ml of RPMI-1640 medium with L-glutamine (Gibco BRL) and IU/mL penicillin (Sigma) and 100 µg/mL streptomycin (Sigma) with PHA + LPS were placed into 24-well cell culture plates (Falcon 3047, Becton Dickinson). The serotonergic substances were dissolved in sterile water, whereas sterile water alone served as the corresponding control. Twenty µl of each serotonergic drug solution was added to the wells and gently mixed with the medium. A total of 0.2 ml of whole blood from each of 26 subjects were cultured with 5-HT (150 ng/mL, 1.5 µg/mL and 15 µg/mL), PCPA (5µM), flesinoxan (15 ng/mL and 1.5 µg/mL), mCPP (27 ng/mL and 2.7 µg/mL) and ritanserin (50 ng/mL and 5.0 µg/mL). The viability of cells was checked by ethidium-bromide-dye exclusion. The samples were incubated for 24 hours in a humidified atmosphere at 37°C, 5% CO₂. Supernatants were taken off carefully under sterile conditions, divided into eppendorf tubes, and frozen immediately at -75°C. IFN γ and IL-10 were quantified by means of ELISA methods (Eurogenetics, Tessenderlo, Belgium) based on appropriate and validated sets of monoclonal antibodies as described by us previously (Maes et al., 1999). All assays of IFN- γ or IL-10 were carried out on the same day in one run by the same operator (AL). The intra-assay CV values for both analytes were less than 8%. In our laboratory, the detection limits are 0.9 U/mL for IFN- γ and 10 pg/mL for IL-10.

Statistics

Repeated measure design analyses of variance (RM ANOVAs) were used to examine the 1) within-subject variability with the effects of serotonergic drugs as temporal condition, i.e. the positive control versus each of the serotonergic drugs at the different concentrations; and 2) between-subject variability with the three study groups (younger volunteers, elderly volunteers and depressed patients) and gender as factors. All results of RM design ANOVAs were corrected for sphericity. Tests on simple effects were employed to clarify main effects or significant interactions. A simple effect is the effect of one variable at one level of the other variable. Differences among treatment means were ascertained by means of the Dunn test. In order to examine the ratio of the secretion of pro-inflammatory (IFN- γ) versus negative immunoregulatory (IL-10) cytokines, the IFN- γ /IL-10 ratio was computed as: z transformed IFN γ

minus z transformed IL-10 (Maes et al., 1998). IFN- γ and IL-10 and their ratio were processed in Box-Cox transformation.

RESULTS

Figure 1 shows the effects of 5-HT on the production of IFN- γ and IL-10. RM design ANOVA showed a significant drug effect on IFN- γ production ($F=11.8$, $df=3/85$, $p=0.00002$), but no significant drug X group interaction ($F=0.92$, $df=6/85$, $p=0.5$). Dunn tests showed significantly lower IFN- γ production in the 5-HT 1.5 $\mu\text{g/mL}$ ($t=2.89$, $p=0.005$) and 15 $\mu\text{g/mL}$ ($t=5.86$, $p=0.000007$), but not 150 ng/mL ($t=2.02$, $p=0.04$) conditions than in the control condition (tested at $p=0.017$). RM design ANOVA showed no significant drug effects on IL-10 production ($F=2.2$, $df=2/74$, $p=0.1$), although there was a significant drug X group effect ($F=2.9$, $df=5/74$, $p=0.018$). Analyses on simple effects showed a significant drug effect in elderly volunteers ($F=5.1$, $df=3/90$, $p=0.003$), but not in younger volunteers ($F=2.2$, $df=3/90$, $p=0.09$) or major depressed patients ($F=0.6$, $df=3/90$, $p=0.6$). In elderly normal volunteers, Dunn tests showed significant suppressant effects of 5-HT at the 1.5 $\mu\text{g/mL}$ ($t=5.41$, $p=0.00001$) and 15 $\mu\text{g/mL}$ ($t=4.23$, $p=0.0002$), but not at 150 ng/mL ($t=1.93$, $p=0.05$) (tested at $p=0.017$).

RM design ANOVA performed on the IFN- γ /IL-10 ratio showed a significant drug effect ($F=13.9$, $df=2/74$, $p=0.00005$), but no significant drug X group interaction ($F=1.8$, $df=5/74$, $p=0.1$). Dunn tests showed that the IFN- γ /IL-10 ratio was significantly lower at the 5-HT 150 ng/mL ($t=4.27$, $p=0.0002$), 1.5 $\mu\text{g/mL}$ ($t=2.64$, $p=0.009$) and 15 $\mu\text{g/mL}$ ($t=6.24$, $p=0.000003$) conditions than in the control condition.

Figure 1 shows the effects of PCPA on the production of IFN- γ and IL-10. RM design ANOVAs showed significant drug effects on IFN γ ($F=110$, $df=1/31$, $p<10^{-5}$) and IL-10 ($F=95.5$, $df=1/30$, $p<10^{-5}$), but no significant drug X group interactions for IFN- γ ($F=2.1$, $df=2/31$, $p=0.1$) or IL-10 ($F=1.6$, $df=2/30$, $p=0.2$). There were no significant effects of PCPA on the IFN γ /IL-10 ratio ($F=0.3$, $df=1/30$, $p=0.6$) and no significant group X drug interaction ($F=0.4$, $df=2/30$, $p=0.7$).

Figure 1

Effects of serotonin (5-HT; 150 ng/mL; 1.5 µg/mL and 15 µg/mL) and p-chlorophenylalanine (PCPA, 5 µM) on the stimulated production of interferon-γ (IFN-γ) and interleukin-10 (IL-10) and on the IFN-γ/IL-10 ratio.

●: Younger normal volunteers; ○: older normal volunteers; ▼: treatment resistant major depressed patients.

RM design ANOVAs did not show any significant effects of flesinoxan (at the two concentrations) on IFN- γ ($F=1.8$, $df=2/61$, $p=0.2$), IL-10 ($F=0.4$, $df=2/51$, $p=0.7$) or the IFN- γ /IL-10 ratio ($F=0.7$, $df=1/41$, $p=0.6$) (data not shown). There were no significant group X drug interaction patterns for IFN- γ ($F=0.2$, $df=4/61$, $p=0.9$), IL-10 ($F=1.4$, $df=3/51$, $p=0.2$) or the IFN- γ /IL-10 ratio ($F=0.7$, $df=3/41$, $p=0.5$).

Figure 2 shows the effects of mCPP and ritanserin on the production of IFN- γ and IL-10. RM design ANOVAs showed that there were no significant effects of mCPP on IFN- γ ($F=2.2$, $df=2/61$, $p=0.1$) and IL-10 ($F=1.5$, $df=2/51$, $p=0.2$). The interaction pattern group X drug was not significant for IFN- γ ($F=1.0$, $df=4/61$, $p=0.4$) and IL-10 ($F=1.5$, $df=3/51$, $p=0.2$). RM design ANOVA showed that there was a significant effect of mCPP on the IFN- γ /IL-10 ratio ($F=3.7$, $df=2/57$, $p=0.03$), but no significant group X drug interaction ($F=1.6$, $df=4/57$, $p=0.2$). Dunn tests (tested at $p=0.025$) showed that the IFN- γ /IL-10 ratio was significantly lower in the mCPP 2.7 $\mu\text{g/mL}$, but not 27 ng/mL , condition than in the control condition.

RM design ANOVA showed a significant effect of ritanserin on the production of IFN- γ ($F=5.2$, $df=2/58$, $p=0.008$), but no significant group X drug interaction ($F=1.7$, $df=4/58$, $p=0.2$). Dunn tests (performed at $p=0.025$) showed a significantly lower IFN- γ production in the ritanserin 5.0 $\mu\text{g/mL}$ ($t=3.15$, $p=0.003$), but not in the 50 ng/mL ($t=0.96$, $p=0.8$) condition than in the control condition. RM design ANOVA did not show significant effects of ritanserin on the production of IL-10 ($F=1.7$, $df=2/58$, $p=0.2$) and no significant group X drug interactions ($F=1.3$, $df=4/58$, $p=0.3$). RM design ANOVA showed a significant effect of ritanserin on the IFN- γ /IL-10 ratio ($F=5.7$, $df=2/55$, $p=0.006$), but no significant group X drug interaction ($F=2.1$, $df=4/55$, $p=0.09$). Dunn tests showed a significantly lower IFN- γ /IL-10 ratio in the ritanserin 5.0 $\mu\text{g/mL}$ ($t=2.98$, $p=0.004$), but not 50 ng/mL ($t=0.15$, $p=0.9$) condition than in the control condition.

RM design ANOVAs showed significant differences in culture supernatant IFN- γ ($F=3.9$, $df=2/31$, $p=0.03$), but not IL-10 ($F=1.8$, $df=2/30$, $p=0.2$) or the IFN- γ /IL-10 ratio ($F=0.4$, $df=2/30$, $p=0.7$) between the study groups. Dunn tests (performed at $p=0.017$) showed significant lower IFN- γ production in elderly than in younger volunteers ($t=2.64$, $p=0.012$) and major depressed patients ($F=3.14$, $p=0.004$). There were no significant differences in IFN- γ production between younger volunteers and major depressed patients ($t=0.51$, $p=0.6$).

Figure 2
Effects of m-chlorophenylpiperazine (mCPP; 27 ng/mL and 2.7 µg/mL), and ritanserin (50 ng/mL and 5.0 µg/mL) on the stimulated production of interferon-γ (IFN-γ) and interleukin-10 (IL-10) and on the IFN-γ/IL-10 ratio.
●: Younger normal volunteers; ○: older normal volunteers; ▼: treatment resistant major depressed patients.

DISCUSSION

The major findings of this study are that 1) 5-HT, 150 ng/mL, 1.5 μ g/mL and 15 μ g/mL, significantly decreased the IFN- γ /IL-10 ratio; 2) PCPA significantly suppressed the production of IFN- γ and IL-10; 3) fleroxan had no significant effects on the production of the above cytokines; and 4) the higher concentrations of mCPP and ritanserin suppressed the IFN- γ /IL-10 ratio. Since IFN- γ is a pro-inflammatory cytokine and IL-10 an anti-inflammatory cytokine, our results suggest that 5-HT, mCPP and ritanserin may have negative immunoregulatory effects. IFN- γ is produced by activated Th1 (T helper-1) lymphocytes and by natural killer cells. IL-10 is produced by a variety of cells, including T-lymphocytes, B-lymphocytes and monocytes. Thus, its expression is not confined to a particular T-cell subset (Th-1 like) as in the rodent (Cavaillon, 1996). IL-10 was originally characterized as a factor which inhibits the secretion of IFN- γ by Th1-cell clones (Murray et al., 1997). Moreover, IL-10 downregulates the production of monocytic cytokines (Cavaillon, 1996). The ratio of IFN- γ to IL-10 in culture supernatants is of critical importance in determining the pro- or anti-inflammatory capacity of culture supernatants (Katsikis et al., 1995).

Previously, it has been found that 5-HT concentrations, close to the physiological concentrations, have immuno-stimulating effects, whereas higher concentrations have immunosuppressive effects (Bonnet et al., 1984; Roszman et al., 1985; Aune et al., 1994). The baseline concentrations of 5-HT in serum are between 50-200 ng/mL, while under pathological conditions such as inflammation, ischemia, or thrombosis, extracellular 5-HT levels may increase to 3 μ M. Thus, in the present study we employed 5-HT at concentrations found in normal serum and during pathological conditions. It has been reported that both T cells and monocytes release 5-HT in culture medium after PHA stimulation and that concentrations of 5-HT between 10^{-8} M to 10^{-7} M, sufficient to activate 5-HT receptors, can be reached in these cultures. We found that 5-HT at all concentrations lowered the IFN- γ /IL-10 ratio, an effect which mainly could be ascribed to the suppressive effects on IFN- γ secretion. These findings extend those of Bonnet et al. (1984) and Devoino and Morozova (1988) who reported that exogenous 5-HT may suppress various aspects of cell-mediated immunity. Devoino et al. (1968) suggested that these changes might be related to central rather than to peripheral 5-HT activities, because lesions of the serotonergic raphe nuclei promoted stimulation in immune responsivity. Nevertheless, our results show that 5-HT has direct effects on peripheral blood immunocytes. Our results also suggest that the suppressive effects of antidepressive drugs on the IFN- γ /IL-10 ratio are probably not related to direct effects of 5-HT. Indeed, the present study showed that 5-HT significantly suppressed the production of

IFN- γ , whereas the common activity of different antidepressants, such as SSRIs, TCAs, HCAs, RIMAs and L-5-HTP is to increase IL-10 secretion, rather than decreasing that of IFN- γ .

The second major finding of this study is that PCPA, an agent which inhibits 5-HT synthesis, dramatically suppressed the production of both IFN- γ and IL-10. Since monocytes and lymphocytes may synthesize 5-HT (Finocchiaro et al., 1988), co-incubation of whole blood with PCPA most likely inhibits 5-HT synthesis in lymphocytes and monocytes. Since we have shown that PCPA inhibits the production of IFN- γ and IL-10, we may hypothesize that endogenous 5-HT exerts an autocrine and necessary role in the secretion of those cytokines. In this respect, Aune et al. (1994) showed that endogenous 5-HT was indispensable for human T cell proliferation. PCPA at the same concentrations as employed in the present study (5 μ M) inhibited the proliferative responses of T cells to polyclonal activators, while the inhibition of T cell proliferation by PCPA was reversed by 5-HTP (Aune et al., 1994). Thus, intracellular 5-HT may be necessary for an optimal synthesis of IFN- γ and IL-10, whereas extracellular 5-HT concentrations at or above serum values may suppress the production of the pro-inflammatory cytokine IFN- γ .

The third major finding of this study revolves around the immunoregulatory effects of 5-HT_{1A} and 5-HT₂ receptor agonists and antagonists. A number of 5-HT receptor subtypes have been identified on immunocytes. Serotonergic receptors on leukocytes were already demonstrated in 1982 by Eliseeva and Stefanovich (1982). Functional, pharmacological, biochemical and molecular analyses have identified the 5HT_{1A} receptor on human NK cells and activated PBMC and T cells (Aune et al., 1993, 1994; Hellstrand et al., 1993). Pharmacological and molecular analyses demonstrated the presence of 5HT_{2C} receptors on human activated T lymphocytes. In the present study, no significant effects of flesinoxan, a 5-HT_{1A} agonist, on IFN- γ or IL-10 production could be found. We found that ritanserin as well as mCPP significantly decrease the IFN- γ /IL-10 production ratio. In the case of ritanserin it is clear that this effect is obtained through inhibition of the IFN- γ production. 5HT_{2A/C} antagonists in the murine model inhibit the passive transfer of DTH reactions (Gershon et al., 1975; Ameisen et al., 1989) and IFN- γ -induced murine macrophage Ia expression (Sternberg et al., 1987). 5-HT_{2A/C} antagonists modulate IFN- γ -induced phagocytosis by murine macrophages (Sternberg et al., 1987). Arzt et al. (1991) reported that 5-HT inhibits - in a concentration dependent way - the LPS-induced production of tumor necrosis factor by human macrophages, and that this effect was blocked by the 5-HT₂ receptor antagonist, ketanserin. On the other hand, 5-HT_{2A/C} receptor antagonists were not able to inhibit PHA-stimulated proliferation of T cells (Nordlind et al., 1992; Aune et al., 1994). Enhancement of murine T-cell

blastogenesis by 5-HT has been shown to occur through 5-HT₂ receptors (Young et al., 1993). The production of Th-1 cytokines, such as IL-2 and IFN- γ , by Ag-stimulated, immune murine spleen cells is inhibited by 5HT_{1A} receptor antagonists, but not by 5HT₂ receptor antagonists (Aune et al., 1994).

Our observations further underscore the role of 5-HT in the production rate of pro-inflammatory (IFN- γ) versus negative immunoregulatory (IL-10) cytokines and may suggest an involvement of the 5HT_{2A/C}, but not 5-HT_{1A}, receptors in 5-HT-mediated IFN- γ versus IL-10 production. However, also the role of 5-HT_{2A/C} related mechanisms for the production of IFN- γ versus IL-10 appears inconclusive since both agonists and antagonists at these receptor sites have similar effects on the IFN- γ /IL-10 production ratio. Perhaps the effects of mCPP may in part be explained by its 5-HT reuptake inhibitory effects (Baumann et al., 1995) or its activity at other 5-HT receptors, such as the 5-HT_{1D} sites (Schoeffer and Hoyer, 1989). Moreover, mCPP has been shown to bind to α -2-adrenergic receptors (Hamik and Peroutka, 1989). Stimulation of α -2-adrenoceptors may result in negative immunoregulatory effects (Maes et al., 2000). Our results that 5-HT_{2A/C} receptor antagonists and agonists do not modulate the production rate of IL-10 suggest that the negative immunoregulatory effects of SSRIs and TCAs, which may in part exert their antidepressant efficacy through their activity at 5-HT_{2A/C} receptors (Maes and Meltzer, 1995), may not be ascribed to 5-HT_{2A/C} mechanisms.

Future research should focus on the effects of antidepressants and serotonergic agents on cyclic AMP (cAMP). Xia et al. (1996) reported that TCAs as well as SSRIs significantly elevate intracellular cAMP concentrations in T lymphocytes and monocytes. Elevation of the intracellular levels of cAMP differently affects the production of IL-10 and IFN- γ (Benbernou et al., 1997). Cyclic AMP-elevating agents inhibit IFN- γ mRNA expression and IFN- γ levels in lymphocytes, and significantly enhance IL-10 mRNA expression and the intracellular levels of IL-10 (Benbernou et al., 1997). In monocytes, cAMP elevating drugs augment the stimulated synthesis of IL-10 at both protein and mRNA levels (Platzer et al., 1995). 5-HT stimulates cAMP formation in homogenates of rat hippocampus in a concentration-dependent manner; 5-HT_{1A}, 5-HT₇ and 5-HT₄ receptors are involved in mediating these responses (Markstein et al., 1999).

In the present study, we found that the production of IFN- γ was significantly lower in older than in younger volunteers and that the production of IFN- γ was significantly higher in fluoxetine-treated TRD patients than in age-matched normal volunteers. The differences between both age-groups may be explained by the previous findings that ageing is associated with various alterations in lymphoid cell functioning, such as decreased IFN- γ production (Candore et al., 1993). The higher IFN- γ production in depressed patients treated with

fluoxetine could perhaps be explained by previous findings that IFN- γ production is significantly increased in depressed patients and that IRS activation is more pronounced in patients with TRD despite antidepressive treatment (Seidel et al., 1995; Maes, 1997). However, the data presented are for a small cohort of subjects. Moreover, the study groups do not include a spectrum of older individuals and drug-free depressed patients. Although the data suggest differences between the younger and older normal volunteers and depressed patients following fluoxetine, a larger cohort of young and old individuals and drug-free depressed patients should be studied in order to establish valid conclusions. In addition, it should be stated that this study employed diluted whole blood stimulated with PHA + LPS to study the effects of drugs on the production of cytokines. It is conceivable that comparable results will be obtained using other polyclonal activators. Nevertheless, diluted whole blood stimulated with LPS + PHA offers the most appropriate and reproducible culture condition in order to measure cytokine production and to study the effects of any drugs on the production of cytokines (De Groote et al., 1992, 1993; Zangerle et al., 1992)

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Chapter 6

Cytokine production in stimulated human whole blood after modulation of the cAMP/PKA-pathway

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Submitted

ABSTRACT

The cyclic adenosine monophosphate dependent protein kinase A (cAMP/PKA) pathway is an important regulator of cytokine production. It has been established that pharmacological elevation of intracellular cAMP-concentrations inhibits the stimulated production of interferon- γ (IFN- γ) and tumor necrosis factor- α (TNF- α), and may enhance the production of interleukin-10 (IL-10) in isolated leukocyte subsets. However, the effects of modulation of the cAMP/PKA-pathway in stimulated whole blood have never been examined. Therefore, we sought to determine the effects of adenylylase, PKA and phosphodiesterase type 4 (PDE4; an enzyme that catalyzes the breakdown of cAMP) on the stimulated production of IFN- γ , TNF- α , IL-6, IL-10 and IL-1 Receptor Antagonist (IL-1RA) in human whole blood. Stimulation of adenylylase by forskolin enhanced the secretion of IL-10 and decreased IFN- γ production at lower concentrations, whereas it had no effect on TNF- α , IL-6 or IL-1RA. The adenylylase inhibitor SQ22536 decreased the production of IFN- γ , TNF- α and both IL-1RA and IL-6 at higher concentrations, but had no effect on IL-10. Direct stimulation of PKA with the cAMP- analogue Sp-8-Br-cAMPS inhibited IFN- γ , TNF- α and IL-1RA production, while it enhanced IL-6 secretion at the highest concentration. Rp-8-Br-cAMPS, an inhibitor of PKA, strongly enhanced the production of IL-10 at the highest concentration, and decreased IFN- γ secretion at lower but increased it at higher concentration. PKA-inhibition enhanced TNF- α production (although not significant) while it had no effect on IL-6 or IL-1RA. Rolipram, an inhibitor of PDE4, dose-dependently inhibited the production of IFN- γ , TNF- α and IL-1RA. At the highest concentration of rolipram, IL-10 secretion was inhibited, while that of IL-6 was enhanced. Rolipram also reduced the IFN- γ /IL-10 ratio, an effect comparable to that of other antidepressants. Clearly, all examined cytokines are differentially affected by inhibitors and stimulators of the cAMP/PKA-pathway. Previous studies using isolated leukocyte subsets and examining only single aspects of the cAMP/PKA-pathway, could not be completely confirmed in stimulated whole blood cultures as employed in this study.

INTRODUCTION

The second messenger cyclic 3',5'-adenosine monophosphate (cAMP) is an important regulator of gene transcription. It is produced by the enzyme adenylyl cyclase upon stimulation of certain membrane bound G-protein coupled receptors. The major effect of cAMP is the activation of protein kinase A (PKA,) which in turn phosphorylates other downstream enzymes, kinases and transcription factors such as cAMP responsive element binding protein (CREB). Receptors that stimulate adenylyl cyclase upon ligand binding include β 2-adrenergic, H_2 -histaminergic, some serotonergic and prostaglandin E2 (PGE₂) receptors, all of which have been described to be expressed on leucocytes. The homeostatic regulation of the intracellular cAMP-concentration is predominantly controlled by phosphodiesterase type 4 (PDE4) that catalyzes the breakdown of cAMP to AMP.

It is known that the cAMP/PKA pathway can influence cytokine expression (for review see Zidek, 1999). In general, pharmacological elevation of intracellular cAMP-concentrations inhibits the pro-inflammatory cytokines tumor necrosis factor- α (TNF- α) and interferon- γ (IFN- γ), while it enhances the production of interleukin-10 (IL-10). For example, the cAMP-analogue dibutyryl-cAMP (db-cAMP) inhibits IFN- γ secretion in human peripheral blood mononuclear cells (PBMCs), human CD4⁺- and CD8⁺-T-cells (Shin et al., 1998), and Jurkat-cells (Benbernou et al., 1997). Histamine, through interaction with H_2 -receptors, inhibits IFN- γ secretion in murine splenocytes and T-helper 1 (Th1) cells (Osna et al., 2001). PGE₂, via stimulation of EP2 receptors, inhibits IFN- γ production in human PBMCs, CD4⁺- and CD8⁺-T-cells (Abe et al., 1997; Shin et al., 1998) and Jurkat cells (Benbernou et al., 1997). Rolipram, which increases cAMP through selective inhibition of PDE4, down-regulates IFN- γ production in human PBMCs (Yoshimura et al., 1998; Jimenez et al., 2001) and in whole blood cultures. The production of TNF- α is also inhibited by rolipram in several culture systems, such as human PBMCs (Yoshimura et al., 1997; Eigler et al., 1998; Jimenez et al., 2001), human whole blood (Brideau et al., 1999), mouse microglia (Yoshikawa et al., 1999) and in rat alveolar epithelial cells (Haddad et al., 2002). Agonists of β -adrenergic receptors inhibit TNF- α secretion (Szabo et al., 1997). Direct stimulation of adenylyl cyclase by forskolin also inhibits TNF- α production in mouse microglia (Yoshikawa et al., 1999). A number of studies examined the influence of cAMP and/or PKA-activation on IL-10 secretion. Most studies found that pharmacological increases of cAMP enhances stimulated IL-10 production in human PBMCs (Meisel et al., 1996; Eigler et al., 1998; Elenkov et al., 1998; Shin et al., 1998; Siegmund et al., 1998), human whole blood cultures (Elenkov et al., 1998; Link et al., 2000), purified human T-cell subsets (Shin et al., 1998), Jurkat-cells (Benbernou et al., 1997) and

mouse microglia (Yoshikawa et al., 1999). However, some reports demonstrated inhibition of IL-10 production by cAMP elevating agents in activated T-cells (Jimenez et al., 2001; Rafiq et al., 2001) and HIV-infected PBMCs (Cole et al., 1998). Less is known about the influence of cAMP and PKA-activation on the expression of IL-1 Receptor Antagonist (IL-1RA). It seems that db-cAMP induces monocytic and neuronal IL-1RA production (Paez Pereda et al., 1995; Yasuhara et al., 1997), but inhibits lipopolysaccharide (LPS)-induced expression and secretion of IL-1RA (Yasuhara et al., 1997). The influence of activation of the cAMP/PKA-pathway on the IL-6 production is less clear and probably dependent on the cell type, stimulus and micro-environment. In leucocytes, PKA-activation seems necessary for optimal IL-6 production, whereby cAMP-accumulation can enhance LPS-induced IL-6 secretion.

Previously, we and others have shown that antidepressants of several classes may have anti-inflammatory effects *in-vitro*, e.g. they inhibit the production of IFN- γ and TNF- α , but may enhance IL-10 production (Xia et al., 1996; Maes et al., 1999; Kubera et al., 2001). The mechanism behind this effect is yet unknown, but it is probably not related to their serotonergic activities (Kubera et al., 2000). There have been some reports that antidepressants may increase intracellular cAMP-concentrations in leucocytes (Xia et al., 1996; Edgar et al., 1999). As mentioned above, pharmacological elevation of intracellular cAMP-concentrations inhibits TNF- α and IFN- γ production, while it can stimulate IL-10 production. We therefore hypothesize that antidepressants exert their effect on cytokine production through stimulation of the cAMP/PKA-pathway. Most studies concerning the relation between cytokine production and the cAMP/PKA-pathway have been performed on isolated PBMCs, or leukocyte subsets. In contrast, the effects of antidepressants on cytokines have mainly been studied in whole blood cultures.

Therefore, the aim of this study is to examine the influence of the cAMP/PKA pathway on the stimulated production of cytokines in human whole blood cultures. Hereto, an both inhibitor and stimulator of adenylate cyclase (SQ22536 and forskolin, respectively) and PKA (Rp-8-Br-cAMPS and Sp-8-Br-cAMPS, respectively), and an inhibitor of PDE4 (rolipram) were added to the cultures, before stimulation with a mixture of LPS and phytohaemagglutinin (PHA). Cytokines in the culture supernatant were then determined by enzyme linked immunosorbent assays (ELISA).

METHODS

Reagents

Lipopolysaccharides (*E.coli* 026:B6) and SQ22536 were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Forskolin and rolipram were obtained from ICN-Biomedicals (Zoetermeer, The Netherlands), phytohaemagglutinin (PHA) came from Abbott Diagnostics (Hoofddorp, The Netherlands). Sp-8-Br-cAMPS and Rp-8-Br-cAMPS were purchased from Biolog Life Science Institute (Bremen, Germany).

Subjects

Blood was drawn from 20 healthy volunteers (at 9 a.m. $\pm$ 30min.) after an overnight fast. All subjects gave written informed consent after full explanation of the study design. The mean age of the subjects was 25.2 ± 1 years and the male/female ratio was 9/11. Exclusion criteria for the subjects were: i) age under 18 or over 40; ii) a past or present history of psychiatric disorder (axis-I); iii) subjects who ever had been taken major psychotropic medication, such as antidepressants, antipsychotics and anxiolytics; iv) subjects with alcohol or any other drug of dependence abuse; v) smokers; vi) subjects with major somatic disorders, such as diabetes, autoimmune diseases, acquired immunodeficiency syndrome; vii) subjects who currently (two weeks prior to blood sampling) suffered from an infectious, allergic or inflammatory response. The subjects abstained from caffeine and alcohol for at least 10 hours prior to blood sampling.

Whole blood stimulation

The influence of substances that inhibit or stimulate the cAMP/PKA pathway, was examined using stimulated diluted human whole blood as described previously (De Groote et al., 1992; Maes et al., 1999). Briefly, blood was diluted four times in RPMI 1640 medium (Gibco, Life Technologies, Belgium) supplemented with L-Glutamine and Penicilline-Streptomycine (Gibco, Life Technologies, Belgium). This blood suspension was plated in 24-well tissue culture plates pre-filled with concentrated solutions of SQ22536, forskolin, Sp-8-Br-cAMPS or Rp-8-Br-cAMPS. After a pre-incubation of 30 min. (37°C, 5% CO₂), a concentrated solution of LPS/PHA was added to the cultures. Plates were placed on gyratory shaker for 10 min. to mix the content of the wells, before incubation at 37°C, 5% CO₂. The final concentrations of the substances were: SQ22536 (1 μ M – 10 μ M – 100 μ M); forskolin (0.2 μ M – 2 μ M – 20 μ M); Sp-8-Br-cAMPS (1 μ M – 10 μ M – 100 μ M); Rp-8-Br-cAMPS (1 μ M –

10 μM – 100 μM); rolipram (0.2 μM – 2 μM – 20 μM). All cultures were stimulated with LPS and PHA in a final concentration of 1 $\mu\text{g}/\text{ml}$ and 5 $\mu\text{g}/\text{ml}$ respectively. All culture conditions were performed in duplicate. The supernatant of one set was collected after 24h (for measurement of $\text{TNF-}\alpha$), the other after 72h (for measurement of $\text{IFN-}\gamma$, IL-10, IL-6 and IL-1RA). To collect culture supernatant, the plates were gently mixed for 15 min. and centrifuged at 1200xg for 15 min. Supernatant was aspirated and stored at -20°C until analysis of cytokines.

Cytokine determination

Cytokines were determined using sandwich based enzyme-linked immunosorbent assays (ELISA; Eurogenetics, Tessenderlo, Belgium). All assays were performed according manufacturer instructions. In our laboratory, the detection limits are as follows: $\text{IFN-}\gamma$: 1 U/ml; IL-10: 3 pg/ml; IL-6: 10 pg/ml; IL-1RA: 0,1 ng/ml; $\text{TNF-}\alpha$: 10 pg/ml.

Statistics

Repeated measures design analysis of variance (RM design ANOVAs) was performed to examine the within-subject variability with the effect of treatment as temporal condition. Differences among treatment means were ascertained by means of the Dunn-test. The $\text{IFN-}\gamma/\text{IL-10}$ ratio was computed as: z-transformed $\text{IFN-}\gamma$ minus z-transformed IL-10.

RESULTS

Influence of adenylate cyclase activity on cytokine production

The effect of adenylate cyclase (AC) function on cytokine production was assessed by the addition of an inhibitor (SQ22536) or an activator (forskolin) of AC. Results of RM-design ANOVAs are presented in Table 1. There was a significant effect of AC-inhibition on the production of $\text{IFN-}\gamma$, $\text{TNF-}\alpha$, and IL-1RA (see Figure 1). There was no effect on IL-10. Post-hoc analyses indicated that SQ22536 at 10 μM and 100 μM significantly reduced $\text{IFN-}\gamma$ and $\text{TNF-}\alpha$ production. The IL-1RA secretion was reduced by 1 μM and 100 μM of SQ22536. IL-6 production was only moderately affected by AC-inhibition: 100 μM SQ22536 slightly reduced IL-6 production.

Activation of AC by forskolin differentially affected the secretion of $\text{IFN-}\gamma$ and IL-10. Whereas 0.2 μM and 2 μM forskolin significantly inhibited $\text{IFN-}\gamma$

production, 2 μ M and 20 μ M forskolin significantly enhanced IL-10 production. There was no effect of forskolin treatment on TNF- α , IL-6 or IL-1RA.

Overall, inhibition of AC inhibits IFN- γ and TNF- α production, and moderately reduces IL6 and IL-1RA expression. In contrast, stimulation of AC enhances IL-10 production and only marginally affects pro-inflammatory cytokines (reduction of IFN- γ secretion).

Table 1

Results of RM design ANOVAs, showing the effects of each cAMP/PKA modulating agent on the stimulated production of cytokines.

	SQ22536			Forskolin			Sp-8-Br-cAMPS			Rp-8-Br-cAMPS			Rolipram		
	F	df	p	F	df	p	F	df	p	F	df	p	F	df	p
IFN- γ	67.12	29	<10 ⁻⁶	12.3	2/36	0.0002	60.7	2/37	0.00001	15.6	2/46	0.00004	108	1/22	<10 ⁻⁵
IL-10	2.6	2/46	0.09	15.7	2/42	0.00005	6.7	2/35	0.004	66.5	2.45	<10 ⁻⁶	46.5	2/43	<10 ⁻⁵
TNF- α	25.42	31	0.00001	0.8	3/50	0.5	26.2	1/27	0.00009	1.6	1/21	0.2	49.5	1/23	0.00001
IL-6	3.57	2/37	0.04	1.4	2/30	0.3	24.6	2/34	0.00001	1.4	2/30	0.2	21.6	2/29	0.00002
IL-1RA	41.02	37	<10 ⁻⁵	1.8	2/36	0.2	34.2	2/40	<10 ⁻⁶	1.74	2/36	0.2	51.3	1/28	0.00001

Influence of PKA activity on cytokine production

To modulate PKA-activity, two different isomers of the cAMP analogue 8-Br-cAMPS were employed. The Sp-isomer stimulates PKA, whereas the Rp-isomer is a cAMP antagonist that inhibits PKA-activity. These compounds were chosen for 1) their high selectivity, 2) their high membrane permeability due to the Br substitution, and 3) their resistance to PDEs due to the phosphorothioate group.

Stimulation of PKA by Sp-8-Br-cAMPS treatment significantly affected the production of all cytokines examined (see Table 1 and Figure 2). Post-hoc analyses revealed that 10 μ M and 100 μ M Sp-8-Br-cAMPS strongly inhibited the production of IFN- γ and TNF- α . IL-1RA secretion was reduced at 100 μ M. Sp-8-Br-cAMPS moderately enhanced IL-10 production at 10 μ M and stimulated IL-6 production at 100 μ M.

There was a significant effect of PKA-inhibition on the production of IFN- γ and IL-10, but not on the other cytokines examined. Rp-8-Br-cAMPS at 100 μ M strongly enhanced IL-10 production, but 1 μ M and 10 μ M had no effect. Rp-8-Br-cAMPS had a dual effect on IFN- γ secretion. Whereas IFN- γ secretion was reduced by 1 μ M and 10 μ M, it was enhanced at 100 μ M Rp-8-Br-cAMPS. Although Rp-8-Br-cAMPS enhanced TNF- α production as well (see Figure 2), this effect did not reach statistical significance.

These results indicate that stimulation of PKA strongly inhibits IFN- γ and TNF- α production, whereas PKA inhibition predominantly stimulates IL-10 secretion.

Effect of PDE4 inhibition on cytokine production

RM design ANOVA showed that there was a significant effect of rolipram on the secretion of IFN- γ , IL-10, TNF- α , IL-6 and IL-1RA (see Table 1 and Figure 3). Post-hoc analysis revealed that rolipram 0.2 μ M significantly decreased the secretion of IFN- γ . At a concentration of 2 μ M, rolipram significantly reduced the production of all cytokines examined. When compared to control cultures, 20 μ M rolipram significantly inhibited the production of IFN- γ , IL-10, TNF- α and IL-1RA, but significantly increased that of IL-6.

Further, rolipram significantly reduced the IFN- γ /IL-10 ratio at all concentrations examined ($F=11.4$, $df=2/35$, $p=0.0003$). This effect is comparable with that of other antidepressants.

DISCUSSION

We have examined the role of the cAMP/PKA-pathway on cytokine production in stimulated diluted human whole blood. The major findings are: 1) blockade of adenylylate cyclase activity reduces the production of the pro-inflammatory cytokines IFN- γ , TNF- α and IL-6, but does not affect IL-10 production; 2) stimulation of adenylylate cyclase enhances IL-10 and reduces IFN- γ production, but does not affect secretion of TNF- α , IL-6 or IL-1RA; 3) direct stimulation of PKA inhibits IFN- γ , TNF- α and IL-1RA production, while increasing IL-6 and IL-10; 4) PKA-inhibition by Rp-8-Br-cAMPS reduces IFN- γ production at lower concentrations, but enhances IL-10 at higher concentrations, while there is no effect on the other cytokines examined; 5) inhibition of PDE4 by rolipram dose dependent decreases IFN- γ , TNF- α , IL-10 and IL-1RA production, while IL-6 production is decreased at lower, but strongly enhanced at higher concentrations.

Figure 1

Influence of SQ22536 and forskolin on stimulated production of cytokines in whole blood. Concentrations employed were: 1 μ M (A), 10 μ M (B) and 100 μ M (C) for SQ22536; and 0.2 μ M (A), 2 μ M (B) and 20 μ M (C) for forskolin.

Figure 2
Influence of Sp-8-Br-cAMPS and Rp-8-Br-cAMPS on stimulated cytokine production in whole blood. Concentrations employed were: 1 μ M (A), 10 μ M (B) and 100 μ M (C) for both substances.

Figure 3
Influence of rolipram on stimulated cytokine production in whole blood.

To the best of our knowledge, the effects of adenylate cyclase inhibition on stimulated cytokine production, have never been examined before. We found that the adenylate cyclase inhibitor SQ22536 dose-dependently inhibited the production of IFN- γ and TNF- α , and reduced IL-6 production at the highest concentration used. This indicates that cAMP-formation is indispensable for optimal pro-inflammatory cytokine production after stimulation with LPS/PHA. Indeed, intracellular cAMP-concentrations are increased in white blood cells after stimulation with LPS/PHA or other immunological stimuli (Wang et al., 1978; Shenker and Gray, 1979; Oksenberg et al., 1989; Kenis et al., 2003), and is required for optimal T-cell activation (Wang et al., 1978). Apparently, blocking of this initial cAMP-increase negatively influences the pro-inflammatory cytokines IFN- γ , TNF- α and IL-6, while production of the anti-inflammatory cytokine IL-10 is not affected.

To study the influence of adenylate cyclase (AC) activity on cytokine production, we used a well-known stimulator of AC, forskolin. This substance directly stimulates AC to form cAMP out of ATP, resulting in increased intracellular cAMP concentrations. Since forskolin was pre-incubated for 30 min. before stimulation with LPS/PHA, the cultures with forskolin have higher cAMP-levels at the time of stimulation. We found that forskolin enhanced IL-10 and IL-6 production but did not affect TNF- α , nor IL-1RA production in stimulated human whole blood. Forskolin-enhanced IL-10 production has been shown previously in human monocytes (Shames et al., 2001), and is probably related to increased intracellular cAMP-concentrations. Others found that forskolin inhibits TNF- α expression and release in purified human T-cells (Sottile et al., 1996) and monocytes (Ollivier et al., 1996; Sottile et al., 1996; Chong et al., 2002), also probably related to a forskolin-induced cAMP-increase. In our whole blood model, however, there was no effect of forskolin on TNF- α . In contrast, the whole blood production of IFN- γ was moderately inhibited only at the lower concentrations of forskolin tested (0,2 – 2 μ M). Indeed, it has been shown that forskolin inhibits IFN- γ expression and secretion in human T-cells (Snijdewint et al., 1993; Sottile et al., 1996; Bodor et al., 2001). Our finding that 20 μ M forskolin does not inhibit IFN- γ production is difficult to explain. Furthermore, forskolin did not affect IL-6 production in stimulated whole blood. Zubiaga et al. also found no effect of forskolin on stimulated IL-6 production in murine Th-2 cells (Zubiaga et al., 1990), while others showed that forskolin up-regulated IL-6 production in pokeweed mitogen stimulated human T-cells (Sottile et al., 1996). To the best of our knowledge, there have been no other reports on the effects of forskolin on IL-1RA production.

It is generally accepted that the downstream effects of cAMP are mediated by PKA. Therefore, we tested the influence of direct activation or inhibition of

PKA on the stimulated production of cytokines. Direct stimulation of PKA with Sp-8-Br-cAMPS dose-dependently inhibited the production of IFN- γ and TNF- α , which could be expected since pharmacological increases in intracellular cAMP or the addition of cAMP-analogues (like 8-Br-cAMP or dibutyryl-cAMP) inhibits IFN- γ (Benbernou et al., 1997; Cole et al., 1998; Shin et al., 1998) and TNF- α (Yoshimura et al., 1997; Wordemann et al., 1998) secretion in several cell culture systems. IL-10 production was significantly enhanced at 10 μ M, but not at 100 μ M Sp-8-Br-cAMPS. An increase in IL-10 production could be expected, since direct stimulation of PKA enhances IL-10 expression and secretion (Platzer et al., 1995; Eigler et al., 1998; Shin et al., 1998). However, it has been shown that IL-10 production is induced by TNF- α (Platzer et al., 1995). This may explain the fact that IL-10 was not further increased after stimulation with 100 μ M Sp-8-Br-cAMPS (as compared to 10 μ M of the PKA-stimulator), because TNF- α production was inhibited by more than 80% at this concentration. IL-6 production was moderately enhanced at 100 μ M Sp-8-Br-cAMPS in our whole blood model. Zubiaga et al. did not find an effect of db-cAMP on IL-6 production in murine Th-cells (Zubiaga et al., 1990). To the best of our knowledge, this is the first report on the influence of PKA-stimulation on IL-1RA. IL-1RA production was inhibited only at 100 μ M Sp-8-Br-cAMPS.

To the best of our knowledge, this is the first report on the effects of direct PKA-inhibition on LPS/PHA-stimulated cytokine production. Blockade of PKA had a concentration dependent effect on IFN- γ production. Rp-8-Br-cAMPS at 1 μ M and 10 μ M moderately decreased IFN- γ , whereas 100 μ M increased IFN- γ production. It is known that activation of PKA inhibits T-cell proliferation, immune cell activation and pro-inflammatory cytokine secretion (including IFN- γ and TNF- α) (Bryce et al., 1999; Zidek, 1999). Prolonged inactivation of PKA will therefore result in increased production of these cytokines upon stimulation. Although not significant, TNF- α production was also increased by 61% at 100 μ M Rp-8-Br-cAMPS. The decreased production of IFN- γ at the lower concentrations of Rp-8-Br-cAMPS are difficult to explain. As mentioned before, increases in intracellular cAMP-concentrations and subsequent PKA-activation are indispensable for normal activation of immune cells.

Surprisingly, IL-10 production was strongly enhanced at 100 μ M of Rp-8-Br-cAMPS. Since it has been shown that cAMP-increases and PKA-activation stimulate IL-10 production (Platzer et al., 1995; Meisel et al., 1996; Benbernou et al., 1997; Eigler et al., 1998; Elenkov et al., 1998; Woiciechowsky et al., 1998), one would expect a down-regulation of IL-10 at high concentrations of the inhibitor. Two recently described phenomena may explain this finding. First, the regulation of IL-10 production by cAMP/PKA is cell-type specific. Thus, it has been reported that the regulation of IL-10 gene expression in human monocytes differs from that in T-cells (Riese et al., 2000). Second, IL-10

production in response to endotoxin, is to a great extent mediated by the initial TNF- α release (Platzer et al., 1995). Thus, it may be speculated that 100 μ M Rp-8-Br-cAMPS increased TNF- α production (by 61 %), resulting in an enhanced production of IL-10. It is important to realize that the concentration of cytokines in the supernatant of stimulated whole blood, is the net effect of cytokine production by all the different cell types present in the blood (i.e. monocytes, T- and B-lymphocytes, neutrophils, Natural Killer cells, mast cells, eosinophils, basophils, etc.). Moreover, these cells also release inflammatory mediators that can influence cytokine production by other cell types. Modulation of adenylate cyclase or PKA-activity may result in an altered release of these mediators or in a different cellular response to these mediators and, hence, resulting in an altered secretion pattern of cytokines. In whole blood, all the natural in-vivo occurring cell types (and in their natural occurring ratios) are present. The results obtained from experiments using whole blood – although they reflect better the in-vivo situation and display the netto effect of drugs on cytokine production, cannot be readily compared with other culture systems that use purified or isolated cell types.

We found that rolipram dose-dependently inhibited the production of IFN- γ and TNF- α at all concentrations examined. These results corroborate those of others, who reported inhibitory effects of rolipram on the production of TNF- α (Gantner et al., 1997; Eigler et al., 1998; Brideau et al., 1999; Yoshikawa et al., 1999). It has also been reported that rolipram reduces the production of IFN- γ in T-cells (Sommer et al., 1995; Essayan et al., 1997; Pette et al., 1999). Our results extend these findings to stimulated whole blood. Interestingly, rolipram, at 2 μ M and 20 μ M, inhibited the secretion of IL-10. The effects of rolipram on *in-vitro* IL-10 production are not clear. In T-cells, rolipram inhibits IL-10 secretion (Crocker et al., 1998; Pette et al., 1999; Jimenez et al., 2001). In contrast, LPS-induced IL-10 production is augmented by rolipram in human PBMCs (Eigler et al., 1998), peritoneal macrophages (Kambayashi et al., 1995) and microglia (Yoshikawa et al., 1999). In all these studies, rolipram was used in the same concentration range as in our experiments. The different effects of rolipram on various culture systems, may be related to a differential regulation of IL-10 expression in different cell types. Activation of the cAMP/PKA-pathway can activate the promoter of the IL-10 gene in monocytes (Platzer et al., 2000), but to a lesser extent in T-cells (Riese et al., 2000). Thus, IL-10 gene expression is differentially regulated in monocytes and T-cells by cAMP elevation. In our whole blood stimulation assay, many IL-10 producing cell types are present. The concentration of IL-10 (and of other cytokines) measured in the culture supernatant, reflects the net effect of rolipram on the stimulated production of cytokines by all these cells.

Another major finding is that rolipram reduces the IFN- γ /IL-10 production ratio. Previously, we have shown that antidepressants like imipramine, fluoxetine, venlafaxine, clomipramine, sertraline and trazodone also decrease the IFN- γ /IL-10 ratio in stimulated whole blood (Maes et al., 1999; Kubera et al., 2001). Thus, antidepressants of various classes have negative immunoregulatory effects. These effects may be of clinical relevance, since several pro-inflammatory cytokines are up-regulated in patients with major depression (Connor and Leonard, 1998; Maes, 1999). Experiments in animals indicate that pro-inflammatory cytokines have profound behavioural effects (such as anhedonia, decreased locomotor activity, sleepdisturbances) (Dantzer et al., 1998), and clinical trials in humans show that cytokine therapy can induce depressive symptoms (Maes et al., 2001). Therefore, we hypothesize that antidepressants exert their effect at least in part by their negative immunoregulatory capacity. This is underscored by the fact that rolipram does not display effects on the central serotonergic system as compared to other antidepressants, e.g. selective serotonin reuptake inhibitors. Thus, rolipram has no effect on serotonin release, the firing rate of serotonergic neurons, does not block serotonin reuptake and reduces serotonin metabolism in several brain regions (Kehr et al., 1985; Schoffelmeer et al., 1985; Scuvee-Moreau et al., 1987). It has been suggested that the antidepressant capacity of rolipram is related to a facilitated neurotransmission at the postsynaptic level due to increased signaling of cAMP-linked noradrenaline and/or serotonin receptors (Kehr et al., 1985; Wachtel and Schneider, 1986).

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Chapter 7

Role of the cAMP/PKA-pathway in the anti-inflammatory effects of paroxetine and imipramine in human whole blood and peripheral blood mononuclear cells

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Submitted

ABSTRACT

Antidepressants have negative immunoregulatory effects *in-vitro* by decreasing the production of interferon- γ (IFN- γ , a pro-inflammatory cytokine), and/or increasing that of interleukin-10 (IL-10, an anti-inflammatory cytokine). The mechanism behind this effect is yet not known, but it may be mediated by the cyclic adenosine monophosphate (cAMP) dependent protein kinase A (PKA) pathway. Indeed, pharmacological elevation of intracellular cAMP-concentrations inhibits the production of pro-inflammatory cytokines and enhances that of IL-10. The present study examines the effects of paroxetine and imipramine on the stimulated production of tumor necrosis factor- α (TNF- α), IFN- γ , IL-10 and IL-6, in diluted human whole blood and in human peripheral blood mononuclear cells (PBMCs). Further, the role of the cAMP/PKA-pathway in the effects of antidepressants on cytokine production was examined by the addition of an adenylate cyclase inhibitor (SQ22536) or an inhibitor of PKA (Rp-8-Br-cAMPS). Paroxetine and imipramine significantly reduced the production of TNF- α , IFN- γ and IL-10 but not IL-6 in whole blood cultures. Rp-8-Br-cAMPS, but not SQ22536, blocked the suppressant effect of paroxetine on TNF- α production. Neither substance could reverse the effects of both antidepressants on IFN- γ or IL-10. In PBMC-cultures, paroxetine and imipramine decreased TNF- α production, which could be reversed by Rp-8-Br-cAMPS but not by SQ22536. Imipramine, but not paroxetine, inhibited IFN- γ and IL-10 secretion by PBMCs. There was no influence of SQ22536 or Rp-8-Br-cAMPS in this effect. It is concluded that the suppressant effect of paroxetine and imipramine on TNF- α production is mediated by PKA, whereas the inhibitory effects on IFN- γ and IL-10 are probably not related to the cAMP/PKA-pathway.

INTRODUCTION

Accumulating evidence suggests that pro-inflammatory cytokines, e.g. interleukin-1 β (IL-1 β), IL-6, interferon- γ (IFN- γ) and tumor necrosis factor α (TNF- α), play a role in the aetiology and pathophysiology of major depression (Connor and Leonard, 1998; Maes, 1999). Indeed, major depressed patients have increased levels of circulating IL-6, IL-1 β and TNF- α (Maes, 1999; Mikova et al., 2001; Owen et al., 2001). These cytokines are thought to mediate "sickness behaviour" induced by the administration of IL-1 β or lipopolysaccharides (LPS) to animals (Bluthé et al., 2000a, 200b; Dantzer, 2001). Sickness behaviour is a syndrome that resembles the clinical symptoms of major depression (Maes et al., 1993), and is characterized by anhedonia, psychomotor retardation, sleep disturbances and decreased social activity and exploration (Yirmiya, 1996; Dantzer, 2001). In humans, immunotherapy with cytokines such as IFN- α induces depressive symptoms and full-blown major depression in psychiatric healthy subjects (Zdilar et al., 2000; Bonaccorso et al., 2001; Schaefer et al., 2002).

If pro-inflammatory cytokines are involved in the pathophysiology of major depression, one may expect that antidepressants reduce these cytokines or counteract their actions. In this respect, it has been shown that antidepressants have negative immunoregulatory effects *in-vitro*. For example, tricyclic antidepressants (TCAs) decrease the production of IL-1 β , TNF- α and IL-6 in purified human monocytes and that of IFN- γ and IL-2 in human T-lymphocytes (Xia et al., 1996). In our laboratory, we demonstrated that TCAs (i.e. imipramine), Selective Serotonin Reuptake Inhibitors (SSRIs, i.e. fluoxetine and sertraline), Serotonin/Noradrenaline Reuptake inhibitors (SNRIs, i.e. venlafaxine), Reversible Inhibitors of Mono-amine Oxidase-A (i.e. moclobemide), the heterocyclic antidepressant trazodone, 5-hydroxy-tryptophan (5-HTP) and lithium, all decrease the production of IFN- γ and/or increase that of IL-10 in stimulated human whole blood (Maes et al., 1999b, 1999a; Kubera et al., 2000, 2001; Lin et al., 2000). IL-10 is an anti-inflammatory cytokine that inhibits activated immune cells and decreases pro-inflammatory cytokine production. Thus, the antidepressant induced decrease in IFN- γ production relative to that of IL-10, indicates that antidepressants have negative immunoregulatory effects *in-vitro*. Moreover, it has been shown that antidepressants may have anti-inflammatory effects *in-vivo* (Kenis and Maes, 2002). For example, antidepressant treatment normalizes increased IL-6 plasma levels in depressed patients (Sluzewska et al., 1996).

The mechanism behind the effects of antidepressants on cytokine production remains unknown. Although serotonin (5-HT) has anti-inflammatory properties and modulates the cytokine network (Kubera and Maes, 2000), the effects of

antidepressants on cytokine production are probably not related to their serotonergic actions (Kubera et al., 2000). We suggest that antidepressants modulate the activity of intracellular transduction pathways that are involved in cytokine regulation.

One of these pathways may be the cyclic adenosine monophosphate dependent protein kinase A (cAMP/PKA) pathway. Cyclic AMP is formed out of ATP by the enzyme adenylate cyclase. Adenylate cyclase activity is positively or negatively regulated by several G-protein coupled receptors (including serotonergic and adrenergic receptors). The major effect of cAMP is the activation of PKA. This kinase, in turn, regulates the activity of other kinases, enzymes and transcription factors. Activation of the cAMP/PKA-pathway inhibits the production of several pro-inflammatory cytokines (including IFN- γ and TNF- α) and stimulates that of IL-10 (Platzer et al., 1995; Benbernou et al., 1997; Eigler et al., 1998). Furthermore, it has been shown that TCAs and fluoxetine may increase intracellular concentrations of cAMP in human white blood cells (Xia et al., 1996; Edgar et al., 1999). Thus, since activation of the cAMP/PKA-pathway and exposure to antidepressants have comparable effects on cytokine production, and since antidepressants may increase cAMP in white blood cells, it is hypothesized that antidepressants modulate cytokine production through activation of the cAMP/PKA-pathway.

Recently, Musselman et al. demonstrated that paroxetine pretreatment is effective in the prevention of IFN- α induced depression (Musselman et al., 2001). It has been shown that IFN- α treatment activates the cytokine network (Brassard et al., 2002), leading to psychiatric disturbances, including major depression (Bonaccorso et al., 2001). We hypothesize that paroxetine inhibits pro-inflammatory cytokine production and, hence, prevents cytokine-induced depressive symptoms.

The purpose of this study is to examine the effect of paroxetine and imipramine on cytokine production, and whether these effects are mediated by cAMP/PKA. Therefore, the specific aims of this study are 1) to examine the effect of paroxetine and imipramine on the stimulated production of TNF- α , IFN- γ , IL-10 and IL-6 in diluted human whole blood and in human peripheral blood mononuclear cells (PBMCs); 2) to examine the role of the cAMP/PKA pathway in this effect by pretreatment of the cultures with SQ22536 (an adenylate cyclase inhibitor) or Rp-8-Br-cAMPS (an inhibitor of PKA).

METHODS

Reagents

Imipramine, SQ22536 and LPS (*E. coli* 026:B6) were purchased from Sigma-Aldrich (Bornem, Belgium). Phytohaemagglutinin was purchased from BioTrading (Bierbeek, Belgium). Paroxetine was a kind gift of GlaxoSmithKline (The Netherlands). Rp-8-Br-cAMPS was acquired from BIOLOG Life Science Institute (Bremen, Germany).

Subjects

After an overnight fast, blood was drawn from 20 healthy volunteers (10 males and 10 females) between 8:30 and 9:30 a.m. All subjects gave written informed consent after full explanation of the study design. This study was approved by the Medical Ethical Committee of the Academic Hospital of Maastricht. The mean age of the subjects was 27.0 ± 5.3 years. Exclusion criteria for the subjects were: i) age under 18 or over 40; ii) a past or present history of psychiatric disorder (axis-I); iii) subjects who ever had been taken major psychotropic medication, such as antidepressants, antipsychotics and anxiolytics; iv) subjects with alcohol or any other drug of dependence abuse; v) smokers; vi) subjects with major somatic disorders, such as diabetes, autoimmune diseases, acquired immunodeficiency syndrome; vii) subjects who currently (two weeks prior to blood sampling) suffered from an infectious, allergic or inflammatory response. The subjects abstained from caffeine and alcohol for at least 12 hours prior to blood sampling.

Culture and stimulation of whole blood

Blood was diluted four times in RPMI 1640 culture medium (BioWhittaker, Verviers, Belgium) supplemented with L-glutamine and antibiotics (100 U/ml penicilline and 100 µg/ml streptomycine). The suspension was homogenized and plated in a 24-well tissue culture plate (Costar, The Netherlands) at 1 ml/well. The wells were pre-filled with concentrated solutions of SQ22536 or Rp-8-Br-cAMP. The final concentration of both substances was 1 µM. After the addition of the blood suspension, the plates were incubated for 30 min at 37°C and 5% CO₂. Subsequently, concentrated solutions of the antidepressants were added to the respective wells. For paroxetine, the final concentrations were 0.01 µM, 0.1 µM and 1 µM, whereas for imipramine 1 µM and 10 µM was used. The plates were placed on a gyratory shaker for 10 min. to mix to contents of the wells, and afterwards incubated for 20 min. at 37°C and 5% CO₂. Then, the cultures were stimulated with LPS and PHA (final concentrations of 10 µg/ml

and 1 µg/ml, respectively). Plates were mixed for 10 min. and transferred to the incubator. Two sets of cultures were performed: one set for the determination of TNF- α (supernatant collected after 24h) and one set for the measurement of IFN- γ , IL-10 and IL-6 (supernatant collected after 72 h). After incubation, the plates were mixed for 10 min. before centrifugation at 800xg and 4°C for 15 min. Supernatant was carefully aspirated and transferred to labeled eppendorf tubes. Samples were stored at -20°C until cytokine analysis.

Purification and culturing of PBMCs

Heparinized human whole blood was layered on top of a separation gradient (Histopaque-1077[®], Sigma, Bornem, Belgium) and centrifuged according to the manufacturers instructions. The PBMC-layer was removed and washed once in culture medium (RPMI-1640, Life Technologies, Ghent, Belgium), and an additional two times in culture medium supplemented with 10% autologous plasma. Cells were counted and the cell suspension was adjusted to 2×10^6 cells/ml. The cells were plated in a 96-well tissue culture plate at 50 µl (1×10^5 cells) per well. Concentrated solutions of the adenylate cyclase inhibitor (SQ22536) and the PKA-inhibitor (Rp-8-Br-cAMPS) were added. The plates were incubated at 37°C and 5% CO₂ for 30 min. before the addition of concentrated solutions of paroxetine and imipramine. Plates were pre-incubated for an additional 30 min. before stimulation with LPS/PHA. The final culture volume was 100 µl and the final concentrations of LPS and PHA were 10 µg/ml and 1 µg/ml, respectively. After an incubation period of 48h, the plates were centrifuged for 10 min. at 800xg and 4°C. Supernatants were carefully aspirated and transferred to Eppendorf tubes. The samples were stored at -20°C until cytokine determination.

Cytokine determinations

Cytokines were determined using commercially available enzyme linked immunosorbent assays (ELISA) from DiaMed-Eurogen (Turnhout, Belgium). All assays were performed according manufacturer instructions. Determinations were performed in a single run by the same operator. The intra-assay variations were lower than 8% for all assays.

Statistics

Repeated Measures (RM) design analyses of variance (ANOVAs) were used to examine the within-subject variability with the effects of paroxetine or imipramine and SQ22536 and Rp-8-Br-cAMPS alone or together versus the positive control as temporal (treatment) condition, and between-subject

variability with gender as factor. The results of all RM design ANOVAs were corrected for sphericity. Post-hoc analysis was performed using Fisher's Least Significant Difference (LSD) test to check for significant differences between treatment conditions.

RESULTS

Effects of paroxetine on cytokine production in whole blood

Figure 1 shows the effects of paroxetine with and without SQ22536 or Rp-8-Br-cAMPS on the production of TNF- α , IFN- γ , IL-10 and IL-6. RM design ANOVA showed a significant effect of paroxetine on the production of TNF- α ($F=4.5$, $df=2/35$, $p=0.02$). Fisher's LSD test showed suppressant effects of paroxetine at 0.01 μM and 0.1 μM . RM design ANOVA showed that treatment with paroxetine alone, and together with SQ22536 or Rp-8-Br-cAMPS, significantly affected the stimulated production of TNF- α ($F=3.6$, $df=4/61$, $p=0.013$). Fisher's LSD test showed that Rp-8-Br-cAMPS significantly reversed the inhibitory effect of all tested paroxetine concentrations on TNF- α production. Pre-incubation with SQ22536 did not significantly increase TNF- α production in the paroxetine treated cultures, although the significant reduction of TNF- α by 0.01 μM paroxetine, was lost by the addition of SQ22536.

Paroxetine significantly affected the production of IFN- γ ($F=5.0$, $df=3/45$, $p=0.007$). Fisher's LSD test showed suppressant effects of paroxetine at 0.01 μM , 0.1 μM and 1 μM . RM design ANOVA showed a significant effect of paroxetine alone, or together with SQ22536 or Rp-8-Br-cAMPS on the stimulated production of IFN- γ ($F=3.2$, $df=3/54$, $p=0.03$). Fisher's LSD test showed that neither pre-incubation with SQ22536 or Rp-8-Br-cAMPS reversed the suppressant effects of paroxetine.

There was a significant effect of paroxetine on the production of IL-10 ($F=5.7$, $df=2/31$, $p=0.01$). Fisher's LSD test showed suppressant effects of paroxetine at 0.01 μM , 0.1 μM and 1 μM . However, there was no significant effect of paroxetine with SQ22536 or Rp-8-Br-cAMPS on the stimulated production of IL-10 ($F=2.3$, $df=2/27$, $p=0.1$).

Although paroxetine inhibited the production of both IFN- γ and IL-10, RM design ANOVA showed that 0.1 μM and 1 μM paroxetine decreased the IFN- γ /IL-10 ratio ($F=3.4$, $df=2/44$, $p=0.034$; data not shown).

There were no significant effects of paroxetine ($F=0.9$, $df=2/30$, $p=0.4$) or paroxetine with SQ22536 or Rp-8-Br-cAMPS ($F=2.0$, $df=2/35$, $p=0.1$) on the stimulated production of IL-6.

The above results indicate that the inhibitory effect of paroxetine on TNF- α production is probably mediated by PKA, independent of cAMP formation. There is no evidence that cAMP/PKA is involved in the inhibitory effects of paroxetine on IFN- γ or IL-10 production.

Figure 1
Effects of paroxetine with or without SQ22536 or Rp-8-Br-cAMPS on the stimulated production of TNF- α , IFN- γ , IL-10 and IL-6 in diluted human whole blood. Blood from 20 healthy volunteers was diluted and stimulated with LPS (10 μ g/ml) and PHA (1 μ g/ml), in the presence or absence of paroxetine 0.01 μ M, 0.1 μ M or 1 μ M. SQ22536 and Rp-8-Br-cAMPS, both used at 1 μ M, were pre-incubated for 30 min. After addition of antidepressants, the cultures were incubated for 30 min. before stimulation with LPS/PHA. Supernatant was collected after 24h (for TNF- α determination) or 72h (for determination of IFN- γ , IL-10 or IL-6). Bars represent means, error bars SEM.

Effects of imipramine on cytokine production in whole blood

Figure 2 shows the effects of imipramine with and without SQ22536 or Rp-8-Br-cAMPS on the production of TNF- α , IFN- γ , IL-10 and IL-6. Imipramine significantly reduced the production of TNF- α at both 1 μ M and 10 μ M ($F=6.5$, $df=2/28$, $p=0.005$). RM design ANOVA showed a significant effect of treatment with imipramine with or without SQ22536 and Rp-8-Br-cAMPS on the production of TNF- α ($F=5.6$, $df=3/53$, $p=0.002$). Fisher's LSD test showed that SQ22536 did not reverse the decrease in TNF- α production, but pre-incubation with Rp-8-Br-cAMPS significantly reversed the imipramine-induced inhibition of TNF- α production.

The secretion of IFN- γ was significantly decreased by imipramine 1 μ M and 10 μ M ($F=5.5$, $df=2/33$, $p=0.008$). RM design ANOVA showed a trend towards significant effects of imipramine with or without SQ22536 or Rp-8-Br-cAMPS on IFN- γ production ($F=2.4$, $df=3/51$, $p=0.076$).

RM design ANOVA showed a significant effect of imipramine on IL-10 production ($F=5.5$, $df=2/30$, $p=0.008$). Fisher's LSD-test showed that imipramine 1 μ M and 10 μ M significantly suppressed the production of IL-10. Imipramine did not change the IFN- γ /IL-10 production ratio (data not shown).

There was a significant effect of treatment with imipramine with or without SQ22536 or Rp-8-Br-cAMPS on the production IL-10 ($F=3.4$, $df=4/60$, $p=0.015$). Fisher's LSD test showed that this significant effect is attributable to the imipramine-induced suppression of IL-10 production.

There was no effect of imipramine ($F=2.8$, $df=1/25$, $p=0.1$) or imipramine with or without SQ22536 and Rp-8-Br-cAMPS ($F=1.4$, $df=2/38$, $p=0.3$) on the production of IL-6.

The above results indicate that a) PKA may play a role in the inhibition of the production of TNF- α by imipramine; b) adenylate cyclase activity seems to have no influence in this effect; and c) the effects of imipramine on IFN- γ and IL-10 are probably independent of cAMP and PKA.

Figure 2

Effect of imipramine with or without SQ22536 or Rp-8-Br-cAMPS on the stimulated production of TNF- α , IFN- γ , IL-10 and IL-6 in diluted human whole blood. Blood from 20 healthy volunteers was diluted and stimulated with LPS (10 μ g/ml) and PHA (1 μ g/ml), in the presence or absence of imipramine 1 μ M or 10 μ M. SQ22536 and Rp-8-Br-cAMPS, both used at 1 μ M, were pre-incubated for 30 min. before stimulation with LPS/PHA. Supernatant was collected after 24h (for TNF- α determination) or 72h (for determination of IFN- γ , IL-10 or IL-6). Bars represent mean, error bars SEM.

Influence of paroxetine and imipramine on the production of cytokines in human PBMCs

The effects of paroxetine (with or without SQ22536 or Rp-8-Br-cAMPS) on the stimulated production of TNF- α , IL-10 and IFN- γ are shown in Figure 3. First, the effect of paroxetine alone was analysed with RM design ANOVA. Paroxetine significantly affected the stimulated production of TNF- α ($F=3.8$, $df=2/26$, $p=0.038$), but not that of IL-10 ($F=2.5$, $df=3/35$, $p=0.082$) or IFN- γ ($F=0.5$, $df=2/27$, $p=0.598$). Fisher's LSD test showed that 1 μ M paroxetine significantly decreased the production of TNF- α . Addition of SQ22536 or Rp-8-Br-cAMPS did not affect cytokine production.

On the other hand, as shown in Figure 4, there was a marked effect of imipramine on the production of all cytokines examined (TNF- α : $F=19.1$, $df=2/30$, $p<10^{-3}$; IL-10: $F=20.9$, $df=1/21$, $p<10^{-3}$; IFN- γ : $F=25.7$, $df=2/28$, $p<10^{-3}$). Post-hoc analysis showed that this was entirely due to the highly suppressant effect of imipramine 10 μ M, whereas there was no effect of imipramine 1 μ M on either cytokine examined. Remarkably, as shown in Figure 4, the production of IL-10 and IFN- γ was completely inhibited by co-incubation with 10 μ M imipramine. Pre-incubation with SQ22536 or Rp-8-Br-cAMPS did not change the inhibitory effect of imipramine on either cytokine examined.

DISCUSSION

The major findings of this study are that paroxetine decreases the stimulated secretion of TNF- α in human whole blood at 0.01 μ M and 0.1 μ M, and of IFN- γ and IL-10 at 0.01 μ M, 0.1 μ M and 1 μ M. Imipramine significantly reduces the production of TNF- α , IFN- γ and IL-10 at 1 μ M and 10 μ M. Both antidepressants do not influence the secretion of IL-6. The inhibitory effect of both antidepressants on TNF- α production is probably mediated by PKA, since addition of the selective PKA-inhibitor Rp-8-Br-cAMPS restored TNF- α production. On the other hand, no significant role of cAMP and/or PKA in the effect of paroxetine or imipramine on IFN- γ and IL-10 production could be found. In human PBMC-cultures, 1 μ M of paroxetine decreased the production of TNF- α , whereas there was no effect on IFN- γ or IL-10. Imipramine at 10 μ M strongly inhibited the production of TNF- α , IFN- γ and IL-10 in PBMC-cultures. These effects could not be reversed by inhibition of adenylate cyclase or PKA.

Figure 3

Effects of paroxetine alone or together with SQ22536 or Rp-8-Br-cAMPS on the stimulated production of TNF- α , IFN- γ and IL-10 in human PBMCs. PBMCs from 20 healthy volunteers were plated at 1×10^5 cells/well and stimulated with LPS (10 μ g/ml) and PHA (1 μ g/ml), in the presence or absence of paroxetine 0.01 μ M, 0.1 μ M or 1 μ M. SQ22536 and Rp-8-Br-cAMPS, both used at 1 μ M, were pre-incubated for 30 min. After addition of antidepressants, the cultures were incubated for 30 min. before stimulation with LPS/PHA. Supernatant was collected after 48h and frozen for cytokine determination. Bars represent mean, error bars SEM.

Figure 4

Effects of imipramine alone or together with SQ22536 or Rp-8-Br-cAMPS on the stimulated production of TNF- α , IFN- γ and IL-10 in human PBMCs. PBMCs from 20 healthy volunteers were plated at 1×10^5 cells/well and stimulated with LPS (10 $\mu\text{g/ml}$) and PHA (1 $\mu\text{g/ml}$), in the presence or absence of imipramine 1 μM or 10 μM . SQ22536 and Rp-8-Br-cAMPS, both used at 1 μM , were pre-incubated for 30 min. After addition of antidepressants, the cultures were incubated for 30 min. before stimulation with LPS/PHA. Supernatant was collected after 48h and frozen for cytokine determination. Bars represent mean, error bars SEM.

The modulation of the cytokine network is best studied in diluted whole blood (De Groote et al., 1992; Zangerle et al., 1992; Yaqoob et al., 1999). Whole blood cultures are more reproducible and have lower intra-assay variations when compared to cultures of purified PBMCs. Moreover, diluted whole blood reflects better the *in-vivo* conditions, contain the humoral immunoregulatory factors present in plasma and preserve the normal monocyte/lymphocyte ratio. However, when examining the influence of pharmacological agents on cytokine secretion, it may be warranted to use PBMC-cultures, since red blood cells may act as scavengers for the agents, and the purified white blood cells are less influenced by other immunomodulatory mediators. Therefore, in this study we compared whole blood and PBMC-cultures to examine the effects of antidepressants on cytokine production.

This is the first report on the effects of antidepressants on the stimulated production of TNF- α in diluted human whole blood. We found that paroxetine at 0.01 μ M and 0.1 μ M inhibits TNF- α secretion in stimulated diluted whole blood. In PBMC-cultures, paroxetine reduces TNF- α secretion at all concentrations, reaching significance at 1 μ M. Imipramine suppressed TNF- α production in whole blood cultures at 1 μ M and 10 μ M, and in PBMC-cultures only at 10 μ M. Although these results suggest that paroxetine inhibits TNF- α secretion *in-vitro*, they also indicate that it is not a marked effect. Others failed to show an effect of paroxetine on the LPS-induced TNF- α release *in-vivo* (Shen et al., 1999). Fluoxetine, another SSRI, did not change splenic TNF- α expression after LPS administration (Yirmiya et al., 2001). On the other hand, the inhibition of TNF- α by imipramine 10 μ M was more pronounced. This is in agreement with previous studies, which showed that various TCAs, including imipramine at 5 μ M, inhibit the secretion of TNF- α and other pro-inflammatory cytokines in human monocytes (Xia et al., 1996). In addition, also some *in-vivo* studies show inhibitory effects of imipramine and other TCAs on TNF- α . For example, treatment with amitriptyline significantly decreased the *ex-vivo* TNF- α secretion in depressed patients (Lanquillon et al., 2000). Chronic treatment with amitriptyline or nortriptyline, significantly increased the p75 subunit of the soluble TNF- α receptor (Hinze-Selch et al., 2000), an endogenous TNF- α antagonist. Chronic administration of desipramine (a TCA) attenuated the *in-vivo* production of TNF- α after an LPS-challenge (Shen et al., 1999; Connor et al., 2000). Thus, TCAs inhibit the production of TNF- α , both *in-vivo* and *in-vitro*, whereas the effect of SSRIs has remained unclear. We found that both paroxetine and imipramine decrease the stimulated TNF- α secretion *in-vitro*. Another finding of this study is that paroxetine and imipramine, at all concentrations examined, decrease the production of IFN- γ and IL-10. We previously showed that antidepressants of several classes inhibit the production of IFN- γ in stimulated human whole blood (Maes et al., 1999a; Kubera et al.,

2001). In these studies, antidepressants had either no effect or even enhanced IL-10 production, but also decreased the production of IFN- γ relative to that of IL-10 (Maes et al., 1999a; Kubera et al., 2001). Here, both paroxetine and imipramine decreased the stimulated production of IL-10. However, paroxetine reduced the IFN- γ /IL-10 production ratio.

A second major finding of this study is that the effects of paroxetine and imipramine on TNF- α production could be mediated by PKA. It is known that, pharmacological elevation of intracellular cAMP-concentrations and the subsequent activation of PKA inhibits TNF- α expression (Platzer et al., 1995; Eigler et al., 1998; Zidek, 1999). We found that blockade of cAMP-formation by the adenylate cyclase inhibitor SQ22536 had no effect on the inhibition of TNF- α by both imipramine and paroxetine. However, inhibition of PKA by Rp-8-Br-cAMPS did reverse this reduction. Thus, the antidepressant-induced inhibition of TNF- α could be mediated by an up-regulation of PKA-activity. Inactive PKA is a tetrameric complex composed of two catalytic subunits bound with two regulatory subunits. Upon binding of cAMP with the regulatory units, these are released from the catalytic subunits rendering the latter free to phosphorylate several target proteins, including other kinases and transcription factors (Shaywitz and Greenberg, 1999). Since blockade of cAMP-formation does not reverse the effect of the antidepressants on TNF- α production, the up-regulation of PKA-activity is probably not mediated by an increase in cAMP. Therefore, it is suggested that antidepressants – being lipophilic molecules that can readily cross the plasma-membrane – interfere with either the catalytic or the regulatory subunit of PKA in such a way that PKA activation is facilitated, resulting in a down-regulation of TNF- α expression.

Increases in cAMP are known to inhibit IFN- γ secretion (Benbernou et al., 1997). However, in the present study, inhibitors of adenylate cyclase or PKA could not reverse the inhibitory effect of the antidepressants on IFN- γ production, indicating that this effect is not mediated by cAMP or PKA. Therefore, other intracellular signal transduction proteins may be involved. Interestingly, it has been reported that fluoxetine may reduce Protein Kinase C (PKC) activity in lymphocytes (Edgar et al., 1998, 1999), an effect that is probably mediated by calcium mobilization. PKC-activation is an important step in pro-inflammatory cytokine production (Kontny et al., 1999). Thus, it may be suggested that down-regulation of PKC mediates the inhibitory effect of antidepressants on IFN- γ and/or TNF- α production.

Another finding of this study is that the effect of antidepressants on whole blood cytokine production differs from that in PBMC-cultures. Although there is an inhibitory effect of paroxetine and imipramine on TNF- α production in both cultures, it could not be reversed by SQ22536 or Rp-8-Br-cAMPS in PBMC-cultures. Only imipramine 10 μ M inhibited the secretion of IFN- γ and IL-10 in

PBMC-cultures, whereas all examined paroxetine and imipramine concentrations inhibited this in whole blood cultures. Other cells present in whole blood cultures (including neutrophils, granulocytes and platelets) can produce cytokines and release immunomodulatory mediators that influence cytokine production by other cell types. Thus, the inhibitory effect of paroxetine and imipramine on cytokine production in whole blood may be related to the influence of these drugs on cells not present in PBMC-cultures. Further, the ratio between lymphocytes and monocytes is preserved in whole blood cultures, whereas purification of PBMCs alters this ratio. Both cell types influence one another, and changing the ratio between them will result in a different cytokine response. Since whole blood cultures reflect better the *in-vivo* environment, are more reproducible and have a lower variability, the use of diluted whole blood instead of PBMC-cultures is advised.

It is concluded that paroxetine and imipramine decrease the production of TNF- α , IFN- γ and IL-10, but not that of IL-6, in stimulated, diluted whole blood. The inhibitory effect on TNF- α is probably related to PKA-activation, whereas no role of cAMP/PKA in the effect on IFN- γ or IL-10 could be demonstrated. In PBMC-cultures, the highest concentration of paroxetine and imipramine inhibits TNF- α production, but this effect is not mediated by the cAMP/PKA-pathway. IFN- γ and IL-10 production in PBMCs is strongly inhibited by 10 μ M imipramine, which cannot be reversed by inhibitors of adenylate cyclase or PKA.

These data are important because negative immunoregulatory effects of antidepressants may play a role in the effectiveness of paroxetine in the prevention of depressive side effects related to IFN- α therapy in Hepatitis C patients (Musselman et al., 2001). IFN- α therapy induces the production of other cytokines including TNF- α and IFN- γ (Bonaccorso et al., 2001; Brassard et al., 2002), two pro-inflammatory cytokines that are implicated in the pathophysiology of major depression (Connor and Leonard, 1998). It is suggested that paroxetine, through lowering the induction of pro-inflammatory cytokines, attenuates the effects of these cytokines on the development of depressive symptomatology. Further research should focus on intracellular transduction cascades that mediate the effects of antidepressants on cytokine production. This may help to clarify the mechanism of action of antidepressants and to elucidate the biology of mood disorders.

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Chapter 8

Influence of antidepressants on intracellular levels of cyclic adenosine monophosphate in human peripheral blood mononuclear cells

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ABSTRACT

This study examines the effects of paroxetine and imipramine on intracellular concentrations of cyclic adenosine monophosphate (cAMP) in human peripheral blood mononuclear cells. It was found that imipramine and paroxetine had no effect on basal cAMP-levels. Stimulation with lipopolysaccharides and phytohaemagglutinin increased intracellular cAMP-concentrations. However, pre-incubation with imipramine or paroxetine did not influence this increase. These data do not support the hypothesis that cAMP may be related to the *in-vitro* anti-inflammatory effects of antidepressants.

INTRODUCTION

Antidepressants have *in-vitro* anti-inflammatory effects. It was found that tricyclic antidepressants decrease the stimulated production of interleukin-1 β (IL-1 β), IL-6 and tumor necrosis factor α (TNF- α) in human monocytes and that of IL-2 and interferon- γ (IFN- γ) in human T-lymphocytes (Xia et al., 1996). These cytokines enhance inflammatory reactions and stimulate T-cell proliferation and, hence, are termed pro-inflammatory cytokines. Some antidepressants enhance the production of IL-10 (Maes et al., 1999), a potent anti-inflammatory cytokine. In stimulated whole blood, antidepressants of several classes have negative immunoregulatory effects as shown by a reduction of the IFN- γ /IL-10 production ratio (Maes et al., 1999; Kubera et al., 2001). The mechanism whereby antidepressant drugs modulate cytokine expression is yet not known. One explanation may revolve around the second messenger cyclic adenosine monophosphate (cAMP). Indeed, antidepressants can increase intracellular cAMP-concentrations in purified human monocytes and lymphocytes (stimulated with lipopolysaccharides (LPS) and phytohaemagglutinin (PHA) respectively) and in concanavalin-A stimulated human peripheral blood mononuclear cells (PBMCs) (Xia et al., 1996; Edgar et al., 1999). Further, pharmacological elevation of cAMP inhibits the production of various pro-inflammatory cytokines, including IFN- γ and TNF- α , and stimulates the expression of IL-10 (Platzer et al., 1995; Benbernou et al., 1997; Zidek, 1999). Thus, it is suggested that antidepressants may enhance cAMP-responses in white blood cells, leading to changes in cytokine expression profiles. The effects of antidepressants on cytokine production have been studied in diluted whole blood stimulated with LPS and PHA. However, the effects of antidepressants on cAMP-concentrations in leukocytes after stimulation with LPS/PHA have not yet been examined.

Therefore, the aim of this study was to determine the influence of the antidepressants imipramine and paroxetine on intracellular cAMP-levels in human PBMCs, with or without LPS/PHA-stimulation.

METHODS

Reagents

Imipramine and LPS (E. coli 026:B6) were purchased from Sigma-Aldrich (Bornem, Belgium). PHA was purchased from BioTrading (Bierbeek, Belgium). Paroxetine was a kind gift of Smith-Kline Beecham (The Netherlands).

Blood collection

After an overnight fast, venous blood was drawn from seven healthy volunteers using Vacutainer®-systems (BD, Erembodegem, Belgium). Subjects abstained from alcohol and caffeine at least 12h prior to blood sampling.

PBMC isolation

Heparinized human whole blood was layered on top of a separation gradient (Histopaque-1077®, Sigma, Bornem, Belgium) and centrifuged according to manufacturer's instructions. The PBMC-layer was removed and washed once in culture medium (RPMI1640, Life Technologies, Ghent, Belgium), and an additional two times in culture medium supplemented with 5% autologous plasma. Cells were counted and the cell suspension was adjusted to 2×10^7 cells/ml. The cells were plated in a 96-well tissue culture plate at 50 μ l (1×10^6 cells) per well. Concentrated solutions of the antidepressants and/or stimulants were then added to a final culture volume of 100 μ l. The plates were incubated at 37°C and 5% CO₂ for 30 min. before the addition of the antidepressants.

Culture conditions

All culture conditions were performed in triplicate. Imipramine was used at final concentrations of 1 μ M and 10 μ M, paroxetine at 0.01 μ M, 0.1 μ M and 1 μ M. These concentrations were chosen since the therapeutic plasma level of imipramine is around 1 μ M and that of paroxetine around 0.1 μ M. Both antidepressants were dissolved in PBS and further diluted in culture medium. The antidepressants were pre-incubated for 30 min. before stimulation with LPS/PHA. The final concentration of LPS and PHA was 10 μ g/ml and 1 μ g/ml respectively.

Intracellular cAMP determination

After the addition of LPS/PHA, the plates were incubated for 30 min. at 37°C and 5% CO₂. After incubation, the plates were put on ice and 100 μ l of an ice-cold solution of 10 % trichloro-acetic acid (TCA) was added to each well. The suspension in each well was transferred to eppendorf tubes. Subsequently, each well was rinsed with 200 μ l of an ice-cold 5% TCA-solution and this suspension was added to the corresponding eppendorf tube. The tubes were centrifuged at 14000 rpm in an Eppendorf centrifuge for 10 min. The lysates were transferred to glass tubes, and TCA in the samples was extracted using diethyl ether. One ml of H₂O-saturated diethyl ether was added to each sample, thoroughly mixed for 20 seconds, and, after separation, the upper ether-phase was aspirated. This procedure was repeated 4 times. Finally, the samples were

put at 50°C for 30 min. to evaporate remaining ether. Samples were stored at -20°C until analysis of cAMP.

Cyclic AMP-concentrations were determined with a radio-immunoassay (RIA) according to the method of Brooker et al. (1979), using antibodies as described by Steiner et al. (1972).

Statistics

Repeated Measures (RM) design analysis of variance (ANOVA) was used to examine the effect of treatment. Dunn-tests were performed to check for significant differences between culture conditions. The results of the RM design ANOVA were corrected for sphericity.

RESULTS AND DISCUSSION

The intracellular cAMP-concentrations are depicted in Figure 1. RM design ANOVA showed that there was a significant effect of culture treatment ($F = 19.2$; $df = 11/245$; $p < 10^{-5}$). Stimulation with LPS/PHA significantly increased intracellular cAMP-concentrations (Dunn-test: $t = 5.11$, $p = 0.00002$). However, there was no effect of the antidepressants on cAMP-concentrations. Moreover, pre-exposure of the LPS/PHA-stimulated cultures to the antidepressants does not have an additional effect on intracellular cAMP-concentrations.

We found that stimulation of human PBMCs with LPS/PHA increases intracellular cAMP-concentrations. This is in agreement with previous studies which describe an increase in intracellular cAMP-concentrations in lymphocytes following exposure to various immunological stimulants (Wang et al., 1978; Shenker and Gray, 1979; Oksenberg et al., 1989). An increased adenylate cyclase activity, followed by an increase in cAMP, seems to be an important step in T-cell activation (Oksenberg et al., 1989). We hypothesized that antidepressants may further enhance the stimulation-induced cAMP increase, which could lead to altered gene expression (e.g. cytokine production). However, in contrast to the results of Xia et al. (1996), we were not able to show that antidepressants of two different classes enhanced intracellular cAMP-levels. Several differences in cultures conditions may explain these discrepancies. In the study of Xia et al. (1996), separated monocytes and lymphocytes were pre-incubated for 24h with tricyclic antidepressants before stimulation with 10 µg/ml LPS and 5 µg/ml PHA respectively, both for 15 min.

Figure 1

Intracellular cAMP-concentrations in human PBMCs after exposure to imipramine and paroxetine, with (light bars) or without (dark bars) stimulation with LPS/PHA. Antidepressants were added 30 min. before stimulation with LPS/PHA. Cells were lysed and cAMP-levels determined 30 min. after the addition of LPS/PHA.

In the present study, PBMCs (containing both monocytes and lymphocytes) were pre-incubated for 30 min. before stimulation with LPS and PHA (10 μg/ml and 1 μg/ml respectively) for 30 min. These culture conditions were chosen since the same conditions were employed in our studies concerning cytokine production in human whole blood. After stimulation, intracellular cAMP-concentrations reach maximum after 30 min. (Wang et al., 1978; Oksenberg et al., 1989) and, hence, intracellular cAMP-concentrations were measured at this time point. Recently, it has been shown that the influence of antidepressants on cAMP may be dependent on the concentration of the mitogenic stimulant (Edgar et al., 1999). Together with the differences in culture conditions, this may explain the differences between the study of Xia et al. (1996) and ours.

Cyclic AMP is formed by adenylate cyclase, an enzyme that is stimulated by several G-protein coupled receptors, including β-adrenergic and some serotonergic receptors (i.e. 5-HT₄, 5-HT₆ and 5-HT₇). The intracellular cAMP-concentration is controlled through its breakdown by phosphodiesterases (PDEs). A possible mechanism by which antidepressants may alter cAMP-concentrations is therefore a direct interaction of antidepressants with adenylate cyclase or PDEs. However, indirect mechanisms should be considered. For

example, increased serotonin concentrations in the culture medium (through inhibition of serotonin reuptake) may stimulate serotonergic receptors that are positively coupled to adenylate cyclase. In this case, longer incubation times are required to detect the cAMP increase. However, we have shown previously that the effects of antidepressants on cytokine production are not related to their serotonergic action (Kubera et al., 2000). This possibility was therefore excluded and we measured intracellular cAMP after 30 min., which is sufficient to detect pharmacological elevation of cAMP-concentrations. Further, it may be hypothesized that the anti-inflammatory effects of antidepressants in diluted whole blood, results from mediators released by other cell types that are not present in PBMCs. However, the anti-inflammatory effects of antidepressants have also been shown in PBMC-cultures (Xia et al., 1996). Thus, antidepressants influence cytokine production by a direct effect on PBMCs.

It is concluded that stimulation with LPS/PHA increase intracellular concentrations of cAMP in human PBMCs. Paroxetine and imipramine do not influence basal cAMP-concentrations. Moreover, pre-exposure of PBMCs with antidepressants does not enhance the cAMP response to LPS/PHA stimulation. Our data do not support the hypothesis that an increase in intracellular cAMP-concentrations is related to alterations in white blood cell cytokine production after exposure to antidepressants *in-vitro*.

It has recently been demonstrated that fluoxetine may inhibit the activity of Protein Kinase C (PKC) in human T-cells (Edgar et al., 1999). Inhibition of PKC may result in a decreased production of pro-inflammatory cytokines (Kontny et al., 1999). Therefore, it may be hypothesized that inhibition of PKC by antidepressants results in an inhibition of pro-inflammatory cytokine production in whole blood. The possible role of PKC in the antidepressant altered cytokine production is subject of future research.

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Chapter 9

Imipramine inhibits interleukin-6 and tumor necrosis factor- α production in primary rat whole brain cell cultures

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Submitted

ABSTRACT

Antidepressants of several classes influence cytokine production by human blood cells. They inhibit Interferon- γ (IFN- γ) and Tumor Necrosis Factor- α (TNF- α), two important pro-inflammatory cytokines, and/or stimulate Interleukin-10 (IL-10), an anti-inflammatory cytokine. We have used re-aggregating whole brain cell cultures to assess the effects of antidepressants (imipramine) on the production of cytokines by brain cells. After stimulation with 1 $\mu\text{g/ml}$ or 10 $\mu\text{g/ml}$ of lipopolysaccharides (LPS), high concentrations of TNF- α , IL-10 and IL-6 were found in the culture supernatant of the whole brain spheroids. IFN- γ could not be detected. Imipramine at 30 μM significantly decreased the production of IL-6, but did not affect IL-10 production. TNF- α production was significantly reduced by imipramine 30 μM after stimulation with 10 $\mu\text{g/ml}$ LPS. There was no effect of 10 μM imipramine on the cytokines examined. This is the first study to show that antidepressants may influence stimulated cytokine production in a multi-cellular culture system of brain cells. It is concluded that imipramine inhibits the production of IL-6 and to a lesser extent that of TNF- α . We hypothesize that antidepressants exert part of their effect through modulation of central cytokine production.

INTRODUCTION

There is accumulating evidence that pro-inflammatory cytokines may play a role in the aetiology and/or pathophysiology of mood disorders. Pro-inflammatory cytokines are mainly produced by activated monocytes, macrophages or other immune cells, and include Interleukin-1 β (IL-1 β), IL-6, Tumor Necrosis Factor- α (TNF- α) and Interferon- γ (IFN- γ). Evidence for their potential role in psychiatric disorders recently emerged from both human studies and animal experiments. Depressed patients have higher circulating levels of pro-inflammatory cytokines as compared to healthy controls (Sluzewska et al., 1996; Berk et al., 1997; Maes, 1999; Mikova et al., 2001; Owen et al., 2001). Hypersecretion of these cytokines has profound behavioural effects and may contribute to the pathophysiology of major depression (Connor and Leonard, 1998). The potential impact of cytokines on the aetiology of major depression is highlighted by the fact that psychiatric healthy patients treated with cytokines (for example Hepatitis C patients treated with IFN- α) can suffer from mood-disturbances and even full-blown major depression (Capuron et al., 2000; Zdilar et al., 2000; Bonaccorso et al., 2002). In animals, administration of pro-inflammatory cytokines or lipopolysaccharides (LPS) induces "sickness behaviour", a syndrome that resembles some key features of clinical depression like anhedonia, reduced social exploration, reduced food intake and sleep disturbances (Yirmiya, 1996; Dantzer, 2001). The molecular mechanisms underlying the behavioural effects of cytokines are yet not known, but may involve stimulation of the afferent vagal nerve and induction of cytokine production in several brain structures (Konsman et al., 1999; Dantzer, 2001).

Indeed, it is known that cytokines can be produced by virtual all brain cells in both normal and pathological conditions (Szelenyi, 2001). Neurons constitutively produce TNF- α (Ignatowski et al., 1997) and IL-6 (Gadient and Otten, 1997; Lemke et al., 1999), which can also be expressed by astrocytes and microglia and are up-regulated under insulting conditions (Munoz-Fernandez and Fresno, 1998; Orzylowska et al., 1999; De Simoni et al., 2000). Brain IL-10 expression has also been found, mainly by astrocytes (Jander et al., 1998; Hulshof et al., 2002). It has even been proposed that some cytokines play a physiological role in the normal brain (Vitkovic et al., 2000).

If hypersecretion of pro-inflammatory cytokines is involved in the pathophysiology of major depression then antidepressants should inhibit the production or counteract the actions of these cytokines. In this respect, it has been shown that antidepressants inhibit pro-inflammatory cytokine and may stimulate anti-inflammatory cytokine production (for review see Kenis and Maes, 2002). This has been demonstrated *in-vitro* using cultures of whole blood or purified peripheral blood mononuclear cells (Xia et al., 1996; Maes et al.,

1999; Lin et al., 2000; Kubera et al., 2001), and in animal models (Shen et al., 1999; Connor et al., 2000; Kubera et al., 2000a, 2000b). However, the effects of antidepressants on cytokine production by brain cells have, to the best of our knowledge, not yet been examined.

Re-aggregating whole brain cell cultures have been used in toxicological and neurodevelopmental studies (Honegger and Schilter, 1995; Teunissen et al., 2000, 2002). Brain cells, obtained by dispersion of fetal rat whole brains, are cultured while continuously rotating on a gyratory shaker. This procedure allows the cells to re-aggregate and form spherical structures. These so-called spheroid cell cultures consist of a three-dimensional network of several functional cell types including neurons, astrocytes, microglia and oligodendrocytes (Teunissen et al., 2000). Since cytokines secreted by one cell can influence the functioning of surrounding cells, it is better to study the cytokine network in a culture system comprising all cells present in the brain. Therefore, we used whole brain spheroids to examine the effects of antidepressants on cytokine production by brain cells. The specific aims of the study were to examine the production of IL-6, TNF- α , IL-10 and IFN- γ in rat whole brain spheroids stimulated with LPS, and to investigate the influence of the tricyclic antidepressant imipramine on the production of these cytokines.

METHODS

Animals

Time pregnant Lewis rats were bred in the animal centre of the University of Maastricht. Experiments were approved by the local committee for animal welfare at the university, according the guidelines of the European Communities Council Directive (86/609/EEC).

Re-aggregating brain cell cultures

Whole brain spheroids were prepared as follows. Whole brains from 12-10 fetuses (gestation day 15) from two rats were isolated and transferred to a nylon gauze bag (Nytal[®], 200 μ m, Merck, The Netherlands) in ice-cold Hank's D2 solution (5.5 mM D-glucose, 41.32 mM sucrose, 138 mM NaCl, 5.4 mM KCl, 0.17 mM Na₂HPO₄, 14 μ M phenol red, 1.8 mM CaCl₂, 0.8 mM MgCl₂). Subsequently, the brains were washed three times in Hank's D1 solution (5.5 mM D-glucose, 46.52 mM sucrose, 138 mM NaCl, 5.4 mM KCl, 0.17 mM Na₂HPO₄, 0.22 mM KH₂PO₄, 14 μ M phenol red), and thereafter gently extruded through the nylon gauze. The cell suspension was then filtered through a nylon mesh (Nytal[®], 150 μ m, Merck, The Netherlands) and washed three times by

centrifugation at 170xg for 5 min. in Hank's D1 solution. Finally, cells were plated out in 25 ml DeLong flasks (Bellco Glass) at a density of 1×10^7 cells/ml (3.5 ml/flask) in DMEM supplemented with 1% L-glutamine and 10% foetal calf serum. The flasks were placed on a gyratory shaker (85 rpm, Innova 2000, New Brunswick, USA) in an incubator at 37°C, 9% CO₂ in humidified air. After 2 days, macroscopic spherical cell aggregates have formed. The cultures were then transferred to 50-ml DeLong flasks and 5 ml of medium was added. Thereafter, medium was changed three times a week. After 1 week of culture, D,L- α -tocopherol (0.1 mM, ICN Biomedical, Aurora, USA) was added to the medium.

Culture maintenance and experiments

After 18 days in culture, the spheroids were plated in 6-well tissue culture plates. Fifty microliter of a concentrated solution of imipramine (Sigma-Aldrich, Zwijndrecht, The Netherlands) was added. The final concentrations of imipramine were 10 μ M and 30 μ M. Medium served as the corresponding control. The plates were then incubated for 4 hours while continuously shaking at 37°C and 9% CO₂ in a humidified atmosphere. Afterwards, concentrated solutions of LPS (*E.coli* strain 026:B6; Sigma-Aldrich, Zwijndrecht, The Netherlands), or medium as control, were added to the cultures. Final concentrations of LPS were 1 μ g/ml and 10 μ g/ml. The plates were then incubated for 72 hours. After incubation, culture supernatants were harvested and stored at -20°C until cytokine analysis.

Cytokine determinations

Cytokines in the culture supernatant were determined by enzyme-linked immunosorbent assays (ELISA) using specific and validated sets of monoclonal or polyclonal antibodies. Antibodies and standard solutions for the development of TNF- α and IFN- γ ELISAs were obtained from U-CyTech (Utrecht, The Netherlands), those for IL-10 and IL-6 from BD-Biosciences (Erembodegem, Belgium). Capture antibodies were diluted in coating buffer (for IL-6, TNF- α and IFN- γ : 50 mM citric acid, 100 mM phosphate buffer, pH 7.2; for IL-10: 50 mM carbonate buffer, pH 9.6) to 2 μ g/ml (IL-6, TNF- α and IFN- γ) or 1 μ g/ml (IL-10). Diluted antibodies were transferred to 96-well ELISA microtiterplates (Greiner, Germany) at 100 μ l per well, and incubated overnight at 4°C. Plates were washed twice in phosphate buffered saline (PBS) with 0.1 % Tween-80 (PBS/T-80) and blocked by adding 150 μ l of 3% bovine serum albumine (BSA) in PBS to each well. After 1 hour incubation at 37°C, plates were washed twice with PBS/T-80. Then, 100 μ l of the samples or standards were added to the wells, and the plates were subsequently incubated for 2 hours

as follows: IL-6 and IFN- γ plates at 37°C; IL-10 and TNF- α plates at room temperature (RT), while shaking at 500 rpm on a microtiterplate-shaker. After incubation, plates were washed five times, followed by the addition of biotinylated detection antibodies (100 μ l/well) diluted in Tris-buffered saline with 0.05 % Tween-20 (TBS-T) and 2.5% BSA (TBS-T/BSA). Again, IL-6 and IFN- γ plates were incubated at 37°C; IL-10 and TNF- α plates at RT while shaking (1 hour for all plates). Then, plates were washed 5 times and incubated at 100 μ l/well with streptavidin conjugated Horse Radish Peroxidase (DiaMed-Eurogen, Turnhout, Belgium) for 20 min. at 37°C. After washing 5 times, 100 μ l of a mixture of H₂O₂ and tetramethylbenzidine (DiaMed-Eurogen, Turnhout, Belgium) was added to the wells and incubated for 20 min. at RT in the dark. Reaction was stopped after 20 min. by the addition of 2 N H₂SO₄. Absorption (at 450/600 nm) was determined using a microtiterplate reader. Standard curve was constructed and cytokine concentrations in the samples calculated using accompanying software.

Using the above described protocol, detection limits were 10 pg/ml for IL-10 and TNF- α , and 200 pg/ml for IL-6. In our laboratory, inter-assay variation was below 10%. All assays were performed on the same day (for each cytokine) by a single operator using the same batch of reagents.

Statistics

Repeated measures design analyses of variance (RM-ANOVAs) were used to evaluate the effect of imipramine on cytokine production. Data were normalized where necessary, and adjusted for sphericity violation using Greenhouse-Geisser corrections. Post-hoc analyses were performed with Least Significant Difference tests (LSD). All analyses were performed using the statistical software package SPSS version 10.0.

RESULTS

Spheroid cultures stimulated with 1 μ g/ml or 10 μ g/ml LPS produced high levels of TNF- α , IL-10 and IL-6 (see Figure 1). IFN- γ could not be detected after stimulation with LPS (data not shown). Additional experiments indicated that neither phorbol-myristate acetate (PMA), Concanavalin A (ConA) or phytohaemagglutinin (PHA) were able to induce IFN- γ production in these cultures (data not shown). In cultures stimulated with 1 μ g/ml, imipramine significantly affected the production of IL-6 ($F=8.9$, $df=1/4$, $p=0.04$), but not that of IL-10 ($F=3.7$, $df=2/5$, $p=0.101$). There was a trend towards a significant effect on TNF- α production ($F=7.3$, $df=1/3$, $p=0.07$). Post-hoc analysis

indicated that imipramine at 30 μ M significantly reduced the production of IL-6, but there was no significant effect of 10 μ M imipramine. Although RM-ANOVA did not show an effect of imipramine treatment on TNF- α , post-hoc comparison indicated that 30 μ M imipramine significantly reduced TNF- α production.

Similar results were found when spheroids were stimulated with 10 μ g/ml LPS. There was a significant effect of imipramine on the production of TNF- α ($F=10.4$, $df=2/5$, $p=0.02$) and IL-6 ($F=46.1$, $df=1/3$, $p=0.005$), but not on that of IL-10 ($F=2.6$, $df=2/5$, $p=0.178$). Post-hoc analysis showed that 30 μ M imipramine significantly reduced the production of TNF- α and IL-6. There was no effect of imipramine 10 μ M on any of the cytokines examined.

In view of the limited number of experiments, non-parametric statistical analyses were concomitantly performed. However, Friedman-tests to assess the effects of imipramine on cytokine production in cultures with either 1 μ g/ml or 10 μ g/ml LPS, showed the same results as with RM-ANOVAs.

DISCUSSION

The present study shows that whole brain spheroids can be stimulated with LPS to produce cytokines. TNF- α , IL-10 and IL-6 were found in high concentrations in the culture supernatant, while IFN- γ could not be detected. Imipramine, a widely used tricyclic antidepressant, inhibited the stimulated production of IL-6 and to a lesser extent that of TNF- α in these whole brain spheroids. IL-10 production was not affected by imipramine.

We found that whole brain spheroids are capable of producing cytokines upon stimulation with LPS. It is known that LPS can stimulate various brain cells to produce cytokines. For example, LPS induces TNF- α production in rat astrocytes (Lieberman et al., 1989; Chung and Benveniste, 1990; Forloni et al., 1997) and microglia (Nakamura et al., 1999; Puffenbarger et al., 2000; Facchinetti et al., 2003). IL-6 is produced upon LPS-stimulation by rat astrocytes (Lieberman et al., 1989; Benveniste et al., 1990; Gottschall et al., 1994; Fischer et al., 1997; Forloni et al., 1997) and microglia (Loughlin and Woodroffe, 1996; Forloni et al., 1997; Nakamura et al., 1999). Recently, Ledeboer and colleagues have demonstrated the production of IL-10 in astrocyte-microglia co-cultures and in enriched astrocyte and microglia cultures, upon stimulation with LPS (Ledeboer et al., 2000, 2002). The effects of LPS are probably mediated by CD14 and Toll-like receptor 4, both of which have found to be expressed in the rat brain (Laflamme and Rivest, 2001). In view of the above, it is likely that the major source of TNF- α , IL-10 and IL-6 in our spheroid cultures are LPS-activated astrocytes and microglia.

Figure 1
Concentrations of TNF- α , IL-10 and IL-6 in culture supernatant of LPS (1 μ g/ml and 10 μ g/ml) stimulated whole brain spheroid cultures, in the absence or presence of 10 μ M or 30 μ M imipramine. Bars represent means of 4 independent experiments; error-bars represent S.E.M.

A second major finding is that the antidepressant imipramine inhibits TNF- α and IL-6 production, but has no effect on IL-10. Interestingly, TNF- α and IL-6 have been implicated in the pathophysiology of major depression. Both cytokines have profound behavioural effects. For example, intra-peritoneal administration of TNF- α in rats reduced the consumption of a palatable solution, an indication of anhedonia (Hayley et al., 1999; Brebner et al., 2000), and intracerebroventricular administration induces anxiogenic-like effects

(Connor et al., 1998). IL-6 seems to have less potent effects on behaviour (Connor et al., 1998; Lenczowski et al., 1999; Brebner et al., 2000), although prolonged elevation of IL-6 may reduce food and water intake (Sakic et al., 2001). Further, IL-6 may act synergistically or enhance the behavioural effects of other cytokines implicated in sickness behaviour (Lenczowski et al., 1999). Another biological feature of major depression in which TNF- α and IL-6 have been implicated, is the hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis. Indeed, both cytokines are potent activators of the HPA-axis (Hayley et al., 1999; Brebner et al., 2000; Dunn, 2000). Moreover, TNF- α and IL-6 can modulate brain functioning by altering monoamine metabolism in various brain regions (Zalcman et al., 1994, 1998; Connor et al., 1998; Brebner et al., 2000). Disturbances in monoaminergic systems have often been implicated in major depression (Maes and Meltzer, 1995). In our model, imipramine inhibited the production of TNF- α and IL-6, and may thus alleviate the behavioural, neurochemical and neuroendocrine effects associated with these cytokines.

Current research on the mechanism of action of antidepressant drugs focuses on their possible neurogenic and/or neuroprotective properties (Malberg et al., 2000; Coyle and Duman, 2003). TNF- α and IL-6 have been implicated in both neurodegeneration and neuroprotection. It seems that while they support neuronal functioning and have proliferating effects in normal physiological conditions, they can induce neuronal cell death at higher concentrations, for example during inflammation or other major insults such as ischaemia (Munoz-Fernandez and Fresno, 1998). On the other hand, the anti-inflammatory cytokine IL-10 seems to have opposing effects to TNF- α and IL-6. First, while the pro-inflammatory cytokines can be neurotoxic and lead to neurodegeneration, IL-10 is neuroprotective and improves the survival and proliferation of neurons and microglia (Strle et al., 2001). Second, IL-10 inhibits peripheral and central pro-inflammatory cytokine production by microglia (Di Santo et al., 1997; Heyen et al., 2000). Third, the behavioural effects of systemic or central administration of LPS are prevented by IL-10 (Bluthé et al., 1999). Our results indicate that the antidepressant imipramine may change the balance between potential neurotoxic and neuroprotective cytokines, and may thus prevent neurodegeneration and/or enhance neurogenesis.

The relevance of our data is underscored by the fact that the effects of imipramine occur in clinically relevant concentrations. Indeed, while steady state serum and plasma concentrations of imipramine reach 0.5 - 1 μ M (Vandel et al., 1992; Levitt et al., 1999), brain concentrations reach much higher levels (Fisar et al., 1996). Moreover, accumulation of antidepressants in brain tissue is not limited to imipramine (Karson et al., 1993; Bolo et al., 2000). Further, while the

presence and effects of cytokines in the brain have mainly been studied in animals, it is known that also human brain cells can produce a broad range of cytokines and express several cytokine receptors (Lee et al., 2002).

This study shows that imipramine – in pharmacological concentrations – reduces the LPS-induced production of IL-6 and TNF- α in a stable multi-cellular culture system of brain cells. In view of the possible role of IL-6 and TNF- α in the pathophysiology of major depression, we hypothesize that imipramine exert its antidepressant effect, at least in part, by modulating the brain cytokine network.

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Chapter 10

General discussion

1. EFFECTS OF ANTIDEPRESSANTS ON CYTOKINE PRODUCTION

Although it was long thought that depression was associated with a state of immunosuppression, Maes et al. (1990) and Smith (1991) were the first to propose that chronic activation of some aspects of the immune system (i.e. macrophages) and the concurrent excessive secretion of pro-inflammatory cytokines underlies the pathophysiology of major depression. Since then, evidence has accumulated to confirm a possible role of cytokines in major depression. This evidence includes:

- increased concentrations of circulating cytokines in depressed patients
- administration of pro-inflammatory cytokines to animals induces sickness behaviour - a syndrome that resembles several clinical features of major depression
- human cytokine therapy can induce major depression in psychiatric healthy subjects

Moreover, it is now clear that cytokines have a dramatic effect on neurotransmitter function and on neuro-endocrine homeostasis. Several possible pathways by which cytokines modulate brain function have been revealed. These include:

- passive or active transfer of cytokines across the blood-brain barrier
- induction of cytokine synthesis in the brain through stimulation of endothelial cells and peri-vascular macrophages
- cytokine stimulation of the afferent vagus nerve

Hence, several possible mechanisms of how cytokines can precipitate depression have been described. These include:

- alterations in neurotransmission and neurotransmitter turn-over
- cytokine-induced catabolism of tryptophan
- induction of HPA-axis hyperactivity
- induction of "glucocorticoid resistance", resulting in sustained HPA-axis hyperactivity and cytokine secretion

If pro-inflammatory cytokines play a role in the aetiology and pathophysiology of major depression, then antidepressants should interfere in their synthesis and/or function. This intriguing hypothesis provides alternative research possibilities to verify the theory of cytokine-induced depression. To study the effect of antidepressants on cytokine production, one can measure circulating cytokine concentrations in depressed patients before and after treatment with antidepressants. As reviewed in Chapter 2, many studies on this topic report inconsistent findings. Several methodological issues may underlie this inconsistency. Due to the limited sample size in most studies, it is not possible to differentiate subgroups of depressed patients (i.e. patients with different

phenotypes of depression). It has been shown that cytokine patterns may differ between patients with melancholic or non-melancholic depression. Differences were also found in patients with dysthymia versus major depression. Furthermore, patients were sometimes treated with different kinds of antidepressants, and medication status at baseline is not always clear. The latter, together with other possible confounding factors (such as smoking), may contribute to the divergent study results. In addition, it is conceivable that not all depressed patients have increased levels of cytokines. Depression is a syndrome with a multi-causal aetiology including genetic, environmental and somatic factors. Patients should therefore be divided in groups with or without elevated cytokine levels. Sluzewska et al. (1996) showed that in patients with elevated IL-6 levels before treatment, these levels returned to control after six weeks of fluoxetine treatment. Besides the heterogeneity of the study population, methodological differences in cytokine measurement may contribute to inconsistency of studies. Cytokine concentrations in serum or plasma should be measured using specific, high-sensitive and well-standardized immuno-assays (e.g. ELISA). Standardization helps to compare results between different studies, while the use of high-sensitive assays is a prerequisite to accurately detect the low concentration of circulating cytokines.

Animal models may be another suitable tool to examine the influence of antidepressants on cytokines. Chronic treatment with TCAs inhibits pro-inflammatory cytokines and enhances IL-10 production (reviewed in Chapter 2). While the effects of other antidepressants on cytokine secretion is not so clear, all antidepressants do counteract the behavioural responses associated with pro-inflammatory cytokines, as do the anti-inflammatory cytokines IL-10 and IL-1RA.

Concerning the effects of antidepressants on cytokine production *in-vitro*, Xia et al. (1996) found that TCAs inhibit monocytic pro-inflammatory cytokines and Th1-derived cytokines. In that study, purified monocytes or lymphocytes were used. However, when examining the effects of pharmacotherapeutics on cytokines, the use of diluted whole blood offers a reliable and more reproducible method (De Groote et al., 1992; Zangerle et al., 1992; Yaqoob et al., 1999). Whole blood cultures contain all cells and immunoregulatory factors that are normally present in plasma and thus reflect better the *in-vivo* environment than purified cells. Further, immune cells are present in their natural occurring ratios. This is important because secretion of cytokines is influenced by cellular contact with other cells and by soluble factors secreted by these cells. Changing the ratio between the different cell types may result in altered cytokine patterns. Stimulated diluted whole blood thus better reflects the *in-vivo* situation, and by addition of a drug to these cultures, one can examine the net effect of this substance on cytokine secretion. Moreover, whole blood

cultures are more reproducible and exhibit smaller inter- and intra-assay variability (De Groote et al., 1992; Yaqoob et al., 1999). Using this model, it was shown that clomipramine, sertraline and trazodone significantly reduce IFN- γ secretion (Maes et al., 1999). Clomipramine also increased that of IL-10. That study was extended to other antidepressants in this thesis (Chapter 4). Imipramine, venlafaxine, 5-hydroxytryptophan (5-HTP, the immediate precursor of serotonin) and fluoxetine all significantly enhanced IL-10 secretion. Fluoxetine also decreased the production of IFN- γ . Another study, using similar whole blood cultures, reported that moclobemide increased stimulated IL-10 production (Lin et al., 2000). Thus, antidepressants of different classes have anti-inflammatory effects *in-vitro* by reducing the secretion of IFN- γ and/or by increasing that of IL-10. The question arises whether these findings are relevant for the *in-vivo* antidepressant properties of these drugs. It is obvious that anti-inflammatory properties of antidepressants are in accordance with the hypothesis that pro-inflammatory cytokines are involved in depression pathology, and with the hypothesis formulated in this thesis. As explained in detail before, peripherally produced cytokines can have dramatic effects on central nervous system function (Dunn et al., 1999; Dunn, 2000), and these peripheral cytokines may precipitate major depression (Connor and Leonard, 1998; Maier and Watkins, 1998). Consequently, it is likely that a reduction in peripherally produced cytokines would contribute to the relief of depressive symptomatology. The reduction of IFN- γ secretion indicates that antidepressants temper a Th1-like immune response, which is associated with activation of monocytes/macrophages and the accompanying production of pro-inflammatory cytokines. On the other hand, IL-10 is an important de-activator of both Th1-lymphocytes and monocytes/macrophages, and inhibits pro-inflammatory cytokine secretion. Antidepressant-induced increases in IL-10 could thus normalize activated immune cells and reduce hypersecretion of pro-inflammatory cytokines. Another emerging question deals with the fact that the whole blood cultures reflect acute effects of antidepressants on cytokine production. In view of the delayed clinical onset of antidepressants, one could argue that only studies dealing with chronic antidepressant exposure are relevant. However, it is conceivable that acute effects on cytokine production, that are maintained during prolonged treatment, are reflected in clinical improvement only after the biological processes related to the behavioural effects of cytokines are restored.

Although circulating pro-inflammatory cytokines may precipitate major depression, it is possible that their effects on emotion and behaviour are mediated by induction of central pro-inflammatory cytokine secretion (Dantzer, 2001). Further, cytokines like IL-1 β and TNF- α may have physiological roles in the normal brain (Vitkovic et al., 2000). In addition, antidepressants occur in

higher concentrations in the brain than in blood. Therefore, the effects of antidepressants on cytokine production by brain cells were explored in Chapter 9. Hereto, re-aggregating whole brain cell cultures were used. These so-called spheroid cultures are based on the capacity of foetal cells to form macroscopic three-dimensional networks (spheres) of differentiated cells, provided that they are cultured under continuous shaking. Brain-spheroid cultures contain mature and functional neurons, astrocytes, microglia and oligodendrocytes (Teunissen et al., 2000). As such, these cultures comprise all the cell types of the normal brain and may reflect better the *in-vivo* micro-environment as compared to other primary cell cultures. It was found that imipramine decreased the production of IL-6 and TNF- α in LPS-stimulated spheroids, while IL-10 secretion was not influenced. The results indicate that antidepressants may selectively reduce pro-inflammatory cytokine secretion by brain cells. These results concur with the findings in human whole blood cultures. Obviously, more experiments with other antidepressants are warranted. Nevertheless, the current data are intriguing, especially in the light of an emerging new theory of depression, the so-called "neurogenesis hypothesis of major depression".

Briefly, this hypothesis states that a prolonged reduction in the generation of new-born neurons in the hippocampus of the adult brain may underlie several psychiatric disorders, including major depression (Jacobs et al., 2000; Kempermann and Kronenberg, 2003). There is indeed evidence that depression is associated with hippocampal atrophy and hippocampal volume loss. In addition, accumulating evidence suggests that chronic treatment with antidepressants increases proliferation of progenitor cells in the hippocampus and promotes neuronal survival (Malberg et al., 2000; Duman et al., 2001; Santarelli et al., 2003). Chronic treatment with antidepressants also increases the activity of survival pathways and stimulate the secretion of several trophic factors, including brain derived neurotrophic factor (BDNF) (Nibuya et al., 1996). The underlying mechanisms of the reduction in neurogenesis has yet to be elucidated, but the negative influence of glucocorticoids on neuronal survival have been implicated (Jacobs et al., 2000). However, it may be suggested that also pro-inflammatory cytokines contribute to this phenomenon (Monje et al., 2003). Indeed, TNF- α , IL-1 β and IL-6 are potent neurotoxic molecules (Szelenyi, 2001). As discussed before, hypersecretion of pro-inflammatory cytokines can induce cytokines in the brain, which has deleterious effects on brain function and on neuronal viability. The findings that antidepressants selectively inhibit pro-inflammatory cytokine secretion by brain cells may thus explain part of the neuroprotective effects of these drugs.

This thesis thus provides evidence that several types of antidepressants modulate the secretion of cytokines, both in the peripheral immune system and in the central nervous system. It is suggested that the reduction of pro-

inflammatory cytokine secretion is part of the mechanism of the antidepressant action of these drugs.

Besides antidepressants, also other psychotropic drugs can modulate the secretion of cytokines. The effect of antipsychotic drugs on cytokines has been subject of a few studies. However, the results are inconsistent and differ between the drugs examined. For example, in *in-vitro* experiments, haloperidol increases the secretion of IL-2, IFN- γ (Rudolf et al., 2002) and IL-1RA (Song et al., 2000), and inhibits TNF- α and IL-1 β (Moots et al., 1999). Clozapine enhances the secretion of IL-2, IFN- γ , IL-6, IL-1RA and sIL-2R (Hinze-Selch et al., 1998; Song et al., 2000; Rudolf et al., 2002). Chlorpromazine has no effect on the *in-vitro* production of TNF- α , IL-1 β , IL-1RA or IL-10 (Bleeker et al., 1997), but in animal experiments, it decreases pro-inflammatory cytokines and enhances IL-10 secretion (for review see Pollmacher et al., 2000). The stimulatory effect of haloperidol and clozapine on Th1-cytokines (IFN- γ , and IL-2) is opposite to the effect of antidepressants. In patients, subchronic treatment with clozapine increases pro-inflammatory cytokines such as TNF- α (Pollmacher et al., 1996), sIL-2R (Maes et al., 1994), and IL-6 (Maes et al., 1997), while prolonged treatment may decrease TNF- α levels (Monteleone et al., 1997). Treatment with risperidone increases IL-12 (Kim et al., 2001), while haloperidol treatment decreases IL-2 levels (Kim et al., 2000). In conclusion, antidepressants and antipsychotics exert differential effects on the cytokine network, and the effect on cytokine secretion of the latter is not uniform among the different drugs.

2. INTRACELLULAR MECHANISMS UNDERLYING ANTIDEPRESSANT ALTERED CYTOKINE EXPRESSION

If the antidepressant altered cytokine secretion contributes to clinical recovery from depression, then uncovering the intracellular mechanisms by which antidepressants change cytokine gene expression would undoubtedly lead to a better understanding of antidepressant action and to the development of new and better drugs. It could also reinforce the cytokine hypothesis of major depression, since a direct effect of antidepressants on cytokine synthesis implies that changes in cytokine patterns during treatment are related to the action of the pharmacological agent itself, and that these changes do not merely reflect an improvement in the patient's condition.

Several mechanisms that underlie the effect of antidepressants on cytokines can be proposed. First, it is possible that the primary biochemical effect of antidepressants (i.e. reuptake inhibition of serotonin and/or noradrenaline) is responsible for a change in cytokine expression. Lymphocytes, monocytes and

macrophages express both the receptors and transporters of several neurotransmitters (Gordon and Barnes, 2003). Moreover, serotonin and noradrenaline have profound effects on immune cell function and cytokine secretion (Mossner and Lesch, 1998; Elenkov et al., 2000). Hence, it may be suggested that the anti-inflammatory properties of antidepressants are related to their serotonergic or noradrenergic activities. Chapter 5 presents data of a study that examined the effect of serotonin and other serotonergic agents on cytokine production in stimulated whole blood. The results point to a complex effect of serotonin on cytokine secretion. Serotonin itself decreases IFN- γ production, but depletion of intracellular serotonin by PCPA inhibits both IFN- γ and IL-10 secretion. Thus, increases in extracellular serotonin concentrations has anti-inflammatory effects through inhibition of IFN- γ , but intracellular serotonin may be necessary for optimal cytokine production. It could not be determined which receptor subtype mediates the effect of serotonin on IFN- γ production. Neither flesinoxan, a 5-HT_{1A} agonist, nor mCPP, a 5-HT_{2A/2C} agonist, had an effect on IFN- γ . Ritanserin, a 5-HT_{2A/2C} antagonist, decreased IFN- γ secretion. It is possible that other 5-HT receptor subtypes (5-HT₄, 5-HT₇, which are also expressed on immunocytes) are involved.

The inhibitory effect of serotonin on IFN- γ and the concomitant reduction of the IFN- γ /IL-10 ratio, is comparable to the effects of antidepressants. However, it was concluded that modulation of cytokine expression by antidepressants is not mediated by serotonin, because antidepressants predominantly increase IL-10, rather than inhibiting IFN- γ . Nevertheless, the role of serotonin cannot be completely excluded. A recent study reported that serotonin inhibits the production of TNF- α in human PBMCs (Cloez-Tayarani et al., 2003). In Chapter 7, it is reported that imipramine and paroxetine also reduce TNF- α secretion in stimulated human whole blood and PBMCs. Clearly, the exact role of serotonin in the effects of antidepressants on cytokines needs further investigation.

The effects of noradrenaline on cytokine expression have been extensively described by others (Elenkov et al., 2000; Elenkov and Chrousos, 2002). Noradrenaline inhibits pro-inflammatory cytokines (TNF- α and IFN- γ), while it stimulates anti-inflammatory cytokine secretion (IL-10 and transforming growth factor β). In addition, the IFN- γ /IL-10 ratio is reduced in whole blood cultures pre-incubated with noradrenaline or noradrenergic agonists (Maes et al., 2000). Thus, antidepressants and noradrenaline have comparable effects on cytokine secretion, and, hence, noradrenaline may be involved in the action of antidepressants on cytokines.

Besides the above mentioned indirect mechanisms (through serotonin and/or noradrenaline) of antidepressants altering cytokine production, more direct effects may be involved. Antidepressants are lipophilic molecules that readily

can cross the cell membrane. It may therefore be proposed that antidepressants modulate intracellular targets, such as kinases or transcription factors, which regulate cytokine gene expression. The effects of antidepressants on cytokine production are comparable to that of drugs that stimulate the cAMP/PKA-signalling pathway. Therefore, it is hypothesized that antidepressants increase cAMP/PKA-signalling, resulting in a decrease of pro-inflammatory (IFN- γ and TNF- α) and an increase in anti-inflammatory (IL-10) cytokine secretion. In order to examine this hypothesis, inhibitors of the cAMP/PKA-pathway were co-incubated with antidepressants in stimulated whole blood (Chapter 7). If antidepressants stimulate cAMP/PKA-signalling, then these inhibitors should reverse the effects of antidepressants on cytokine production. Blockade of cAMP-formation by inhibiting adenylate cyclase did not change the effects of antidepressants (imipramine and paroxetine) on TNF- α , IFN- γ and IL-10. On the other hand, inhibition of PKA reversed the inhibitory effect of both antidepressants on TNF- α production, but not on that of IFN- γ or IL-10. These results indicate that: i) the effects of antidepressants on cytokine production are not mediated by an increase in cAMP (through enhanced activity of adenylate cyclase); and ii) increased PKA-activity is involved in the decrease in TNF- α production, but not in the effects on IFN- γ or IL-10. Further, in Chapter 8 it is shown that antidepressants do not increase intracellular cAMP-concentrations in white blood cells. Taken together, these data indicate that increases in intracellular cAMP-concentrations are not involved in the effect of antidepressants on *in-vitro* cytokine secretion. Yet, the effects of antidepressants on TNF- α are related to enhanced PKA-activity, which is generally thought to be a consequence of increased cAMP-formation. Our data thus show that antidepressants may enhance PKA-activity, independent of increases in cAMP. It is hypothesized that antidepressants may facilitate the interaction of cAMP with the regulatory subunit of PKA, thereby increasing PKA-activity without influencing intracellular cAMP-concentrations. Alternatively, antidepressants may directly activate PKA by interacting with the regulatory subunits. Both possibilities require more detailed investigation.

Although cAMP/PKA-signalling is an important and well-known pathway in the regulation of cytokine expression, many other signalling cascades are involved. It is therefore conceivable that antidepressants target other pathways that modulate cytokine expression. For example, it has been reported that fluoxetine may reduce Protein Kinase C (PKC) activity in lymphocytes (Edgar et al., 1998, 1999). PKC-activation is an important step in pro-inflammatory cytokine production (Kontny et al., 1999). Hence, it may be suggested that down-regulation of PKC mediates the inhibitory effect of antidepressants on pro-inflammatory cytokines. Recently, research has focused on the effects of antidepressants on glucocorticoid receptor functioning. Sustained hyperactivity

of the HPA-axis in depressed patients probably results from reduced glucocorticoid receptor function, resulting in impaired negative feed-back regulation (Holsboer, 2000; Pariante and Miller, 2001). Several antidepressants potentiate glucocorticoid receptor signalling by inducing translocation of the receptor from cytoplasm to the nucleus, independent of the presence of glucocorticoids, and by enhancing glucocorticoid-induced gene transcription (Pariante et al., 1997, 2001, 2003; Okuyama-Tamura et al., 2003). Interestingly, glucocorticoids inhibit pro-inflammatory cytokine secretion (TNF- α and IFN- γ) and stimulate the production of anti-inflammatory cytokines (IL-10) (for review see Elenkov and Chrousos, 2002). Thus, it may be suggested that antidepressants modulate cytokine secretion by enhancing glucocorticoid mediated signalling.

3. FUTURE DIRECTIONS

It is clear that many intracellular pathways are possible targets for antidepressants. Rather than modulating all these pathways and seeing whether they reverse or enhance the effects of antidepressants, a more target-directed approach to find the pathways involved may be preferred. Micro-array technology offers a convenient tool for the simultaneous screening of a vast number of genes that are potentially up- or down-regulated following antidepressant treatment. Both acute and long-term effects (reflecting adaptive changes) on gene expression can be examined using gene expression arrays.

Still, the question remains how antidepressants modulate gene expression. The primary target of these drugs is probably not the genome itself. Rather, antidepressants induce changes in activity of kinases or transcription factors, or modulate the function of membrane-associated and intracellular receptors. These acute changes ultimately lead to altered gene-expression. A possible approach to examine effects of antidepressants on kinase activity is the use of phospho-specific antibodies that recognize (phosphorylated) substrates of specific kinases. Later, the proteins that are primarily activated by these kinases can be determined by other techniques like two-dimensional gel-electrophoresis or mass-spectrometry.

These procedures offer a more appropriate tool to look for intracellular targets of antidepressants, and may give an answer to what signalling pathways are involved in the effects of antidepressants on cytokine synthesis. They will also render additional information on how other common adaptive changes that are relevant for antidepressant action, are established. Finally, the ability to modulate specific signalling pathways with more selective drugs may offer new and improved treatment approaches for major depression.

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Summary

Chapter 1 is a general introduction to this thesis and outlines the scientific background of the hypothesis and the presented experiments. Current views on the mechanism of action of antidepressants and the different antidepressant classes are briefly discussed. This is followed by an introduction to the following topics: cytokines and the immune system, the putative role of cytokines in the pathophysiology of major depression, and intracellular signalling through the cyclic AMP/Protein Kinase A (cAMP/PKA) pathway. The first part of the hypothesis states that antidepressants have anti-inflammatory effects by inhibiting the production of pro-inflammatory cytokines and/or by stimulating that of anti-inflammatory cytokines. The second part states that these effects are mediated by the cAMP/PKA pathway.

Chapter 2 provides a review of the current literature concerning the effects of antidepressants on the production of cytokines. These effects have been examined in depressed patients before and after treatment with antidepressants, in animal models of depression, and in *in-vitro* experiments. The results of the human studies are not always conclusive. The methodological shortcomings underlying these inconsistencies are discussed. From animal experiments it is clear that tricyclic antidepressants have anti-inflammatory effects, while antidepressants of all classes have anti-inflammatory effects *in-vitro*.

In **chapter 3** the stability of cytokines was examined using an accelerated stability testing protocol. We found that samples for the determination of interleukin-6 (IL-6), soluble IL-6 Receptor and CC16 can be stored at -20°C for a longer period of time (years). IL-10 is less stable at -20°C and should be stored at -70°C .

In **chapter 4**, the effects of several antidepressants (imipramine, venlafaxine and fluoxetine) and 5 hydroxytryptophan (5-HTP) on the production of interferon- γ (IFN- γ) and IL-10 were examined. Whole blood from healthy volunteers and treatment resistant depressed patients was stimulated with lipopolysaccharides (LPS) and phytohaemagglutinin (PHA). This stimulates white blood cells to produce cytokines. By incubating the blood in the presence or absence of antidepressants, one can examine the effects of these pharmacological agents on the cytokine network. All examined antidepressants and 5-HTP reduced the IFN- γ /IL-10 production ratio, and, hence, have anti-inflammatory effects.

Antidepressants clearly act on the central serotonergic system by inhibiting the reuptake of serotonin (5-HT) or by stimulation of serotonergic receptors. White blood cells are also capable of synthesizing serotonin and express serotonergic

receptors on their cell membrane. In order to examine whether serotonin mediates the effects of antidepressants on cytokine secretion, the effects of serotonergic agonists and antagonists on the production of cytokines in stimulated whole blood were explored in **chapter 5**. Stimulation of 5-HT_{1A}-receptors (using flesinoxan) did not influence cytokine secretion. Both an agonist (m-chlorophenylpiperazine) and an antagonist (ritanserin) of 5-HT_{2A/C} receptors reduced the IFN- γ /IL-10 ratio. Serotonin itself also decreases the IFN- γ /IL-10 ratio. Intracellular depletion of serotonin (using para-chlorophenylalanine) inhibited both IFN- γ and IL-10 production. It is concluded that intracellular serotonin is necessary for the optimal production of IFN- γ and IL-10 and that 5-HT_{1A} receptors are not involved in the effects of antidepressants on cytokine production. Further, the reduction of the IFN- γ /IL-10 ratio by 5-HT was due to the inhibition of IFN- γ secretion, while previous studies indicated that antidepressants predominantly increased the production of IL-10. Therefore, it is concluded that the effects of antidepressants on cytokines are probably not mediated by serotonin.

In **chapter 6**, the effects of agonists and antagonists of the cAMP/PKA-pathway on *in-vitro* cytokine production are described. The major findings are that stimulation of adenylate cyclase decreases the production of IFN- γ , while it enhances that of IL-10. Stimulation of PKA inhibits both IFN- γ and TNF- α production. Blocking of adenylate cyclase reduces the IFN- γ and TNF- α secretion, while inhibition of PKA stimulates TNF- α and IL-10. We also examined the effects of rolipram on cytokine secretion. Rolipram is an inhibitor of phosphodiesterase type 4, and inhibits the breakdown of cAMP and, hence, increases intracellular cAMP-concentrations. Rolipram, which is also known as an antidepressant, inhibited the secretion of IFN- γ and TNF- α , and to a lesser extent also that of IL-10. However, rolipram reduced the IFN- γ /IL-10 ratio, an effect that is comparable with that of other antidepressants. Clearly, *in-vitro* cytokine production is differentially affected by inhibitors and activators of the cAMP/PKA-pathway.

In **chapter 7**, we examined whether the effects of antidepressants on cytokine production are mediated by activation of the cAMP/PKA-pathway. Stimulated whole blood was incubated with paroxetine and imipramine in the presence or absence of SQ22536 (an inhibitor of adenylate cyclase) or Rp-8-Br-cAMPS (an inhibitor of PKA). We found that both paroxetine and imipramine reduced the secretion of TNF- α , IFN- γ and IL-10. Paroxetine also decreased the IFN- γ /IL-10 ratio. The effects of both antidepressants on TNF- α secretion was reversed by Rp-8-Br-cAMPS. Pre-incubation with SQ22536 had no effect. It is concluded that PKA mediates the effects of antidepressants on TNF- α , but not the effects on IFN- γ or IL-10.

Chapter 8 presents results of the effects of antidepressants on cAMP-concentrations in white blood cells. Both antidepressants affected neither basal cAMP-concentrations nor those after stimulation with LPS and PHA.

The effects of antidepressants on the production of cytokines by brain cells has never been examined before. In **chapter 9**, we used primary rat whole brain cell cultures to examine the effects imipramine on the production of IL-6, TNF- α , and IL-10. The cultures consist of a three-dimensional network of differentiated neurons, astrocytes and microglia. In this way, one can examine the net effect of imipramine on cytokine secretion by all types of brain cells. Imipramine reduced the production of IL-6 and TNF- α . We concluded that imipramine inhibits pro-inflammatory cytokine production by brain cells, and, hence, that it may modulate cytokine expression in the brain.

In **chapter 10**, all the results presented in this thesis are critically discussed in the light of current views on the mechanism of action of antidepressants.

Samenvatting

Hoofdstuk 1 is een algemene introductie en beschrijft de wetenschappelijke achtergrond van de hypothese en de uitgevoerde experimenten. Eerst wordt de huidige visie op de werkingsmechanismen van antidepressiva belicht. Daarna volgt een inleiding tot de volgende onderwerpen: cytokines en het immuunsysteem, de mogelijke rol van cytokines in de pathofysiologie van depressie, en intracellulaire signaaloverdracht via cyclisch AMP/Proteïne Kinase A (cAMP/PKA). Het eerste deel van de hypothese luidt dat antidepressiva anti-inflammatoire effecten hebben door het remmen van pro-inflammatoire cytokines en/of door het stimuleren van anti-inflammatoire cytokines. Het tweede deel stelt dat deze effecten gemedieerd worden door de cAMP/PKA-pathway.

Hoofdstuk 2 is een literatuuronderzoek betreffende de effecten van antidepressiva op de productie van cytokines. Deze effecten werden zowel in depressieve patiënten (voor en na behandeling met antidepressiva), in diersmodellen van depressie, als in *in-vitro* experimenten onderzocht. De resultaten van de humane studies zijn niet eenduidig en de methodologische gebreken van deze studies worden besproken. Uit de diersmodellen blijkt dat voornamelijk de tricyclische antidepressiva een anti-inflammatoir effect hebben, terwijl antidepressiva van alle klassen anti-inflammatoire eigenschappen vertonen *in-vitro*.

Hoofdstuk 3 beschrijft een versnelde stabiliteitsstudie van cytokines in serum. We kwamen tot de conclusie dat interleukine-6 (IL-6), de oplosbare IL-6 receptor en CC16 gedurende lange tijd (verschillende jaren) op -20°C kunnen bewaard worden. IL-10 blijft maar enkele maanden stabiel bij -20°C en wordt best bij -70°C bewaard.

Met de experimenten beschreven in **hoofdstuk 4** onderzochten we de effecten van verschillende antidepressiva (imipramine, venlafaxine en fluoxetine) en 5-hydroxy-tryptofaan (5-HTP) op de gestimuleerde productie van interferon- γ (IFN- γ) en IL-10 *in-vitro*. Bloed van gezonde vrijwilligers en therapieresistente patiënten werd gestimuleerd met lipopolysacchariden (LPS) en phytohemagglutinine (PHA). Hierdoor worden de witte bloedcellen aangezet tot cytokine productie. Door het bloed zowel in aan- als afwezigheid van de antidepressiva te incuberen, krijgen we een idee van de effecten van deze farmacologische stoffen op het cytokine netwerk. Alle onderzochte antidepressiva en 5-HTP reduceren de IFN- γ /IL-10 ratio, en hebben dus anti-inflammatoire effecten.

Antidepressiva beïnvloeden het centrale serotonine systeem door het blokkeren van de heropname van serotonine of door stimulatie van serotonine receptoren. Witte bloedcellen synthetiseren serotonine en brengen serotonine receptoren tot expressie. Om na te gaan of serotonine betrokken is in het effect van antidepressiva op cytokine secretie, werd in **hoofdstuk 5** gekeken naar de invloed van serotonine en agonisten/antagonisten van serotonine op de productie van cytokines. We gebruikten hiervoor gestimuleerd volbloed. Stimulatie van 5-HT1A-receptoren (met flesinoxan) heeft geen invloed op de secretie van cytokines. Zowel een agonist (m-chlorophenylpiperazine) als een antagonist (ritanserin) van 5-HT2A/C receptoren verlagen de IFN- γ /IL-10 ratio. Serotonine zelf verminderde de IFN- γ /IL-10 ratio, net zoals antidepressiva. Intracellulaire depletie van serotonine (door incubatie met parachlorophenylalanine) onderdrukt de secretie van zowel IFN- γ en IL-10. Uit deze experimenten besluiten we dat intracellulair serotonine noodzakelijk is voor een optimale productie van IFN- γ en IL-10, en dat 5-HT1A receptoren niet betrokken zijn in de effecten van antidepressiva op cytokines. Verder is de reductie in de IFN- γ /IL-10 ratio door serotonine voornamelijk te wijten aan een onderdrukking van de IFN- γ productie, terwijl uit eerdere studies bleek dat antidepressiva voornamelijk de IL-10 stimuleren. Daarom werd besloten dat de effecten van antidepressiva op cytokines waarschijnlijk niet gemedieerd worden door serotonine.

Hoofdstuk 6 beschrijft de effecten van agonisten en antagonisten van de cAMP/PKA-weg op de *in-vitro* cytokine productie. De belangrijkste bevindingen zijn dat stimulatie van adenylaat cyclase de secretie van IFN- γ remt en deze van IL-10 stimuleert. PKA-activatie remt de productie van IFN- γ en TNF- α . Het blokkeren van adenylaat cyclase vermindert de IFN- γ en TNF- α productie, terwijl inhibitie van PKA zowel TNF- α als IL-10 stimuleert. Bij deze experimenten werd ook het effect van de phosphodiesterase remmer rolipram onderzocht. Rolipram, wat ook een antidepressivum is, remt de afbraak van cAMP en verhoogt zo de intracellulaire cAMP-concentraties. Rolipram verminderde de secretie van IFN- γ en TNF- α , en in mindere mate deze van IL-10. Net zoals andere antidepressiva verminderde rolipram de IFN- γ /IL-10 ratio. Activatie of inhibitie van de cAMP/PKA-pathway heeft dus diverse effecten op de secretie van cytokines.

In **hoofdstuk 7** werd nagegaan of de effecten van antidepressiva op de *in-vitro* cytokine secretie gerelateerd zijn aan activatie van de cAMP/PKA-pathway. Volbloed werd geïncubeerd met paroxetine of imipramine, telkens in aan- of afwezigheid van SQ22536 (een remmer van adenylaat cyclase), of Rp-8-Br-cAMPS (een inhibitor van PKA). We vonden dat paroxetine en imipramine de productie van TNF- α , IFN- γ en IL-10 reduceerden, waarbij paroxetine ook de IFN- γ /IL-10 reduceerde. De effecten van beide antidepressiva op TNF- α

secretie werd tegengegaan door pre-incubatie met Rp-8-Br-cAMPS. Dit was niet het geval met de andere cytokines. Pre-incubatie met SQ22536 had geen effect. PKA medieert dus de effecten van antidepressiva op TNF- α , maar niet op deze van andere cytokines.

Hoofdstuk 8 geeft de resultaten weer van de effecten van antidepressiva op cAMP-concentraties in witte bloedcellen. Er was geen effect van paroxetine en imipramine op de basale en de gestimuleerde cAMP-concentraties.

De effecten van antidepressiva op cytokine productie in hersencellen werd nog nooit onderzocht. In **hoofdstuk 9** maakten we gebruik van een primaire cultuur van foetale rat hersencellen om de effecten van imipramine op IL-6, TNF- α en IL-10 na te gaan. Het voordeel van een dergelijke cultuur is dat er macroscopische sferen, bestaande uit gedifferentieerde astrocyten, microglia en neuronen gevormd worden. Dit laat ons toe om het effect van imipramine op alle celtypen zoals ze in de hersenen voorkomen te onderzoeken. Imipramine verminderde de productie van IL-6 en TNF- α , maar niet deze van IL-10. We besluiten hieruit dat imipramine de productie van pro-inflammatoire cytokines door hersencellen remt, en dat imipramine dusdanig de secretie van cytokines in de hersenen kan beïnvloeden.

In **hoofdstuk 10** worden de resultaten uit de verschillende hoofdstukken kritisch besproken. Hierbij worden de resultaten ook besproken in het licht van andere gangbare hypothesen betreffende de werkingsmechanismen van antidepressiva.

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