IMMUNOPHARMACOLOGY OF INFLAMMATORY BOWEL DISEASE

Effects of glucocorticoids and nicotine on cytokine production

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Effects of glucocorticoids and nicotine on cytokine production

Immunofarmacologie van chronische inflammatoire darm aandoeningen

Effecten van glucocorticosteroiden en nicotine op cytokine produktie

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Ter nagedachtenis aan mijn ouders

Voor Tonny

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PART 1

Introduction

Chapter 1

Inflammatory bowel disease

1.1 Clinical aspects of inflammatory bowel disease

There are a number of different definitions for inflammatory bowel disease (IBD) to be found in the literature. The definition accepted by most researchers and clinicians is that the term inflammatory bowel disease comprises two, in practice, distinct entities: ulcerative colitis (UC) and Crohn's disease (CD)[1]. Both disorders are characterized by chronic relapsing inflammation of parts of the gastrointestinal tract of unknown origin, causing abdominal pain and diarrhoea. Because of the recurrent pattern both diseases show signs of acute inflammation (e.g. influx of granulocytes out of the blood into the mucosa of the intestines) as well as signs of chronic inflammation (e.g. infiltrates of lymphocytes and macrophages).

Ulcerative colitis was 're-discovered' in the previous century by Wilks[2] and it became clear that UC was different from other known ulcerating diseases occurring in the large intestine such as dysentery and tuberculosis. UC is restricted to the large bowel and characterized by severe ulcerations, predominantly located in the rectum (90 % of the patients) and rectosigmoid (40 %), but sometimes affecting the entire colon (20 %)[3]. The inflammation is limited to the mucosa, rendering it hyperaemic, edematous and friable in the acute phase. In most cases small mucosal haemorrhages and aphthoid ulcers can be seen macroscopically. Coalescence of these ulcers leads to irregular large ulcers. In the more serious cases parts of the mucosa may disappear and be replaced by granulation tissue which, in a later stage, may be covered with new epithelium. Islands of granulation tissue covered with epithelium may protrude into the lumen giving a cobblestone appearance (pseudopolyps). The mucosa bleeds easily giving rise to the main characteristic of UC, loss of blood with or without faeces. Histology shows infiltrates of inflammatory cells between

the crypts, diminished number of Goblet cells in the crypts and at the colon surface, edema of the mucosa and crypt abscesses (infiltrates of granulocytes in the wall and the lumen of the crypts). Particularly important in active colitis are the epithelial changes that appear in the margins of ulcers and often in the intervening mucosa. They consist of mucous depletion and inflammatory metaplasia. Even more important are varying degrees of atypical dysplasia, which may be classified as low to high grade[4]. The next step is the development of carcinoma.

Symptoms depend on the extent and seriousness of the inflammation. In the case of proctitis the patients do not suffer from diarrhoea but their stools contain blood and mucus. In the case of a more extensive inflammation patients suffer from diarrhoea that often consists of a mixture of faeces, blood and mucus, and colicky lower abdominal pain together with variable extraintestinal symptoms including fever and loss of weight. Grossly bloody stools and extraintestinal complications are far more common than with CD and the blood loss may be considerable[5].

In 1932 Crohn and associates described -what they considered- a new pathologic and clinical entity, regional ileitis[6]. It is a non-specific, chronic, recurrent, granulomatous disease, affecting mainly young adults and characterized by a necrotizing ulcerating inflammatory process. In the long lasting cases formation of strictures is an important feature[7]. Nowadays the consensus is that CD may involve any portion of the gastrointestinal tract from mouth to anus, but most often the small intestine and colon are affected[8]. Diseased segments are separated by macroscopic normal tissue. This phenomena is called skip lesions. In contrast to UC, in CD the inflammation extends through the entire wall of the intestine into the mesentery. In the transmural inflammation the inflammatory cell population consists primarily of lymphocytes and plasma cells. Noncaseating granulomas are found in 50 to 70 % of the cases during histologic examination of diseased intestinal segments or regional lymph nodes. If the terminal ileum is frequently involved in the inflammation the increase of fibrous tissue in the wall may cause it to thicken. The fibrous tissue shrivels, causing stenosis of the lumen and the intestine may become a more or less

stiff tube. The mucosa may be marked by numerous linear ulcers in the long axis of the bowel, creating deep fissures, separated from each other by nodular mucosal thickenings that give a "cobblestone" appearance. Penetrating ulcers may give rise to fistulous communications to adherent loops of bowel. These fistulas, so characteristic for CD, may penetrate to the skin or through the umbilicus or perineum[9].

The clinical presentation of CD is highly variable, depending on the age of the patient, the location and extent of the diseased bowel, and the acuteness of onset of the illness. If only the colon is affected diarrhoea will be the dominant symptom, often without any visible blood loss. In the case of a regional ileitis abdominal pain is prominent and diarrhoea often absent. The pain will be more profound if there is a clear stenosis of the intestine present, independent of the location of the inflammation.

Patients suffering from IBD often have extraintestinal symptoms and manifestations, examples are: erythema nodosum, pyoderma gangrenosum, intestinal peripheral arthritis, ankylosing spondylitis, sacroilitis, sclerosing cholangitis, chronic active hepatitis, anterior uveitis and even pericarditis or myocarditis[10,11]. This might suggest that IBD is a systemic, rather than a purely gastrointestinal disorder. Some of these extraintestinal manifestations may even be life threatening.

There are many similarities between UC and CD, but there are also some clear differences[12].

Characteristic	Crohn's disease (CD)	Ulcerative colitis (UC)
Small intestinal involvement alone	30-35%	Never
Combined small and large	50-60%	Only large intestine
Large intestinal involvement alone	20%	Always
Extraintestinal complications	Yes	Yes
Familial predisposition	Yes	Yes
Age, race, sex distribution	Same as UC	Same as CD
Causation	Unknown	Unknown
Nature of inflammatory reaction		
Ulcerations	Numerous, linear,	Coalescent, irregular,
Transmural fibrosis	++++	Rare
Granulomas	+++	Rare
Secondary cancers	+	+++

Table 1.1 Comparison of Crohn's disease and Ulcerative Colitis.

1.2 Epidemiology of inflammatory bowel disease

Inflammatory bowel disease has a world wide distribution, albeit with significant differences in prevalence and incidence between different areas of the globe. The incidence rates are highest in the northern countries such as the Scandinavian countries, Great Britain, U.S.A. and Canada, followed by Central Europe. In Africa, South America and Asia the incidence of IBD is very low[13]. This implies the existence of a gradient from north to south. Even within the U.S.A. the reported rates in the northern states are higher than those of the southern states[14,15]. Another striking difference is that between urban and rural areas, with

the first having higher incidence rates than the second. Other epidemiological factors that influence the incidence and prevalence of IBD are age, race, gender and socioeconomic status. Both UC and CD are primarily diseases of young adults, as far as the age of onset is concerned. Nevertheless some studies report a bimodal distribution, with the largest peak between the ages of 15 and 25 and a smaller one in the sixth decade of life [16,17]. Some authors, however, suggest that the second peak might be an artefact caused by uncommon presentations of other gastrointestinal diseases such as ischemic bowel disease or diverticulitis[18].

Although the manifestations are similar, Whites seem to be affected more frequently than Blacks, Indians and Hispanics in the U.S.A.[19,20]. Inflammatory bowel disease affects women and men similarly, but the incidence rates for CD are approximately 20 % higher in women, and for UC 20 % higher in men[16].

People with higher socioeconomic status have a greater risk of developing IBD[21,22]. Environmental factors such as the use of oral contraceptives have also been associated with IBD[23,24]. Cigarette smoking seems to have a differentiated effect in IBD, it increases the risk for CD and protects against UC[25,26].

The incidence of CD has shown a steady increase during the last decades in the western countries[27,28,29]. The number of hospital admissions for CD in the Netherlands for instance has almost doubled in the period from 1970 - 1977, whereas the admission rate for UC has hardly changed[30]. For Sweden an increase and level-off at a relatively high incidence value has been described[31,32,33,34].

In UC, in contrast, the incidence rates have remained fairly constant[17,34,35,36,37]. The relation between incidence of UC and CD seems to be relatively constant at about 2:1[38].

1.3 Etiology and pathogenesis

As is the case for many other aspects of IBD the search for the cause of UC and CD has produced similarities between these diseases, the most important one being that the

etiology remains unknown for both. The epidemiology described in the previous paragraph suggests that both disorders are the result of a genetical susceptibility, immunological dysfunction and some environmental agent or agents. Research has mainly focused on genetics, pathology, infection, animal models, immunology and mediators of inflammation.

	Crohn's disease (CD)	Ulcerative colitis (UC)
Disease location	All regions of gastrointsetinal tract	Colon only
Association with smoking	Positive	Negative
Inflammatory response	Transmural, granulomatous	Mucosal
Lymphokine profile	↑ IL-2, IFN-γ (Th1)	↑ IL-4, IL-10 (Th2)
Immunoglobulin profile	IgG_2	IgG_1
Epithelial IgG and complement deposition	No	Yes
Autoantibodies	No	Yes
HLA haplotype	DR-1, DQw5	DR-2

IL=Interleukin; IFN=Interferon; Th1=T Helper lymphocyte subset 1.

Table 1.2 Clinical, immunologic, and genetic differences between Crohn's disease and Ulcerative Colitis.

1.3.1 Genetics

Racial and ethnic differences in the prevalence of IBD have been reported by many authors[19,20,39]. Another striking finding is the increased prevalence of IBD among first degree relatives of patients suffering from UC or CD[40,41,42]. Twin studies are indispensable when studying the genetics of any disorder. The concordance found for monozygotic twins in comparison to dizygotic twins is a strong evidence for genetic susceptibility in IBD[43].

Research suggesting an association between F allotype of C3 (complement system) and CD of the small bowel[44] has been strengthened by studies of first degree relatives of CD patients, reporting an enhanced production of complement components by small intestine in these healthy individuals[45]. These results could indicate that abnormal complement secretion may play a causative role in CD. These abnormalities were not found
for UC, but Podolsky *et al.*[46] reported a selective deficiency in subclass IV mucin glycoproteins from UC patients. Mucin isolated from the colon of CD patients did not differ in subclasses from healthy controls. Results from Tysk and associates[47] confirm Podolsky's findings and suggest that this deficiency might be a primary mechanism, rendering an individual more susceptible to UC.

The association of IBD with the HLA associated disorders ankylosing spodylitis and primary sclerosing cholangitis has prompted the idea that the HLA region may play a role in determining the severity of IBD Multiple studies have reported an association of UC with HLA, although the results are not consistent over all studies[48]. Especially the DR allele would appear to be a marker of disease susceptibility in certain ethnic groups[49].

Studies at the beginning of this decade reported a subset of antineutrophil cytoplasmic antibodies (ANCA) to be an indirect marker of predisposition in UC[50,51]. However, the presence of ANCA seems to be independent of the severity of the disease[52,53]. Even healthy relatives of UC patients have an increased frequency of positive ANCA compared to healthy controls[54].

1.3.2 Immunology

The immune system is a complex and indispensable defense system, and individuals with deficient immune responses die early in life, while those with a partially hampered immune system are at great risk of developing chronic illnesses. There is no doubt that the immune system plays a major role in the etiology of IBD Evidence for this view are the histopathological findings in both disorders, the laboratory findings and most convincing the effectiveness of immunosuppressive drugs in the treatment of IBD

The mechanisms leading to clinical symptoms in IBD may be summarized as initiating events, being perpetuated in a genetically susceptible individual leading to tissue damage and eventually to clinical symptoms.

Initiating events can range from any infectious agent to for instance drugs or toxic food additives. Drugs can directly influence the intestinal functions but can also exert their influence indirectly by changing the intestinal flora[55,56].

Recent clinical and experimental observations have strongly suggested an important role for luminal products in the perpetuation of the inflammation. Decreasing exposure of the inflamed intestinal segments to microorganisms by surgical diversion or antibiotics usually diminishes the inflammation. Furthermore, in animal models of chronic inflammation the inflammation will not develop in a germ free environment, while in some models bacterial products are used to generate the intestinal inflammation[57].

The 'immunological' theories about the cause of IBD can be divided in two major categories[58]. The first category assumes an inappropriate response of the patient's immune system to common agents. This abnormal reaction could be caused by a defect in the mucosal barrier or a defective regulation of the immune system.

The second group of theories originates from the idea that the presence of a harmful agent in the intestinal lumen can cause alterations in the immune system leading to persistence of a specific infection.

IBD, especially CD, is associated with an increased permeability of the mucosa caused by loss of the epithelial integrity, the local effects of inflammatory mediators and the transmigration of neutrophils[59,60]. The increased permeability results in an overly active immune system by facilitating the absorption of toxic agents and antigens present in the lumen of the distal intestine[58]. Several defects of the mucosal barrier have been postulated, such as changes in mucin structure, lectin binding and metabolism of short fatty acids[61,62]. In CD the increased mucosal permeability seems to be a genetically determined factor, rendering certain individuals susceptible for the disease. Indicative for this is the fact that a

proportion of the healthy relatives of CD patients also have an enhanced permeability of their mucosa after taking non-steroidal anti-inflammatory drugs[63,64].

The distal ileum and colon are the domain of many micro-organisms, antigens and all sorts of biological and chemical residues, capable of causing tissue damage. Normally the protective mechanisms of the intestines have no problem eliminating these potential dangers. In the case of actual damage to the intestinal surface the injuries are promptly repaired and the acute inflammatory responses effectively down-regulated[65]. In IBD this immunoregulatory response may be inappropriate due to a genetic abnormality.

A defective immunoregulation may result in multiple abnormal immune responses[58]. One of these abnormal reactions is an excessive aggressive response to elements in the intestinal lumen.

The immune system is a very complex entity with many regulatory mechanisms such as immunoregulatory cells and mediators (pro- and anti-inflammatory). A disturbance of this balance in favour of the pro-inflammatory response, caused by an increase of pro-inflammatory mediators or a decrease of immunosuppressive molecules, may result in chronic inflammation. This suggests that IBD may be caused by a failure to down-regulate an acute, but in itself, common inflammatory response[66]. The inflamed mucosa contains increased numbers of T lymphocytes, but the $CD4^+$, $CD8^+$ ratio does not differ significantly from normal mucosa[67].

Dalton and associates reported an incapability of peripheral blood lymphocytes isolated from UC and CD patients to down-regulate the antigen stimulated proliferation of mononuclear cells[68]. They also found that in contrast to intra-epithelial lymphocytes (I.E.L.) from control patients, I.E.L. from UC patients were unable to down-regulate the proliferative response of peripheral blood T cells to tetanus toxoid[69].

Epithelial cells expressing HLA Class II molecules can present antigens to T cells[70]. Selby *et al.* examined the expression of HLA-DR and HLA-A, B, C antigens by colonic epithelium from IBD patients. They found an increased expression of HLA Class II antigens in inflamed colonic epithelium from CD as well as UC patients in comparison to non-

inflamed epithelium[71]. In UC and CD epithelial cells seem to stimulate $CD4^+$ T cells, whereas enterocytes from normal tissue and non-IBD inflamed tissue predominantly stimulate $CD8^+$ cells[72]. These findings may indicate an intrinsic defect in epithelial cells from IBD patients, causing an up-regulation of the local immune response through activation of $CD4^+$ cells and lack of stimulation of suppressor T cells.

Another hypothesis to explain the cause of IBD is that of persistent infection as a result of a specific agent. For CD two infectious agents are currently receiving considerable attention, mycobacterium paratuberculosis and the measles virus. The first because of the striking resemblance to Johne's disease and ileocecal tuberculosis. Johne's disease is caused by M. paratuberculosis and is a chronic granulomatous enterocolitis occurring in ruminants. Furthermore, M. paratuberculosis has been cultured from patients with CD but not from patients suffering from UC or other gastrointestinal disorders[73]. More recently, Sanderson and associates confirmed the presence of DNA specific for M. paratuberculosis in CD tissue, using PCR and Sweeney *et al.* suggested a possible mode of infection via cow milk [74,75]. There are however, multiple pieces of evidence against the causative role of M. paratuberculosis in CD, such the lack of clinical efficacy of anti-mycobacterial antibiotics and the clinical benefit of corticosteroids. Other 'cons' are the non reproducible cellular and humoral immune responses and the absence of 'person to person transmission' [73].

Evidence for persistence of measles virus in colonic tissue of CD patients has been reported[76]. Together with the increased incidence of CD in individuals born during or just after measles epidemics[77], this has lead to the hypothesis that CD might be the result of a persistent measles viral infection. This hypothesis does not seem to be in accordance with the rising incidence for CD in spite of vaccination against the measles virus. Although vaccination is usually performed by using live measles vaccines, it is unlikely that infection with a wild-type virus would be associated with a lower risk than contact with the attenuated form of the virus. The virulence of the wild-type greatly exceeds that of the attenuated

viruses[78]. For these reasons it seems unlikely that the measles virus would play an important role in the etiology of CD.

Amplification of the immune response leads to tissue damage and eventually fibrosis. The early stage of the inflammatory process coincides with the production of the pro-inflammatory cytokines tumor necrosis factor α (TNFα) and interleukin 1 (IL-1), primarily by activated macrophages. These cytokines not only activate immune cells such as T-cells, but they also stimulate mesenchymal, epithelial and endothelial cells[79,80]. In IBD there is an enhanced production of IL-1. Furthermore the IL-1 concentrations in tissues correlate with disease activity[81]. In rabbit immune complex colitis early administration of IL-1 receptor antagonist (IL-1RA) diminishes the inflammatory reaction in the colon[82]. IL-1RA is a protein that inhibits binding of IL-1 to its receptor and by doing so suppresses the pro-inflammatory effects of IL-1. An imbalance between IL-1 and IL-1RA, in favour of IL-1, would enhance T-cell stimulation and with this amplify the inflammatory response[83].

The IL-1 induced T-cell stimulation enhances the release of interleukin 2 (IL-2) and interferon γ (INFγ) by Th1 cells. In CD, in contrast to UC, IL-2 and INFγ concentrations are increased. IL-2 and INFγ induce cell mediated immunity, whereas the Th2 derived cytokine IL-10, which is increased in UC, induces humoral immunity[84,85,86]. This could be interpreted as an activation of Th1 cells in CD and Th2 cells in UC.

The adherence of neutrophils, monocytes and T lymphocytes to blood vessels and consequently the migration into the inflamed tissue is preceded by expression of adhesion molecules and their ligands on these cells. IL-1, TNFα and INFγ stimulate expression of these molecules[80]. This process of immigration is further stimulated by chemotactic molecules such as IL-8, transforming growth factor-β (TGFβ), leukotriene B_4 and platelet activating factor, complement fragments and bacterial products.

One of the most important complications of CD is obstruction of the intestinal lumen by stenosis. Stenosis is the result of fibrosis and hyperplasia of intestinal smooth muscle cells [87]. Both the proliferation of fibroblasts and of intestinal smooth muscle cells is

stimulated by IL-1, TNFα and insulin-like growth factor-1 (IGF-1), produced by the inflamed tissue. Furthermore, IGF-1 and TGFβ induce collagen synthesis.

1.4 Current therapeutic approach

1.4.1 Introduction

Current medical treatment of IBD largely still consists of corticosteroids and aminosalicylates, either alone or in combination. For patients who are intolerant or whose disease is refractory to these drugs there is an increasing variety of alternative drugs becoming available[88]. Most of these drugs however, are still in an experimental phase, have limited applicability and are at best only suitable for specific groups of IBD patients.

Although glucocorticosteroids are the most important therapeutic agents in the management of IBD they will not be discussed in this section. In Chapter 2 the clinical and pharmacological actions of glucocorticosteroids are discussed extensively.

Recently clinical efficacy has been reported for nicotine in the treatment of UC[89]. The second part of this thesis deals with immunopharmacological actions of this compound.

In this section a brief overview of the aminosalicylates and some of the immunosuppressive drugs is given.

1.4.2 Aminosalicylates

The first aminosalicylate used in the treatment of IBD was sulphasalazine, sulphapyridine linked to 5-aminosalicylic acid (5-ASA) by an azo-bond. This molecule is split into its two compounds by bacteria in the colon[90]. Subsequent research showed 5-ASA to be the active therapeutic moiety of sulphasalazine, whereas the side effects seen during sulphasalazine therapy are predominantly induced by the sulphapyridine component[91]. 5-ASA it thought to exert its effect locally in the intestinal mucosa. Although the exact mechanism of action is not known, it is evident that 5-ASA has multiple anti-inflammatory effects. Both, cyclooxygenase and 5-lipoxygenase pathways are inhibited by 5-ASA,

resulting in inhibition of eicosanoid production[92,93]. Alternative anti-inflammatory mechanisms of 5-ASA are: prevention of lysosomal release and respiratory burst[94], diminishing free radicals concentration[95] and inhibition of antibody synthesis[96].

Aminosalicylates are the drug of choice for patients with mild to moderate UC or mild Crohn's ileitis[97] and adjuvant treatment for severe UC. In addition to its effectiveness in active disease, 5-ASA has also been shown to be effective in maintaining remission in UC[98].

1.4.3 Methotrexate

Methotrexate is the most widely used antimetabolite in cancer chemotherapy, but recently it has been used in the treatment of patients with IBD refractory to corticosteroids[99,100]. It has immunosuppressive and anti-inflammatory properties, and in the treatment of IBD a steroid sparing effect[101]. Methotrexate is ineffective as maintenance therapy in IBD, especially in UC[102,103].

1.4.4 Azathioprine / 6-mercaptopurine

Both these drugs are competitive purine antagonists with strong immunosuppressive and anti-inflammatory effects[104,105]. Although their mode of action is not completely elucidated, it is clear that they inhibit the synthesis of DNA, RNA and protein. *In vivo,* azathioprine is converted into 6-mercaptopurine, which is the active metabolite.

Azathioprine / 6-mercaptopurine have proven to be effective in UC as well as in CD, both in the induction and in the maintenance of remission[106,107,108,109]. In CD 6-mercaptopurine does not only induce remission, but also causes closure of fistula [110]. Furthermore, these drugs have a steroid sparing effect and could therefore have a role in limiting the severe side effects of long term steroid use.

Both azathioprine and 6-mercaptopurine may require 3-6 months before displaying clinical efficacy[110,111]. Although side effects limit the use of these immunosuppressive drugs they are generally moderate and only in about 10% patients necessitate cessation of therapy [106,109,111].

A frequently seen adverse effect is a dose related bone marrow depression, albeit usually of a reversible nature[112]. The increased incidence of various cancers seen in patients receiving azathioprine after organ transplant, does not seem to apply for IBD patients taking this drug[113]. All in all, the long term use of azathioprine / 6-mercaptopurine can be considered relatively save, provided that regular haematological examination is being carried out.

1.4.5 Cyclosporin

Cyclosporin, also known as cyclosporin A, is an 11 amino acids containing cyclic polypeptide with powerful immunosuppressive actions. Its effects are predominantly the result of inhibition of an early step in the activation of T cells. This leads to blockage of the transcription of early genes encoding the cytokines[114].

Cyclosporin, especially when administered intravenously, has a high clinical efficacy in severe UC as well as in severe CD[115]. Lichtiger *et al.* treated 11 patients with severe UC who had failed to respond to high doses of intravenous corticosteroids with intravenous cyclosporin. All patients continued to receive 300 mg/day hydrocortisone intravenously and the aminosalicylic acid-based medications were continued in the patients using them at the start of the study. In this double blind, randomized, placebo controlled trial the response rate in the cyclosporin group was 82 % against 0 % in the placebo group (hydrocortisone ± 5-ASA)[116].

Although trials with patients suffering from CD yielded less positive results cyclosporin is also effective in this disorder[115]. In contrast to 6-mercaptopurine, cyclosporin causes a rapid response. Patients improve clinically within two weeks after the onset of treatment.

1.5 Conclusions

In Summary, inflammatory bowel disease is a chronic, relapsing inflammation of the human gastrointestinal tract or part of it. Symptoms are variable as are the complications, allowing some patients a near to normal quality of life, while rendering others invalid. Although the etiology is still unknown medical treatment of inflammatory bowel disease has made progress during the last decade, albeit at an evolutionary rather than a revolutionary pace.

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PART 2

Corticosteroids

Chapter 2

Glucocorticoids

2.1 Synthesis and metabolism

Glucocorticoids (GC) are effective anti-inflammatory agents which are notorious for the serious adverse effects they may cause when administered in a too high dose or over an extended period of time. Nevertheless, they are physiological hormones secreted by the zona fasciculata of the adrenal cortex and life would be impossible without them[1]. Corticosteroids are synthesised from cholesterol, derived from plasmalipoproteins. First, cholesterol is converted into pregnenolone[2]. This occurs in the mitochondria and is stimulated by adrenocorticotrophic hormone (ACTH). Following this step, the synthesis pathway branches into two routes: one passes through 17-α-hydroxyprogesterone and 1-deoxycortisol to cortisol, while the other proceeds from 17-progesterone and corticosterone to aldosterone. These two different end products also differ in function. This cascade of stimulary events is inhibited by cortisol. Thus, cortisol regulates its own release[3]. The glucocorticoids of which cortisol is the main exponent *in vivo*, play an essential role in coping with stress[4].

The other group of steroids produced by the adrenal cortex are termed minaralocorticoids and are responsible for regulation of the salt and water metabolism. The main mineralocorticoid is aldosterone[2]. As with most classifications, the division in glucocorticoids and mineralocorticoids is artificial and arbitrary, because naturally occurring GC also possess mineralocorticoid activity and vice versa.

Metabolism

Cortisol is predominantly metabolized in the liver and the plasma half-life is approximately 100 minutes. Metabolism by the liver results in a decrease biological activity

and increases the water solubility of the metabolized hormones[5]. The latter is necessary in order to enable excretion by the kidney.

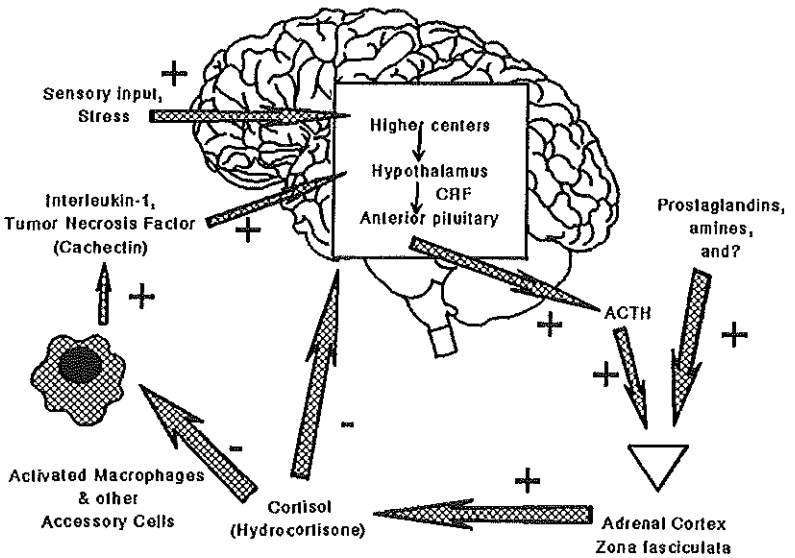

Figure 2.1 Schematic representation of endogenous glucocorticoid production.

2.2 Physiological effects of glucocorticoids

The name glucocorticoids originates from the influence these hormones have on glucose metabolism. However, the physiological effects of these hormones extend far beyond carbohydrate metabolism. Glucocorticoids play an important role in lipid and protein metabolism, regulate the immune system and renal function, and have a strong influence on growth, development, bone metabolism and central nervous system activity[1].

In view of the topic of this thesis, inflammatory bowel disease, the

immunoregulatory actions of GC will be discussed in more detail.

Carbohydrate, protein and lipid metabolism

Glucocorticoids stimulate gluconeogenesis, resulting in increased blood glucose and liver glycogen levels. Underlying this process is the inhibition of protein synthesis by GC, which causes a transfer of amino acids from muscle, fat tissue and bone to the liver[6,7,8]. In the liver amino acids are converted to glucose. Furthermore, GC stimulate the catabolism of lipids in adipose tissue and enhance the action of other lipolytic agents. The result is an increased plasma free fatty acid level[9]. Thus, the effects of GC on glucose metabolism are opposite to those of insulin[6,7,10,11,12].

Immune and anti-inflammatory responses

In contrast to the immunosuppressive and anti-inflammatory actions of GC in therapeutic doses, our knowledge about the role of physiological levels of GC in immunological and inflammatory responses is limited. The fact that surgical removal of the adrenal gland greatly exacerbates the inflammatory responses, strongly suggests an anti-inflammatory role for GC under physiological circumstances.

The endogenous GC level in the circulation varies strongly[13]. Inflammation is characterized by release of numerous inflammatory mediators by inflammatory cells such as interleukin 1 (IL-1) and tumor necrosis factor α (TNFα). These mediators will indirectly activate the hypothalamic-pituitary-adrenal axis by stimulating certain centres in the brain. The result is enhancement of cortisol release, leading to inhibition of the inflammatory reaction[14,15,16,17,18].

The difference in susceptibility to streptococcal cell wall-induced arthritis between Lewis rats and Fisher rats further illustrates the role GC play in preventing the activated defence mechanisms from overshooting[19,20]. The extremely susceptible Lewis rats have impaired plasma ACTH and GC responses to the administration of IL-1 and corticotropin releasing hormone (CRH). The cause of these inadequate responses is a defective regulation

of the CRH gene in the paraventricular nucleus[20]. These observations have been further strengthened by data from experiments with RU 486, a glucocorticoid receptor antagonist. Treatment of Fisher rats with RU 486 increases their susceptibility to the induction of arthritis.

2.3 Mechanism of action

Glucocorticoids exert their actions after binding to specific receptors. These receptors are not located in the cell membrane but within the cytoplasm, at least in the absence of the steroid. The glucocorticoid receptor (GR) belongs to a supergene family

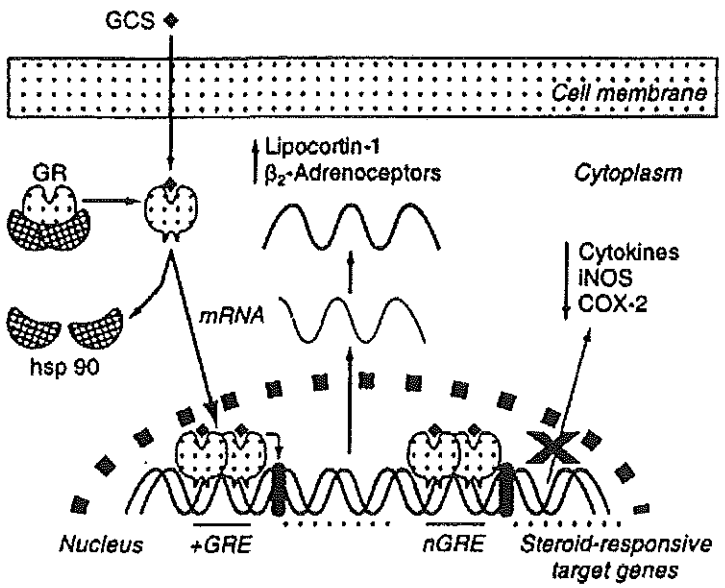

Figure 2.2 Classic model of glucocorticoid action. Glucocorticoid (GCS) enters the cell and binds to a cytoplasmic GR that is complexed with two molecules of a 90 kd heat shock protein (hsp 90). GR translocates to the nucleus where, as a dimer, it binds to a GRE on the 5'-upstream promoter sequence of steroid-responsive genes. GREs may increase transcription, and nGREs may decrease transcription, resulting in increased or decreased mRNA and protein synthesis.

containing cytosolic receptors for various steroids, such as progesterone and oestrogen, and for thyroid hormone, retinoic acid and vitamin D[21,22]. The GR is a heteromer consisting of a steroid- and a DNA-binding subunit and two 90 kD heat-shock proteins[23]. Following binding of the GC to its receptor the activated receptor complex moves to the nucleus of the target cell. In the nucleus this complex binds to the glucocorticoid response elements, formed by specific DNA sequences. This results in the down-regulation of the transcription of pro-inflammatory genes and up-regulation of the transcription of anti-inflammatory genes such as lipocortin, neutral endopeptidase and inhibitors of plasminogen activator. Glucocorticoids may also modulate gene transcription by destabilizing specific messenger RNA molecules[24].

Genes down-regulated by GC	Genes up-regulated by GC
IL-1, IL-6, IL-8	Lipocortin
TNFα, IFNγ, GM-CSF	Neutral endopeptidase
Cyclooxygenase type II	Receptors for cytokine
Nitric oxide synthase	Receptors for hormones
Elastase	Plasminogen activator inhibitor
Plasminogen activator	
Collagenase	

Table 2.1 Gene Modulating actions of glucocorticoids.

2.4 *In vivo* anti-inflammatory effects of glucocorticoids

Effects on inflammatory cells

Glucocorticoid administration not only alters the number of leucocytes but also affects the functions of inflammatory cells. They inhibit cytokine release by both

lymphocytes and monocytes, proliferation of lymphocytes and release of arachidonic acid metabolites by monocytes and macrophages[25,26,27,28]. Granulocyte functions, on the other hand, are less influenced by GC. Chemotaxis, phagocytosis and release of arachidonic acid metabolites by neutrophils are not inhibited by GC, nor is chemotaxis by eosinophils[29,30].

Although data from *in vitro* studies suggest that GC do not inhibit chemotaxis or adherence to endothelial cells, *in vivo* studies have suggested an inhibition of transendothelial migration of leucocytes. Biopsies obtained from the airways of glucocorticoid-treated asthmatic patients exhibit a reduced number of lymphocytes and eosinophils[31]. There is still a lot to be unravelled about the mechanism through which GC reduce the infiltration of inflammatory cells to the inflamed tissue. However, recent *in vitro* studies have shown that cytokines such as IL-1, IL-4 and TNFα activate the endothelium, increasing its adhesive properties and by doing so, stimulate the adherence of leucocytes and their subsequent migration[32]. Animals and human studies have demonstrated inhibition of cytokine release by GC[33,34,35,36], suggesting suppression of endothelial activation. A key factor in the migration of leucocytes are the chemoattractants such as lipid mediators, peptides and chemokines. Glucocorticoids strongly inhibit the production of these chemotactic factors.

Vascular effects of glucocorticoids

Two important aspects of inflammation are increased blood flow and permeability. Both facilitate the transport of leucocytes and plasma proteins to the inflammation sites. A number of inflammatory mediators cause the blood vessel diameter and the permeability to increase. Glucocorticoids inhibit the release of these mediators such as IL-1, TNFα, neuropeptides, nitric oxide and prostaglandins. The inhibition of vascular permeability and diameter by GC, however, is not only a result of this indirect action but also of the direct influence of GC on endothelial cell shape and contractility[37,38,39].

2.5 Glucocorticoids in inflammatory bowel disease

Pharmacokinetics

As with any disease affecting the intestines one should be aware of the potential risk of impaired absorption when treating inflammatory bowel disease patients with oral GC. Plasma concentrations vary considerably after oral ingestion of the same dose of GC by normal subjects or patients with IBD[40]. It is still unclear whether absorption of oral GC in IBD patients is normal or not. Both, an impaired absorption of prednisolone[41,42], especially in the case of extensive small bowel Crohn's disease[43], as well as a normal absorption have been described in IBD patients[44,45,46] in comparison to healthy controls.

2.5.1 Ulcerative colitis (UC)

As early as in the forties data from uncontrolled studies with GC and ACTH suggested a beneficial effect of these compounds in the treatment of UC[47,48,49,50,51,52,53]. Subsequently, the efficacy of GC as therapy for UC was clearly demonstrated by placebo-controlled studies[54,55]. Although some studies showed ACTH to have a slightly better result than GC in the UC patients treated for relapse, it is nowadays hardly ever used[56,57]. The disadvantages of ACTH are the high costs and the fact that it has to be administered parenterally.

For mild to moderate inflammation the optimal dose was found to be 40-60 mg of prednisolone daily[58]. Although the use of enemas has enabled the employment of local therapy it has not been able to eliminate the side effects of treatment with GC entirely. This is due to the fact that even with local application of hydrocortisone hemisuccinate or prednisolone 21 phosphate, a considerable amount of the GC is absorbed[59,60,61,62,63].

Although GC are the most effective drug in the treatment of acute exacerbations of ulcerative colitis, they do not reduce the rate of relapses[56,64,65].

2.5.2 Crohn's disease (CD)

Placebo-controlled trials with GC for the treatment of CD were done much later than those for UC. As is the case for UC, treatment with GC is effective in acute exacerbations of CD [66,67]. In CD, however, low dose GC therapy seems also to reduce the rate of relapses in CD patients brought into remission with GC[66,67,68,69].

2.6 Conclusions

Glucocorticoids are the clinically most effective treatment for IBD available today. However, their influence is not confined to the intestines. Glucocorticoids affect almost every part of the immune system and multiple other systems. Prolonged treatment with GC can therefore cause various side effects. In order to reduce these side effects more and more emphasis is being placed on research efforts to develop rapidly metabolized topical steroids.

Further research concerning the anti-inflammatory effects of GC is warranted because this might enable us to determine which of these effects are necessary to induce remission in IBD. Such knowledge will render the tools for the development of new anti-inflammatory compounds with the selectivity yearned for.

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Chapter 3

Inhibition of the production of mediators of inflammation by corticosteroids is a glucocorticoid receptor-mediated process*

Abstract - In order to find an explanation for corticosteroid resistance we assessed whether inhibition by dexamethason (DEX) of the stimulated production of TNFα, IL_6, PGE_2 and LTB_4 by peripheral blood mononuclear cells (MNC) depends on binding to the glucocorticoid receptor (GR) and whether it is determined by the number or the affinity of the GR of these cells. GR number and affinity of MNC were determined by means of a whole cell DEX binding assay. MNC were incubated with DEX and LPS or A23187 in the absence or presence of RU486, a potent steroid antagonist. DEX caused a concentration dependent inhibition of TNFα, IL_6 and PGE_2 production but had no effect on LTB_4 production. RU486 significantly blocked the effect of DEX, but no correlations were found between the inhibition of mediator release and the K_d or receptor number.

3.1 Introduction

Corticosteroids decrease the severity of the inflammation and cause a subjective improvement in the majority, but not in all patients with non-infectious inflammatory disorders such as inflammatory bowel disease (IBD)[1], asthma[2] and rheumatoid arthritis[3]. The anti-inflammatory effects of corticosteroids are ascribed to inhibition of the production of mediators of inflammation, including eicosanoids and cytokines[4,5,6].

**Based on:* Madretsma G.S., van Dijk A.P.M., Tak C.J.A.M., Wilson J.H.P., Zijlstra F.J. Inhibition of the production of mediators of inflammation by corticosteroids is a glucocorticoid receptor-mediated process. *Mediators of inflammation.* 1996;5:100-103

Several control studies have shown that topical or systemic corticosteroids are much more effective than placebo controls, but that not all patients improve. Depending on the dose and route of application, duration of the treatment, severity of the exacerbation and the parameters under investigation, approximately 10 - 40 % of the patients do not respond or respond insufficiently to corticosteroids[7,8].

Stimulation of mononuclear cells (MNC) by lipopolysaccharide (LPS) or Ca-ionophore enhances the production of a range of inflammatory mediators by these cells such as the cytokines tumor necrosis factor α (TNFα) and interleukin 6 (IL-6), and the eicosanoids prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4)[9]. This provides an *in vitro* model for studying the anti-inflammatory properties of corticosteroids.

In order to clarify some aspects of the mechanism through which corticosteroids exert their anti-inflammatory effects we examined the effect of dexamethasone on the production of mediators of inflammation by human MNC *in vitro* and assessed whether this effect depends on binding of corticosteroids to the glucocorticoid receptor (GR).

The hypothesis of this study was that the inhibition of the production of mediators of inflammation by corticosteroids is a glucocorticoid receptor-mediated process. We furthermore tested if the ability of dexamethasone (DEX) to inhibit the LPS-stimulated production of TNFα, IL-6, PGE_2 and the Ca^{2+}-ionophore enhanced LTB_4 production by MNC is determined by the number or affinity of glucocorticoid receptors (GR) on these cells.

3.2 Materials and Methods

Healthy volunteers

Approval for this study was obtained from the Medical Ethical Committee of the University Hospital Rotterdam.

Venous blood was obtained from 9 healthy adults, ranging in age from 22 to 37 years, who joined voluntarily after full explanation of the nature, significance and scope of

the study. None of the subjects had taken corticosteroids or any other anti-inflammatory drug for a period of at least 4 weeks prior to donating blood.

Isolation of human MNC

Mononuclear cells were isolated from the heparinized venous blood immediately after blood sampling. The method used was a modification of the technique originally described by Boyum[10]. Briefly, the blood was diluted 1:1 with phosphate buffered saline (PBS: Oxoid, U.K.) before fractioning it by an one-step Ficoll-paque gradient (Pharmacia, Sweden) centrifugation at 1100xG for 15 minutes at 20° C. The interphase was washed in PBS and resuspended in Dulbecco's Modified Eagles Medium (DMEM) containing HEPES and foetal calf serum (Gibco, UK), supplemented with penicillin and streptomycin (Flow Lab, UK). Cells were stained by Hemacolor (Merck, Germany), the final yield of MNC was greater than 95 % and the cell viability (tested by Trypan blue exclusion) was over 97 %.

Culturing of MNC and measurement of cytokines and eicosanoids

The cells ($2x10^6$ per well) were cultured in DMEM and incubated in the presence or absence of varying concentrations of DEX (Genfarma, The Netherlands) for 24 hours prior to stimulation with LPS (5 μg/ml, E. coli 0111:B4, Sigma, USA) for 24 hours or Ca^{2+}-ionophore (A23187, 1 μM, Sigma, U.S.A.) for 15 minutes. All incubations were performed at 37 °C in an humidified atmosphere of 5 % CO_2 and 95 % air. DEX was dissolved in culture medium and concentration of DEX varied from 10^{-9} to 10^{-5} M.

TNFα and IL-6 were measured by commercially available ELISA-kits (Central Laboratory for Bloodtransfusion, The Netherlands) whereas PGE_2 and LTB_4 levels were determined by specific radioimmunoassays (Standards: Sigma U.S.A., ^{3}H-labels: Amersham UK and antibodies: Advanced Magnetics Inc., USA).

The effects of different concentrations of DEX on secretion of TNFα, IL-6 and PGE_2 were expressed as percentage inhibition of production in control cultures (cells

incubated with DMEM plus supplements and LPS).

Assessment of glucocorticoid receptor number by a whole cell assay

The method used was that described by Lamberts et al.[11], but a 1000-fold excess of unlabelled RU-486 (Roussel, France), a corticoid receptor antagonist, was used instead of an excess of unlabelled DEX. A stock solution was made by dissolving RU-486 in ethanol. This solution was diluted in culture medium. Final concentration ethanol in the assay samples was less than 0.1 % .

In order to enhance the dissociation of endogenously bound corticoids, the MNC used in the assay were washed 3 times in DMEM, each time allowing for an equilibration period of 30 min at 37° C in a shaking waterbath[12].

Specific binding was determined by subtracting the amount of nonspecifically bound radioligand from the total amount bound. Data were analyzed by constructing a Scatchard plot[13].

Statistics

The effect of DEX on the stimulated release of each mediator was studied in 9 separate experiments, with blood from 9 different donors. Values are expressed as mean ± S.E.M. Control and DEX-treated cell cultures were compared by paired t-test. P-values < 0.05 were considered significant.

Effect of DEX alone versus the effect of DEX after preincubation with RU486 was compared by Manova.

3.3 Results

Mediator production by MNC

After the incubation period LPS-stimulated MNC released the mediators measured in the following concentration, TNFα: 90 ± 25 pg/10^6 cells, IL-6: 1165 ± 320 pg/10^6 cells,

PGE_2: 1420 ± 340 pg/10^6. MNC stimulated with Ca^{2+}-ionophore released : 2970 ± 940 pg $LTB_4/10^6$ cells.

Effect of DEX on mediator release by MNC

The inhibitory effect of DEX on TNFα production was of comparable magnitude to that of IL-6 and PGE_2, with an IC_{50} value (concentration of DEX that causes a 50 % decrease in mediator release) of 65 nM in comparison to 145 and 140 nM for IL-6 and PGE_2 respectively. There was a considerable inter-individual variation in IC_{50} values in response to DEX with a 95 % confidence interval of [10 - 400 nM] for TNFα, [15 - 1095 nM] for IL-6 and [30 - 690 nM] for PGE_2. The effects of DEX on the production of the studied mediators are shown in figure 3.1.

DEX had no effect on Ca^{2+}-ionophore-stimulated LTB_4 release.

Antagonism of the effects of DEX in MNC by RU486

Incubation of MNC with RU486, a steroid receptor antagonist, for 2 hours prior to the addition of DEX diminished the inhibitory effect of the glucocorticoid in a dose dependent manner (figure 3.2).

Assessment of glucocorticoid receptor number and K_d in MNC

Glucocorticoid receptor content was 4430 ± 340 sites / cell (mean ± S.E.M.) and the K_d 9.5 ± 0.7 nM. In figure 3.3 a representative Scatchard plot from one experiment of the binding of ^{3}H-DEX to glucocorticoid receptors of MNC is shown. The slope is equal to the negative reciprocal of the dissociation constant ($-1/K_d$), and the intercept on the abscissa equal to the total concentration of receptors (B_{max}).

No correlations were found between the inhibition of mediator release and K_d or receptor number.

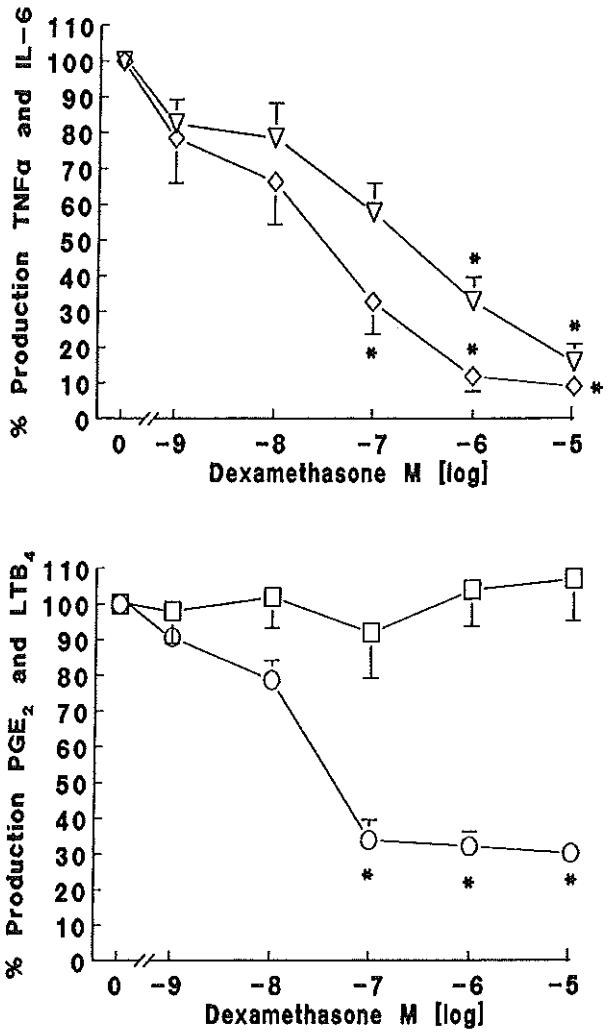

Figure 3.1 Effect of DEX on the secretion of (a) TNFα (◇) and IL-6 (▽), and (b) PGE_2 (○) and LTB_4 (□) by MNC cultures stimulated by LPS (5 μg/ml). Production is presented as a percentage, mean ± S.E.M. (vertical bars), of LPS- or Ca^{2+}-ionophore enhanced release and represents 9 experiments performed on MNC from 9 different donors. * Significance with $P < 0.05$ compared to LPS-enhanced production.

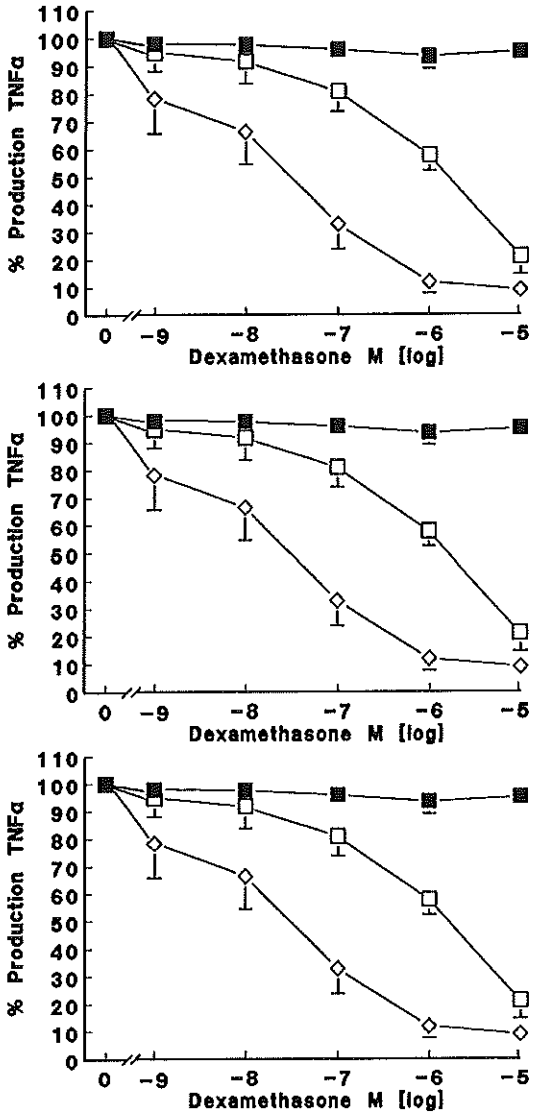

Figure 3.2 Effect of DEX on the secretion of (a) TNFα (◇), (b) IL-6 (▽) and (c) PGE_2 (○) and the effect of DEX after preincubation of MNC with RU486 10^{-8} M (□) or 10^{-6} M (■). RU486 significantly inhibits the effect of DEX in a dose dependent fashion, $P < 0.001$.

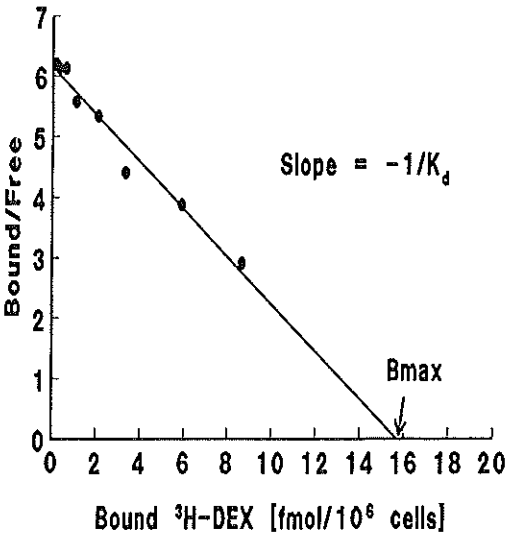

Figure 3.3 Scatchard plot of specifically bound ^{3}H-dexamethasone to MNC. MNC were incubated with 0 - 16 nM ^{3}H-dexamethasone in the absence or presence of a 1000-fold excess of unlabelled RU-486. K_d and B_{max} in the case shown are 12.8 nM and 15.7 fmol / 10^6 MNC respectively.

3.4 Discussion

The inhibitory effect of DEX in this *in vitro* experiment varied considerably from donor to donor. The effect of DEX on the release of TNFα, IL-6 and PGE_2 can not be explained by DEX's effect on the viability of the cells, otherwise the same effects should have been found for LTB_4 release. Instead, no significant differences were found between LTB_4 production in the presence versus absence of DEX. This might indicate that

corticosteroids do not inhibit 5-lipoxygenase in MNC as they do the gene expression of cytokine-induced cycloxygenase 2 in monocytes[14]. Furthermore, culture of MNC with DEX, in the concentrations used in this study, did not effect the viability of these cells as determined by trypan blue exclusion.

The fact that the effect of DEX could be diminished or even abolished by pre-incubation of the MNC with RU486, a steroid receptor antagonist, strongly suggests a glucocorticoid receptor mediated process.

Mononuclear cells play a central role in chronic inflammation. They possess the capacity to produce eicosanoids and cytokines which modulate the inflammatory response[15,16,17]. Corticosteroids exert their anti-inflammatory effects partly by inhibiting the production of inflammatory mediators, through a glucocorticoid receptor-mediated process. This means that the GR plays a crucial role in the anti-inflammatory effects of corticosteroids.

Considering these findings it seems logical that inter-individual variations in reaction to corticosteroids could be explained by variation in the number of GR on the MNC or the affinity of these receptors for DEX. No correlations were found however, between IC_{50} of the mediators under investigation and receptor number or K_d. Thus, the degree of response of MNC of healthy donors to DEX does not seem to be determined by the characteristics of the GR, and other factors must play a role. This finding however does not exclude the possibility that changes in GR number or affinity are important in glucocorticoid resistance in inflammatory diseases.

The MNC used in this study were isolated from blood obtained from healthy volunteers, who presumably have a normal response to corticosteroids. It is to be expected that comparison of MNC obtained from patients who have proven to be non-responders to corticosteroids with MNC of responders may reveal differences in GR characteristics more clearly. Studies with MNC isolated from blood obtained from inflammatory bowel disease patients responding to corticosteroids and patients not responding have been initiated.

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Chapter 4

Glucocorticoid resistance in inflammatory bowel disease*

A glucocorticosteroid receptor-mediated disorder?

Abstract - A minority of patients with inflammatory bowel disease (IBD) do not respond to high doses of glucocorticosteroids. Variation in response to corticosteroids could be due to variation in number or affinity of glucocorticoid receptors (GR) on mononuclear cells (MNC). In order to test this hypothesis we assessed if GR-number and GR-affinity of MNC isolated from venous blood of IBD-patients not responding to corticosteroid therapy differ from those of healthy volunteers and IBD-patients who do respond to this type of therapy. GR-content and K_d for the three groups were respectively, healthy volunteers: 4430 $\pm$ 340 sites per cell and 9.5 $\pm$ 0.7 nmol/l; responders: 3900 $\pm$ 210 sites/cell, 7.9 $\pm$ 0.6 nmol/l; non-responders: 2450 $\pm$ 310 sites/cell, 6.0 $\pm$ 1.4 nmol/l (difference non-responders versus responders and healthy volunteers, $P < 0.01$). Thus, mononuclear cells of IBD-patients not responding to glucocorticosteroids have a low glucocorticoid receptor number.

However, experiments with blood drown from the same nonresponders 9 to 12 months later did not confirm this finding. A possible explanation for this discrepancy could be that corticosteroid resistance in IBD is acquired and reversible, as seems to be the case in asthma.

**Based on:* G. Stanley Madretsma MD, Jeanette P.M. van Dijk BSc, J.H. Paul Wilson MD, Ruud Schouten MD, Dirk-Jan Bac MD, Freek J. Zijlstra PhD. *Submitted.*

4.1 Introduction

Corticosteroids decrease the severity of the inflammation and give a subjective improvement in the majority, but not in all patients with inflammatory bowel disease (IBD). Depending on the dose and route of application, duration of the treatment, severity of the exacerbation and the parameters under investigation, approximately 10 - 40 % of the patients do not respond or respond insufficiently to corticosteroids[1,2].

There are several possible reasons for lack of clinical response to corticosteroids. Individual differences in pharmacokinetics of corticosteroids occur, which can explain part of the variations in responses[3,4]. It should be pointed out, however, that two small studies failed to demonstrate diminished absorption after oral administration in IBD[5,6]. In some patients non-compliance may be the cause of lack of response[7]. Some individuals however, may be poor responders at a cellular level.

Individual differences in the effects of corticosteroids on mononuclear cells have been found[8,9]. Corrigan et al.[8] showed that there is a correlation between *in vitro* inhibition of phytohemagglutinin-induced proliferation of T-lymphocytes by dexamethasone and the clinical response to corticosteroids in asthmatic patients. This finding suggests that it might be possible to identify non-responders before starting treatment by using *in vitro* tests. The anti-inflammatory effects of corticosteroids are ascribed to inhibition of the production of mediators of inflammation, including eicosanoids and cytokines. Previous studies in our laboratory showed this inhibition to be glucocorticoid receptor mediated[10]. Interindividual variation in reaction to corticosteroids in IBD therefore could be explained by variation in the number of glucocorticoid receptors on the MNC or the affinity of these receptors for corticosteroids. In order to test this hypothesis we assessed if patients who had failed to react to corticosteroid therapy have less GR on their mononuclear cells.

4.2 Methods

Approval for this study was obtained from the Medical Ethical Committee of the University Hospital of Rotterdam.

Venous blood was obtained from three groups of individuals. The first group consisted of 9 healthy adults, ranging in age from 22 to 37 years (Controls). The second group were 6 IBD-patients (age: 27 to 66 years) who had undergone colectomy because the inflammation failed to react to medical treatment, even with high doses of corticosteroids (Non-responders). 'High doses of corticosteroids' was defined as 60 mg prednisone or more per day for at least 5 consecutive days. The third group comprised 6 IBD-patients (age: 21 to 59 years) with a history of one or more exacerbations which had responded successfully to corticosteroids (Responders).

All of these responding patients had at least one exacerbation rated as moderate to severe by endoscopic examination. All individuals participated after full explanation of the nature, significance and scope of the study. None of the subjects had taken corticosteroids for a period of at least 4 weeks prior to donating blood.

Isolation of human mononuclear cells

Mononuclear cells were isolated from the heparinized venous blood immediately after blood sampling. The method used was a modification of the technique described by Boyum[11]. Briefly, the blood was diluted 1:1 with Phosphate Buffered Saline (PBS; Oxoid, U.K.) before fractioning it by a one-step Ficoll-paque gradient (Pharmacia,Sweden) centrifugation at 2500 rpm for 15 minutes at 20° C. The interphase was washed in PBS and resuspended in Dulbecco's Modified Eagles Medium (DMEM) containing Hepes and foetal calf serum (Gibco, UK), supplemented with penicillin and streptomycin (Flow Lab, UK). Cells were stained by Hemacolor (Merck, Germany), the final yield of MNC was greater than 95 % and the cell viability (assessed by Trypan blue exclusion) was over 97 %.

Assessment of glucocorticoid receptor number by a whole cell assay

The method used was that described by Lamberts et al.[12], but a 1000-fold excess of unlabelled RU-486 (Roussel, France), a corticoid receptor antagonist, was used instead of an excess of unlabelled dexamethasone. A stock solution was made by dissolving RU-486 in ethanol. This solution was diluted in culture medium, resulting in final concentration ethanol in the assay samples of less than 0.1 % .

In order to enhance the dissociation of endogenously bound corticoids, the mononuclear cells used in the assay were washed 3 times in culture medium, each time allowing for an equilibration period of 30 min at 37° C in a shaking water bath[13].

Specific binding was determined by subtracting the amount of nonspecifically bound radioligand from the total amount bound. Data were analyzed by constructing a Scatchard plot[14].

Plasma cortisol level

An aliquot of the heparinized blood was centrifuged at 4000 rpm for 10 min at 25 °C and the plasma stored at -80 °C until measurement of the cortisol level by radio immuno assay (Coat-a-count cortisol; Diagnostic Products Corporation, The Netherlands).

Statistics

The corticosteroid receptor number, K_d and plasma cortisol levels are expressed as mean $\pm$ S.E.M. The Mann-Whitney U test was used to compare results in the three groups. A P value < 0.05 was considered significant for each comparison.

4.3 Results

Assessment of glucocorticoid receptor number and K_d in mononuclear cells.

In figure 4.1 a representative Scatchard plot from one experiment of the binding of ^{3}H-dexamethasone to glucocorticoid receptors on mononuclear cells is shown. The slope is

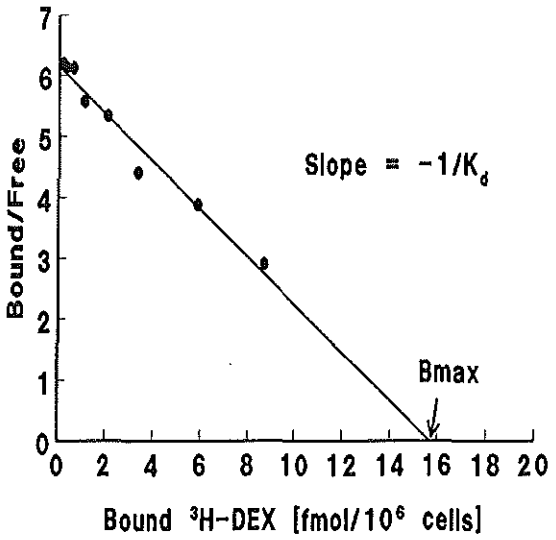

Figure 4.1 Scatchard plot of specifically bound ^{3}H-dexamethasone to mononuclear cells. MNC were incubated with 0 - 16 nmol/l ^{3}H-dexamethasone in the absence or presence of a 1000-fold excess of unlabelled RU-486. K_d and B_{max} in the case shown are 12.8 nmol/l and 15.7 fmol/10^6 MNC respectively.

equal to the negative reciprocal of the dissociation constant (-1/K_d), and the intercept on the abscissa equal to the total concentration of receptors (B_{max}).

The glucocorticoid receptor content for the healthy volunteers was 4430 ± 340 sites/cell (mean ± S.E.M.) with a K_d of 9.5 ± 0.7 nmol/l. For the responders we calculated 3900 ± 210 sites /cell, with a K_d of 7.9 ± 0.6 nmol/l , whereas the non-responders had 2450 ± 310 GR per cell, with a K_d of 6.0 ± 1.4 nmol/l.

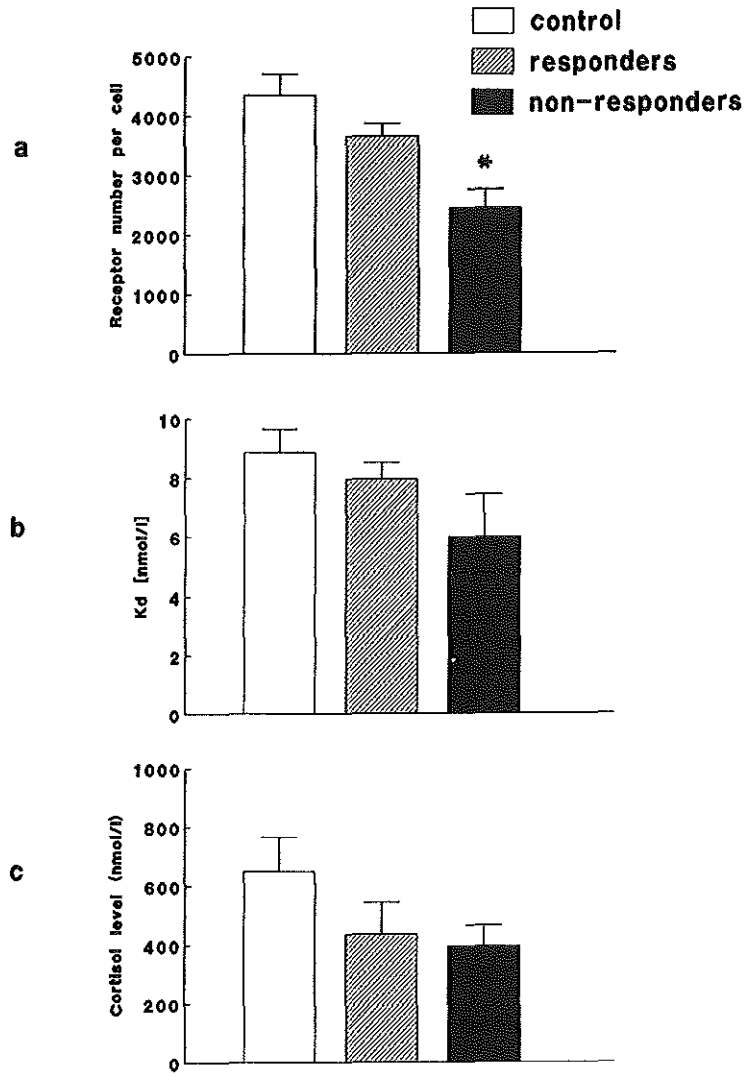

Figure 4.2 Glucocorticoid receptor characteristics of healthy volunteers (n=9), responding (n=6) and non-responding (n=6) inflammatory bowel disease patients. The number of receptors per cell (a), the dissociation constant (K_d) in nmol/l (b) and the plasma cortisol level nmol/l (c), are presented as mean ± S.E.M. (vertical bars). $^*P<0.01$ compared with control or responders.

The mononuclear cells of the non-responders had a significantly lower glucocorticoid receptor number (figure 4.2a), compared to the two other groups ($P< 0.01$). The GR content on MNC of the responders did not differ significantly from that of the healthy volunteers. No significant differences were observed in the K_d of the GR between the three groups (figure 4.2b).

Plasma cortisol level

The measurements of cortisol in plasma are shown in figure 4.2c. Mean cortisol level in healthy volunteers was 390 $\pm$ 95 nmol/l. This did not differ significantly from the plasma cortisol levels measured for the responders 435 $\pm$ 110 nmol/l and non-responders: 395 $\pm$ 70 nmol/l (figure 4.2c).

4.4 Discussion

This study shows that the majority of patients who have not responded to high dose of corticosteroids for IBD have a reduced number of corticosteroid receptors on their mononuclear cells. The ability to recognise IBD-patients who will not respond adequately to treatment with corticosteroids, before starting treatment, is of potential value, as these patients could then be given alternative immunosuppressive drugs at an early stage. The criteria used in this study to classify glucocorticoid resistance are rigorous, but gave us a clear and objective tool to discriminate between responders and non-responders to glucocorticoid therapy.

A 'washout' period of at least 4 weeks was chosen because of the fact that patients taking glucocorticosteroids experience a phenomenon known as 'receptor down regulation'. This means that the number of GR on the cells of these individuals decreases during treatment with glucocorticosteroids. Receptor down regulation occurs within 24 hours, but is a reversible process. After cessation of glucocorticoid administration the number of GR gradually returns to normal[13].

In addition to these two groups of patients there are also patients who respond only to high dose of glucocorticosteroids. We chose to only include the patients from both extremes of the spectrum of corticosteroid response because comparison between these two groups was likely to display the differences more clearly.

Mononuclear cells play a central role in chronic inflammation. They have the capacity to produce eicosanoids (biological active lipids) and cytokines (immuno regulatory proteins) which modulate the inflammatory response[15,16,17]. Glucocorticosteroids exerts their anti-inflammatory effects partly by inhibiting the production of these inflammatory mediators, through a glucocorticoid receptor-mediated process. This means that the GR plays a crucial role in the anti-inflammatory effects of glucocorticoids. A possible explanation for the phenomenon of corticosteroid resistance in IBD therefore could be altered glucocorticoid receptor characteristics, e.g. an alteration of GR-number or affinity.

As far as we know no research has been published on GR studies of MNC of glucocorticoid resistant IBD-patients. In asthmatics who do not respond to aerosol or systemic corticosteroids, *in vitro* reduction in monocyte function has been described, and also a change in glucocorticoid receptor affinity for dexamethasone[18,19]. A reduction in glucocorticoid receptor number has not been reported. Recently, reports have been published describing familial cortisol resistance[14,20] in which a reduced glucocorticoid receptor number and elevated blood cortisol levels were found in some of these patients. The authors concluded that familial cortisol resistance might be caused by a lower glucocorticoid receptor number.

In our patients mononuclear cells from non-responders have a significant lower GR-number in comparison to responders and healthy volunteers, whereas the affinity of these receptors for dexamethasone do not differ. This indicates the absence of a compensation mechanism in these cells, lowering the K_d in order to compensate the decrease in receptor number by an increase of the affinity of the receptors for glucocorticoids. A 50 % reduction in glucocorticoid receptor number, even without a change in receptor affinity is sufficient to change effectiveness of glucocorticoids as shown by Pepin *et al*, who found that

a decrease in GR-number of about 50 % causes symptoms in transgenic mice bearing antisense RNA transgene[21].

In contrast to some forms of familial cortisol resistance the mean blood cortisol levels of the non-responders did not differ significantly from that of the responders or healthy volunteers. Further studies have been initiated to characterize the nature of the defect in our patients.

However, experiments with blood drown from the same nonresponders 9 to 12 months later did not confirm this finding. A possible explanation for this discrepancy could be that corticosteroid resistance in IBD is acquired and reversible, as seems to be the case in asthma[22,23].

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PART 3

Smoking and Nicotine

Chapter 5

Pharmacology and Immunology

5.1 Introduction

Although the resistance against smoking in public has increased considerably during the last decade, smoking still remains the most common form of substance abuse in our society. Tobacco was first introduced in Europe in the sixteenth century by explorers returning from North and South America. There are various forms in which tobacco can be consumed. Smoking it in pipes was most preferred until the second part of the last century. With the manufacturing of cigarettes, at the end of the nineteenth century, tobacco consumption gradually shifted from chewing and smoking pipes to smoking cigarettes. Currently, in most Western countries about 35 % of the adult population consists of smokers[1].

After the second world war it became more and more evident that smoking is harmful to one's health. The main health risks caused by smoking are cancer and cardiovascular disease. Interestingly, it has recently become clear that beside the adverse effects, smoking also has some protective effects against certain disorders[2,3,4,5].

5.2 Pharmacodynamics of smoking and nicotine

Although cigarette smoke contains more than six thousand components, nicotine seems to be the only pharmacologically active substance, present in large enough quantities, to cause systemic effects. The alkaloid nicotine is a tertiary amine composed of a pyridine and pyrrolidine ring. Nicotine binds to various types of acetylcholine receptors. According to their location these receptors can be divided in central nicotinic receptors (located in the central nervous system) and peripheral nicotinic receptors (located in the peripheral nervous

system). Central nicotinic receptors are primarily located in the hypothalamus, hippocampus, thalamus, midbrain, brain stem and certain segments of the cerebral cortex[6,7].

Stimulation of presynaptic central nicotinic receptors leads to activation of several neurohumoral pathways. This results in the release of various neurotransmitters and hormones such as acetylcholine, noradrenaline, dopamine, serotonin, vasopressin, growth hormone and adrenocorticotrophic hormone (ACTH)[8,9,10].

The peripheral nicotinic receptors occur primarily in the autonomic ganglia and the striated muscles. Stimulation of the receptors in the sympathetic ganglia and adrenal medulla causes liberation of catecholamines from the adrenal medulla and release of noradrenaline from the atria and blood vessels[11]. This results in sympaticomimetic effects such as vasoconstriction, tachycardia and increased myocardial contraction.

The nicotinic receptors in striated muscle play an important role in neuromuscular transmission. Short-term stimulation, leading to a brief depolarization of the motor end plate and the membrane of the muscle cell, results in contraction of the striated muscle. Blockage of these receptors by non-depolarizing agents (e.g. pancuronium) causes a temporary paralysis. Paralysis can also be achieved by prolonging depolarization of the neuromuscular endplate with depolarizing compounds (e.g. suxamethonium), causing loss of electrical excitability[12].

Receptors with a high affinity for nicotine have recently also been reported on human lymphocytes[13,14,15]. The exact function of these receptors has not yet been elucidated, but is seems likely that they would have a immunoregulatory role.

5.2.1 Effects of cigarette smoking on the central nervous system

The transport of nicotine across the blood-brain barrier is both a process of passive diffusion as well as active uptake by the choroid plexus[16]. Although the effects of smoking on the central nervous system (CNS) are complex and diverse most smokers experience a state of arousal during and following smoking of cigarettes. Paradoxically, this is accompanied by a feeling of relaxation, especially in stressful situations. Furthermore, most

smokers report an improvement of their mood and ability to concentrate after smoking[17]. One could argue that the enhanced performance and mood are the result of the reduction of withdrawal symptoms. However, improvement in the performance of nonsmokers following the administration of nicotine suggests a true enhancement[18].

As is the case with most substances of abuse, nicotine stimulates the mesolimbic dopaminergic system[19]. This might at least partly explain development of addiction, because this system plays an important role in reward mechanisms[20].

5.2.2 Endocrine system

Several observations have been reported on smoking and stress. Especially female smokers, but also male smokers, albeit to a lesser extent, tend to increase their tobacco consumption in stressful situations[21,22,23,24]. The number of cigarettes smoked and the intensity with which they are smoked increases, resulting in higher plasma nicotine levels than in a relaxing environment[23].

The one component in tobacco smoke that influences stress the most is nicotine[25]. Nicotine enhances the release of corticotropin releasing factor (CRF) and ACTH from the hypothalamus and the pituitary respectively[26,27,28]. Both of these hormones stimulate the release of glucocorticoids from the adrenals. Thus, nicotine induces increased plasma levels of glucorticoids in animals[26,29,30], as well as in humans[31,32,33]. The increase in serum cortisol and corticosterone seems to be dose-dependent[31,33].

5.2.3 Cardiovascular system

Smoking a cigarette has multiple effects on the cardiovascular system of healthy smokers. The amount of nicotine absorbed from one cigarette leads to an increase in blood pressure of 5 to 10 mm Hg, in heart rate of 10 to 20 beats per minute and enhances cardiac output and coronary blood flow[17]. In chronic smokers the cardiovascular effects of nicotine are less pronounced as a result of development of tolerance[34].

5.2.4 Gastrointestinal tract

In the healthy individual nicotine has a stimulatory effect on the gastrointestinal tract, causing enhancement of gastric acid secretion and increase of tone and motility[35]. Nicotine also plays a role in gastrointestinal pathology. Smoking prolongs the oesophageal acid clearance time and increases the number of reflux events, thus it enhances oesophageal acid exposure[36]. Furthermore, nicotine has been implicated in the initiation and maintenance of damage to the gastric mucosa[37].

5.3 Pharmacokinetics of smoking

While smoking a cigarette the nicotine is carried on inhaled tar droplets. The rate of absorption of nicotine correlates positively with the pH of the smoke. Nicotine out of cigarettes causing smoke with a alkaline pH is readily absorbed, starting even in the oral cavity. Once the smoke has arrived in the small airways and alveoli the absorption becomes independent of the pH of the smoke. Smoking one cigarette yields on average 1 mg of nicotine[38].

Nicotine obtained from smoking enters the brain more rapidly than intravenous administered nicotine, but the redistribution to other tissues also occurs at a high rate, resulting in a rapid decrease of the nicotine concentration in the brain. The ability to change the nicotine level so rapidly allows 'fine tuning' of the nicotine effects desired while smoking[17]. This is also illustrated by the fact that when smokers are switched to cigarettes with lower nicotine content they smoke more cigarettes in order to achieve the amount of nicotine needed[39]. Although nicotine is administered intermittently during the part of the day that the smoker is awake, it is being accumulated during this period because of the half-life of two hours. This enables the smoker to maintain a certain nicotine level as he sleeps [40]. Thus, nicotine exposure of a moderate smoker is of a chronic nature rather than of an intermittent one.

The metabolism of nicotine has been studied more extensively in trials with transdermal nicotine systems and will be discussed in the section concerning the pharmacokinetics of those systems (section 5.4.2).

5.4 Transdermal nicotine

The notion that nicotine is the component in tobacco responsible for the addiction to cigarette smoking has lead to the employment of nicotine replacement therapy to aid smoking cessation[41]. The primary reason given for relapse after an attempt to stop smoking are the withdrawal symptoms[42].

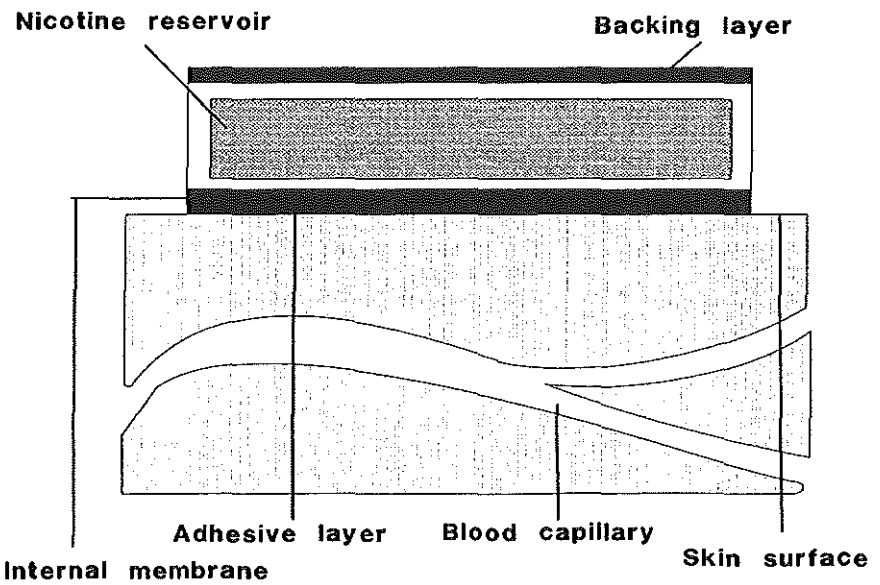

Figure 5.1 Schematic representation of transdermal nicotine dilivery.

The idea is to replace the nicotine that would otherwise have been obtained from the smoking of cigarettes, in order to reduce the withdrawal symptoms. Nicotine can be administered via various methods, such as chewing gum, nasal sprays and nicotine patches.

The amount of nicotine needed to prevent withdrawal symptoms is approximately 50 % of that obtained by smoking.

5.4.1 Pharmacodynamics of transdermal nicotine

Although little research has been published concerning the pharmacodynamics of transdermal nicotine administration, it seems unlikely that it would differ much from the effects of nicotine obtained from cigarettes, discussed in section 5.2.

5.4.2 Pharmacokinetics of transdermal nicotine

Pharmacological research concerning transdermal nicotine has mainly focused on the pharmacokinetic properties. Transdermal delivery has been employed to administer various drugs, such as glycerylnitrate, hyoscine, clonidine and oestrogens. Transdermal nicotine systems consist of a drug containing reservoir incorporated in a stick-on plaster. The amount of nicotine absorbed from these systems ranges from 35 to 90 %, depending on the system used[43,44]. Only a small proportion of the nicotine applied is lost resulting in a bioavailability of more than 80 %[43]. After application, maximum plasma nicotine concentrations are reached within 3 to 12 hours. A dose-related increase in plasma nicotine levels has been reported following single application of different doses[43,44,45]. There is a gradual release of nicotine after application and plasma nicotine concentrations are still elevated at the end of the application period, followed by a gradual decrease after removal of the patch. Repeated transdermal administration of nicotine at 24 hour intervals yields a steady state within 2 to 4 days[46,47], but does not result in accumulation of nicotine, instead accumulation of cotinine occurs.

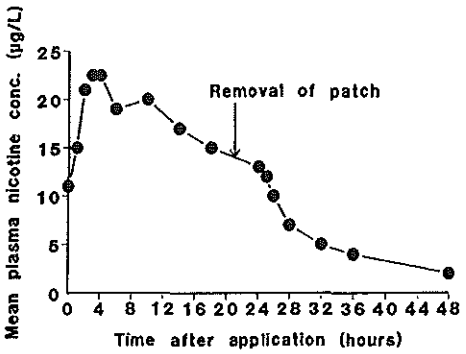

Figure 5.2 Mean plasma nicotine concentrations in 24 smokers following 24-hour application of transdermal nicotine (adapted from Palmer *et al.* 1992).

Studies of skin strips taken from individuals following application of transdermal nicotine patches did not detect cotinine in the epidermis, indicating lack of metabolism of nicotine in the skin[41]. Metabolism of nicotine occurs primarily in the liver and to a lesser extent in the lung[48]. The half-life of nicotine administered via smoking is approximately 2 hours, albeit with a considerable interindividual variation. This is shorter than the 3 to 6 hours reported for transdermally administered nicotine[43,44,46,49]. An explanation for this difference could be the formation of a nicotine skin depot, leading to a sustained absorption of nicotine after the patch has been removed[43]. The major metabolites of nicotine are cotinine and nicotine-N-oxide.

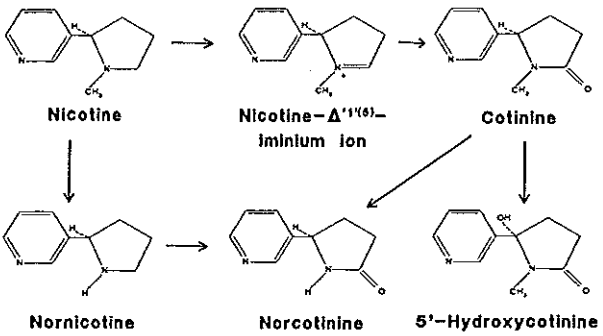

Figure 5.3 Metabolism of nicotine.

In vitro studies have reported pharmacological actions of cotinine[50], but *in vivo* it does not seem to have any cardiovascular effects in humans, nor does it influence the desire to smoke[51]. This indicates that the previous mentioned accumulation of cotinine during repeated nicotine application probably does not have any clinical effect. Little research has been published concerning the pharmacological effects of the other metabolites of nicotine. Less that 10 % of the nicotine administered is excreted unchanged in the urine. Renal excretion however, is negatively correlated with the pH of the urine[52]. In contrast to plasma, the primary metabolite excreted in urine is not cotinine but *trans*-3'-hydroxycotinine[53].

5.5 Immunological aspects of smoking and nicotine

Although the main interest of research on smoking and nicotine has been on the pharmacological effects on the central nervous system and the cardiovascular system, during the last two decades considerable attention has been given to the effects on the immune system.

In this section the effects of voluntary exposure to tobacco smoke on the immune system of animals and humans will be reviewed. Although, involuntary or environmental tobacco smoke exposure may also have significant effects on the immunological response, little information exists about this form of exposure to smoke.

5.5.1 Studies in animals

The effects of tobacco smoke on the immune system depends on various factors such as the species tested, the age of the animals tested, duration of exposure and the level of exposure. The most common test systems employ mice, rats and guinea pigs. The immunological effects of short-term or acute exposure are in general limited and return to normal soon after cessation of exposure. Subchronic or chronic exposure generally has a suppressive effect on the immune system.

Short-term exposure of animals to tobacco smoke

Tobacco smoke has been reported to cause changes in cellular as well as in humoral mediated immunity, however, not all authors agree on the effect on the humoral part. The differences reported may partly be due to differences in the set-up of the studies such as differences in the species used, differences in exposure level and differences in the types of cigarettes used. Thus short-term exposure of animals to tobacco smoke has been found to significantly inhibit, slightly stimulate or to have no effect on the primary and secondary antibody response[54].

On the effect of tobacco smoke on the cellular immunity exists more agreement. Thomas and associates reported a decreased responsiveness to phytohemagglutinin (PHA) of peripheral blood and splenic lymphocytes isolated from mice exposed to cigarette smoke for 5 weeks[55]. In contrast, the local pulmonary immune system was stimulated, resulting in an enhanced responsiveness to PHA of cervical and mediastinal derived lymphocytes. This suggests a differentiated initial response of local and peripheral immune system on antigens.

A very important issue when studying the effects of tobacco smoke is its influence on the host-resistance to tumor formation. In a study by Thomas *et al.* exposure to cigarette smoke for 3 days did not stimulate tumor growth in mice following intratracheal injection of two different types of tumor cells[56].

Chronic exposure of animals to tobacco smoke

Most studies report decreased humoral immune response after chronic exposure to cigarette smoke. Findings reported are a decreased primary and secondary antibody production by cells in the lung, lymph nodes and spleen[55,57] and diminished hemagglutinin and haemolytic antibody titers[58]. In contrast to the differentiated response upon short-time exposure to cigarette smoke, the effects of chronic exposure are similar for the local and the peripheral immune system. Lymphocytes from peripheral blood and spleen as well as from regional lymph nodes, derived from mice chronically exposed to tobacco smoke, show a diminished response to PHA stimulation in comparison to cells from control animals[55,59]. Contrary to the lack of effect on host-resistance against tumor genesis reported for short-time exposure, chronically exposed animals exhibit a severely decreased resistance against the development of tumors following inoculation with tumor cells[60,61,62].

Chronic exposure to cigarette smoke naturally also causes multiple local immunological changes in the lungs, such as an increase in number and in metabolic rate of pulmonary macrophages[63,64,65]. It is however, highly improbable that these local changes in the lung would play an important role in the effects smoking has on inflammatory bowel disease and for this reason they will not be discussed.

5.5.2 Studies in humans

Effects of tobacco smoke on the cellular immune system

As early as 1940 Scheer reported an increased leukocyte count in peripheral blood of smokers[66]. This finding has been confirmed by many authors[67,68,69]. The extent of the leukocytosis caused by smoking seems to depend on race, gender, age and the amount of

cigarettes smoked[67,70]. These variables, in combination with the small sample size, may explain the conflicting reports published about the effects of cigarette smoking on human T-lymphocytes. Some authors report a significant decrease in the CD4+/CD8+ ratio[71,72,73,74], while others find no change or even an increased helper/suppressor ratio[67,75,76,77]. Among studies reporting the effects of smoking on B-lymphocytes there is considerably more agreement. The overall majority of these studies report an increased number of B-cell in smokers[67,75,76,77,78,79].

The effects of smoking on lymphocyte function have, if possible, been the object of even more debate than the influence of smoking on T-cell subsets. The influence of phytohemagglutinin stimulated proliferation seems to depend strongly on the age of the smokers tested, with an enhanced proliferation in young individuals and an inhibition in older smokers, compared to nonsmokers of the same age group[80,81]. Other authors did not observe any influence of cigarette smoking on PHA, concanavalin A or alloantigens-induced proliferation[76,82,83,84].

Tobacco consumers have a far greater change of developing tumors than nonsmokers. This increased incidence does not only apply for malignancies located in the lung and oral cavity, but also for those in organs which do not come in direct contact with tobacco or tobacco smoke[85,86,87]. This suggests suppression of not only the local but also of the more general defense mechanisms against carcinogenesis in these individuals. Natural killer (NK) cells play a major role in tumor resistance and for this reason considerable research has been carried out concerning the effect of tobacco smoke on NK cell activity in smokers. Indeed, several studies have reported a decreased NK cell activity in leukocytes of smokers[68,76,88,89].

Effects of tobacco smoke on the humoral immune system

Most authors agree that the immunoglobulin levels are decreased in smokers, except IgE levels[88,89,90,91,92,93]. The elevated IgE levels found in smokers are often accompanied by

eosinophilia[94,95,96,97]. Interestingly however, the correlation between eosinophilia, IgE levels and skin reactivity seen in nonsmokers, has not been found for smokers[98].

5.5.3 *In vitro* and *in vivo* studies with nicotine

Because tobacco smoke consists of so many components it is difficult to establish which of these components is responsible for the immunoregulatory effects. There are however, many pieces of evidence suggesting a major role for nicotine.

Animal studies

Tobacco smoke can be divided in a particulate and a gaseous phase with the help of a Cambridge filter. These two phases are of a different composition, with nicotine being a major constituent of the particulate phase[99]. The particulate phase of tobacco smoke is essential for the immunosuppressive effect of cigarette smoke[100]. Furthermore, administration of nicotine to animals have been reported to cause various effects on their cellular and humoral immune system[101,102,103,104,105,106].

Human studies

Nair *et al.* studied the *in vitro* effect of nicotine on NK cell activity in human lymphocytes and found a significant suppression[107]. The *in vitro* immunoglobulin production has also been shown to be modulated by nicotine, albeit in a different pattern than by smoking[108]. Furthermore, nicotine has a significant influence on the *in vitro* production of mediators of inflammation. Saareks and associates reported that nicotine causes a dose-dependent increase of prostaglandin E_2 production and a decrease of leukotriene B_4 synthesis by polymorphonuclear leukocytes. The same authors also observed a reduced thromboxane B_2 production in platelet rich plasma following incubation with nicotine[109]. In a study on the effect of nicotine on *in vitro* cytokine production by human mononuclear cells we found a significant inhibition of the PHA-stimulated production of tumor necrosis factor α and interleukin 2, reaching its maximum effect at nicotine concentrations equivalent to nicotine

levels measured in the plasma of smokers[110]. Recently, prolonged neutrophil survival has been reported, after incubation with nicotine. This effect was subscribed to suppression of apoptosis by nicotine[111].

In agreement with these *in vitro* findings are the reports by Pullan *et al.*[112] and Lasher et al.[113] on the beneficial effects of nicotine administered transdermally or orally in ulcerative colitis.

5.6 Conclusion

A therapeutic role for nicotine?

The epidemiological finding that smoking is associated with a decreased incidence of certain disorders suggests that nicotine might have beneficial effects in some diseases. The availability of nicotine in easy administrable forms has lead to the implementation of clinical trials to study the efficacy of nicotine in the treatment of these disorders[112,113,114]. Although, the data of these studies are promising, showing significant clinical improvement and a reasonable tolerability of nonsmokers for nicotine treatment, it has not yet become a established therapy for these patients. Most worrying are of course the potential addictive power, and the cardiovascular and carcinogenic side effects.

In a large randomized, double-blind, controlled trial in patients with active ulcerative colitis, Pullan *et al.* reported no evident withdrawal symptoms after six week treatment with nicotine[112]. The authors ascribe the lack of withdrawal symptoms to the steady release of nicotine by transdermal nicotine patches, which does not cause the sharp increases in plasma nicotine concentrations measured following smoking. In this study, patients did not experience serious adverse effects necessitating medical treatment. Other authors however, have reported serious cardiovascular complications during transdermal nicotine treatment [115,116]. Further investigation is needed to unravel the exact mode of action of nicotine in these disorders. Knowledge of the exact mechanisms involved might enable the development of agonists more acceptable than nicotine itself.

5.7 References

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Chapter 6

Nicotine inhibits cytokine production by mouse colonic mucosa*.

Abstract - We examined the *in vivo* effect of nicotine on the synthesis of mediators of inflammation by mouse colonic mucosa. The synthesis of lipid mediators such as the prostanoids prostaglandin E_2, 6-keto-prostaglandin $F_{1\alpha}$ and thromboxane B_2, the 5-lipoxygenase products leukotriene B_4 and leukotriene C_4 and platelet activating factor was not affected, whereas the synthesis of the pro-inflammatory cytokines interleukin 1ß and tumor necrosis factor α was significantly inhibited.

6.1 Introduction

Recent studies have shown that transdermal application of nicotine in patients with active ulcerative colitis has a beneficial effect on the symptoms of this disease[1].
Exposure of animals to tobacco smoke has been reported to cause a decreased humoral reponse. Findings reported are a decreased primary and secondary antibody production by cells in the lung, lymph nodes and spleen[2,3] and diminished hemagglutinin and haemolytic antibody titers[4]. Lymphocytes from peripheral blood and spleen as well from regional lymph nodes show a diminished response to PHA stimulation in comparison to cells from control animals[2,5].

The effects of nicotine on lipid mediators such as prostaglandins are still controversial. In rabbits prostaglandin production was inhibited by nicotine in a inverse dose dependent manner, whereas in ferrets no significant effects were observed[6].

Based on: Van Dijk A.P.M., Madretsma G.S., Keuskamp Z.J., Zijlstra F.J. Nicotine inhibits cytokine production by mouse colonic mucosa. *Eur. J. Phar.* 1995;278:R11-R12.

This study is based on very strong epidemiological evidence that identifies ulcerative colitis as predominantly a disease of non-smokers[7]. The mechanism underlying this beneficial effect of nicotine is unknown. In order to find an explanation for this response to nicotine we investigated the *in vivo* effect of nicotine on lipid mediators and cytokines produced by colonic mucosa in mice.

6.2 Methods

This study was approved by the Animal Experimental Committee of the Erasmus university of Rotterdam.

Colonic mucosa was obtained from 15 BALB/c female mice (19-21 g). Nine mice were used as controls, six mice were treated with nicotine. Initially, mice received nicotine in drinking water *ad libitum*: 2 days 25 μg/ml, 2 days 50 μg/ml, 2 days 100 μg/ml and 2 days 200 μg/ml. Thereafter nicotine was dissolved in saline and given during 14 days subcutaneously by an Alzet osmotic mini-pump with an infusion rate of 200 μg/day. Controls, nine mice, received tap water and an osmotic pump filled with saline. The concentration of nicotine and cotinine in plasma reflected the dose of nicotine given.

After the mice were killed by cervical dislocation the colon from the hepatic flexure to the rectum was removed immediately. The colon was opened in transverse direction and faeces removed. The weight of the colon was determined and 2 ml Krebs-Henseleit buffer added, after which the tissue (approximately 200 mg) was fragmented using an Ultra-Turrax (Polytron, Switzerland) and centrifuged for 15 min. at 10.000 x g. The amount of protein per mg wet homogenized tissue was determined by a micro-scale method.

Eicosanoids were determined by radioimmunoassay, using antibodies from Advanced Magnetics (MA, USA), tritiated compounds from Amersham (UK) and standards from Sigma Co. (USA). Platelet activating factor was determined by radioimmunoassay (PAF-SPA system, Amersham, UK) and cytokines by enzyme-linked immunosorbent assay:

tumor necrosis factor α from Genzyme Corp. (USA) and interleukin-1ß from Cistron Biotechnology (Eurogenetics, Belgium).

6.3 Results

After 3 weeks of treatment plasma nicotine and cotinine concentrations were 3.6 ± 0.6 (mean ± S.E.M.) and 0.3 ± 0.05 ng/ml in controls and 42 ± 3.0 and 105 ± 11 ng/ml in nicotine treated mice respectively. These data are equivalent to plasma cotinine levels in patients treated with nicotine patches with a release of 15 mg nicotine per day, reflecting 15 cigarettes per day[1].

The synthesis of lipid mediators was unaffected, whereas the production of IL-1ß and TNFα significantly inhibited (table 6.1).

Mediator		Controls	Nicotine
Protein	(μg/mg)	11.8 ± 0.69	12.5 ± 0.52
6-keto-prostaglandin $F_{1\alpha}$	(pg/mg)	3620 ± 428	3500 ± 341
Prostaglandin E_2	(pg/mg)	1020 ± 157	1170 ± 73
Thromboxane B_2	(pg/mg)	416 ± 53	415 ± 32
Leukotriene B_4	(pg/mg)	3.4 ± 0.42	2.9 ± 0.28
Leukotriene C_4	(pg/mg)	1.5 ± 0.27	1.8 ± 0.39
Platelet activating factor	(pg/mg)	54 ± 20	48 ± 14
Interleukin-1β	(pg/mg)	1.3 ± 0.60	< 0.1 *
Tumor necrosis factor α	(pg/mg)	120 ± 58	13 ± 13 *

Table 6.1 Formation of lipid mediators and cytokines by mouse colonic mucosa after nicotine treatment. * $P<0.05$ compared with controls.

6.4 Discussion

This is, as far as we know, the first study in which the direct effect of nicotine on the synthesis of pro-inflammatory cytokines was investigated and shown to be of a strong inhibitory nature. Recently the effect of exposure to cigarette smoke was investigated in alveolar macrophages in rats[8]. As expected tumor necrosis factor α was increased in alveolar macrophages, but surprisingly decreased in peritoneal macrophages 8 hours after the smoking session. In that study peritoneal macrophages were taken as internal control cells and the authors failed to conclude that this unexpected effect could be due to circulating nicotine.

Both TNFα and IL-1ß are thought to be important cytokines involved in the initial events preceding the further development of inflammation in the gastrointestinal tract. Although TNFα and IL-1ß are mainly produced by macrophages[9], other cells also secrete TNFα. It has been shown that TNFα producing cells are increased in the mucosa of inflamed intestine [10].Our results offer a possible explanation for the beneficial effects of nicotine in ulcerative colitis.

6.5 References.

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Chapter 7.

Nicotine inhibits the *in vitro* production of interleukin 2 and tumor necrosis factor α by human mononuclear cells*

Abstract - Smoking protects against ulcerative colitis (UC), and treatment with nicotine patches has a beneficial symptomatic effect in patients with UC. To find an explanation for this response to nicotine in UC, we assessed the effects of nicotine on cytokine production by mononuclear cells (MNC). MNC were isolated from peripheral blood from healthy volunteers. Non-adherent MNC were preincubated with varying concentrations of nicotine or prednisolone for 24 hours followed by addition of phytohemagglutinin (10 μg/ml). The concentrations of interleukin 2 (IL-2) and tumor necrosis factor α (TNFα) in the supernatants were determined by ELISA.

Nicotine as well as prednisolone caused a significant inhibition of IL-2 and TNFα production. The maximum inhibition caused by nicotine was about 50 % of that caused by prednisolone and was reached at concentrations equivalent to nicotine levels measured in plasma of smokers.

These results indicate that nicotine exerts its immunoregulatory role through modulation of the cytokine production by non-adherent mononuclear cells.

7.1 Introduction

During the last decade it has become clear that cigarette smoking not only increases the risk of developing cancer[1] or cardiovascular disease[2], but surprisingly, also has

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*, *Based on:* Madretsma G.S., Donze G.J., van Dijk A.P.M., Tak C.J.A.M., Wilson J.H.P., Zijlstra F.J. *Immunopharmacology*. 1996;35:47-51.

beneficial effects on some chronic diseases such as Alzheimer disease[3] and ulcerative colitis[4,5]. Although the mechanisms underlying these beneficial effects are unknown, there is evidence that smoking influences the cellular[6] as well as the humoral immune system[7,8,9].

Cigarette smoke contains a range of compounds with pharmacological activity. Of these compounds nicotine is the most important and it therefore seems logical to assume it to be responsible for most of the immunoregulatory effects of cigarette smoke. Treatment of patients with active ulcerative colitis (UC), a form of inflammatory bowel disease, with nicotine patches proved to have a beneficial effect[10]. The application of transdermal nicotine significantly improved symptoms in these patients. Nicotine may therefore have anti-inflammatory properties which could be important in the treatment of UC.

Recent studies have indicated an important role for T-cells in inflammatory bowel disease (IBD), especially in the initial phase of the inflammation. Cytokines produced by these cells play a key role in the pathogenesis of IBD. Elevated levels of TNFα and IL-2 have been reported in serum and stool of patients suffering from IBD, for most other cytokines there have been conflicting reports or they have not been adequately investigated[11].

In order to find an explanation for the beneficial effect of nicotine in ulcerative colitis we examined the effect of nicotine on the production of two important mediators of inflammation in inflammatory bowel disease, interleukin 2 (IL-2) and tumor necrosis factor α (TNFα).

7.2 Materials and Methods

Approval for this study was obtained from the Medical Ethical Committee of the University Hospital of Rotterdam.

Isolation of mononuclear cells

Mononuclear cells (MNC) were isolated from 60 ml heparinized venous blood

immediately after blood sampling. The method used was a modification of the technique described by Boyum[12]. Briefly, the blood was diluted 1:1 with Phosphate Buffered Saline (PBS; Oxoid, U.K.) before fractioning it by a one-step Ficoll-Paque gradient (Pharmacia, Sweden) centrifugation at 1100 *g* for 15 minutes at 20 °C. The interphase was washed in PBS and resuspended in Dulbecco's Modified Eagles Medium (DMEM) containing HEPES and foetal calf serum (Gibco, U.K.), supplemented with penicillin and streptomycin (Flow Lab, U.K.).

Following isolation the MNC were incubated in culture medium for 90 minutes in a water saturated atmosphere of 37 °C containing 5 % CO_2. Non-adherent MNC were harvested by repeated rinsing of the culture flasks (Costar, U.S.A.) with DMEM. The viability of the cells, assessed by Trypan blue exclusion, was over 95 %.

Characterization of non-adherent mononuclear cell fraction

The leucocyte subpopulations present in the non-adherent MNC fraction were analyzed by FACS using the monoclonal antibodies Leu 4, Leu11 and Leu19 (Becton Dickinson, Mountain View, CA) that recognize CD3, CD16 and CD19 respectively. CD14 and CD20 positive cells were characterized by My 4 and B4 (Coulter Cytometry, Florida)

Stimulation experiments

Non-adherent MNC were cultured (2 x 10^6 cells/ well) in 24 wells culture plates (Costar, Cambridge MA, USA) in the absence or presence of varying concentrations of nicotine-hydrogen-tartrate (BDH Ltd Poole, U.K.) or prednisolone-di-sodium-phosphate (Genfarma, The Netherlands) for 24 hours, followed by addition of phytohemagglutinin (PHA: 10 μg/ml; Sigma Chemie, Belgium). After incubation for another 24 hours supernatants were harvested and stored at -80 °C until IL-2 and TNFα concentrations were assessed by ELISA (IL-2: Medgenix, Belgium, sensitivity: 70 pg/ml and TNFα: C.L.B., The Netherlands, sensitivity: 3 pg/ml). The concentration of IL-2 and TNFα produced in the presence of PHA and absence of nicotine and prednisolone was used as control and set

at 100 %. All incubations were performed in duplicate.

Statistics

The IL-2 and TNFα production are expressed as mean ± S.E.M.(n= 5). Statistical comparisons between control and drug-treated cell cultures were made by paired t-test. A $P < 0.05$ was considered significant.

7.3 Results

The non-adherent MNC fraction consisted primarily of T-lymphocytes (table 7.1). The PHA-stimulated TNFα production ranged from 3580 to 5430 pg/ml, with a mean ± S.E.M of 4585 ± 360 pg/ml and a median of 4810 pg/ml. For IL-2 we measured a range of 1000 - 2660 pg/ml, with a mean of 1615 ± 295 pg/ml and a median of 1590 pg/ml.

Cluster differentiation	Percentage
CD3	72.1 ±2.3
CD16/CD56	15.1 ±0.8
CD20	7.3 ±1.9
CD14	0.6 ±0.1
Not classified	4.9 ±1.2

Table 7.1. Percentage of different cell types comprising the non-adherent mononuclear cells. Cells were characterized by FACS using the monoclonal antibodies Leu 14, Leu 11, Leu 19, B4 and My 4.

The production of IL-2 and TNFα by these cells was inhibited in a dose dependent manner when nicotine was added 24 hours prior to PHA (figure 7.1). This inhibition was only partial. For IL-2 the maximum effect of nicotine was observed at 10^{-7} M (figure 7.1a)

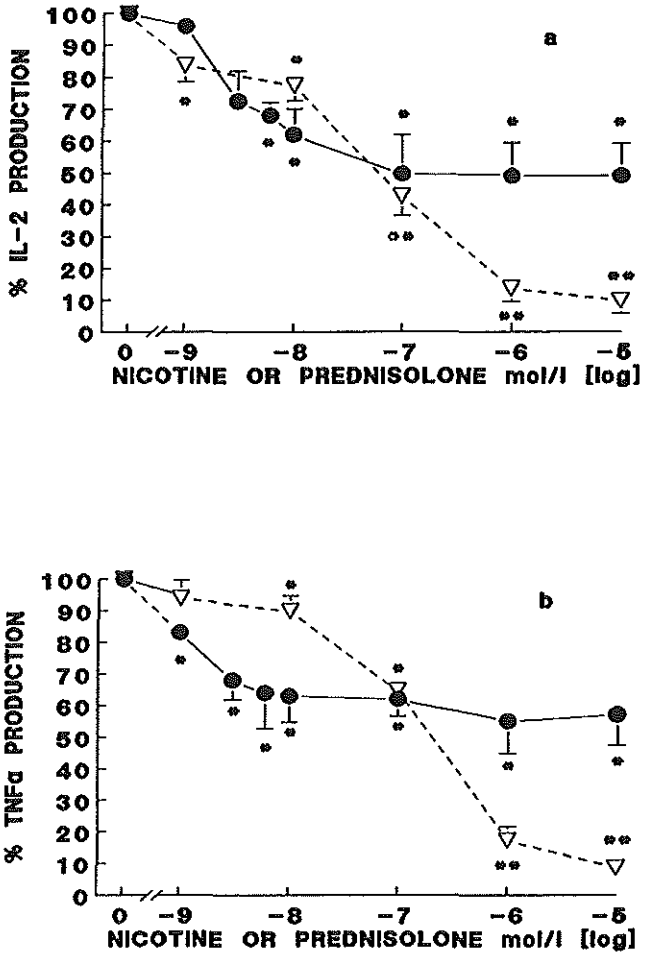

Figure 7.1 Effects of nicotine (solid dot) and prednisolone (open triangle) on the synthesis of interleukin 2 (a) and tumor necrosis factor α (b) in cultures of human peripheral blood lymphocytes stimulated with phytohemagglutinin (PHA: 10 μg/ml). Cytokine production (percentage of control) is presented as mean ± S.E.M. (vertical bars) and represent 5 experiments performed on non adherent mononuclear cells obtained from 5 separate healthy volunteers. The paired t-test was used to compare control and drug-treated cell cultures. Prednisolone and nicotine caused a significant inhibition of cytokine production, * $P < 0.05$; ** $P < 0.01$ compared with control cultures incubated with DMEM and PHA.

and for TNFα at 10^{-8} M (figure 7.1b). A further increase of the nicotine concentration did not enhance the effect. There was a considerable interindividual variation in response to nicotine but enhanced production of the cytokines after addition of nicotine was never observed.

Addition of nicotine and PHA simultaneously or addition of nicotine after PHA, in contrast, had no effect on IL-2 and TNFα production. Nicotine did not influence the viability of the cells, even in the highest concentration used (10^{-5} M).

Treatment of the non-adherent MNC with the corticosteroid prednisolone, also caused a dose dependent inhibition of IL-2 and TNFα production. As with nicotine, the addition of prednisolone had to proceed that of PHA in order to cause a significant inhibition of IL-2 and TNFα production at the lower doses. In contrast to nicotine, the effect of prednisolone did not reach a plateau at the concentrations tested.

7.4 Discussion

The results of this study show that nicotine inhibits the *in vitro* production of IL-2 and TNFα by human non-adherent MNC. This is in agreement with our recent observation of an inhibition of cytokine production by mouse colonic mucosa after treatment with nicotine[13].

The inhibition of IL-2 and TNFα production by human MNC appears to be dependent on the concentration of nicotine, the duration of incubation and the individual donor. The potency of nicotine, however, was about 50 % of that of prednisolone, a potent glucocorticosteroid. It is at present not clear whether nicotine administered transdermally is less effective clinically than prednisolone, as the clinical observation by Pullan *et al* was on an additional anti-inflammatory effect of nicotine added to maintenance therapy with corticosteroids or 5-aminosalicylic acid, in the treatment of ulcerative colitis. In this study, nicotine reached its maximum effect at concentrations equivalent to those measured in plasma of smokers[14].

We chose to use a cell population consisting predominantly of T-cells in this study for two reasons. Recent study has proven cyclosporin to be effective in the treatment of ulcerative colitis refractory to steroid therapy[15]. Cyclosporin exerts its immunosuppressive effect by suppressing the induction and amplification of T-cell responses by inhibiting cytokine gene activation, particularly IL-2[16]. Another finding indicating an important role for the T-cell in the pathogenesis of ulcerative colitis is the observation that appendectomy has a protective effect against ulcerative colitis. The appendix is a helper T-cell organ[17].

Inhibition of TNFα production by nicotine offers not only a possible explanation for the beneficial effect of this drug in UC but could also shed some light on the role smoking plays in the development of cancer. Smoking does not only elevate the risk of lung cancer but also of cancer in organs which do not come in contact with smoke itself[18,19,20]. A possible contributory factor to this carcinogenic effect of smoke 'at a distance' is that nicotine causes a more or less general suppression of the immune system, rendering the individual more susceptible for the development of neoplasms.

We conclude that nicotine has immunoregulatory effects through modulation of the cytokine production by non-adherent mononuclear cells.

7.5 References

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Chapter 8

Nicotine induced inhibition of interleukin 2 and tumor necrosis factor α production by human mononuclear cells is mediated via a noncholinergic receptor*

Abstract - Nicotine has been shown to affect the cellular as well as the humoral immune system of animals and humans, and *in vitro* and *in vivo* nicotine influences cytokine production.

We recently found an inhibition of human mononuclear cell produced IL-2 and TNFα by nicotine.

Human leukocytes possess receptors for nicotine, but these receptors have not yet been characterised. In order to establish an immunological role for these receptors we set out to demonstrate their involvement in the inhibition of cytokine production by nicotine.

Non-adherent MNC were preincubated in the presence or absence of two cholinergic nicotine receptor antagonists, hexamethonium and pancuronium, followed by the addition of nicotine and phytohemagglutinin. The concentrations of IL-2 and TNFα in the supernatants were determined by ELISA. In addition, ligand binding studies and displacement experiments were conducted to confirm the presence of receptors for nicotine on the MNC and to further characterize these receptors.

Interleukin 2 and TNFα production was significantly inhibited by nicotine. However, this inhibition could not be blocked by hexamethonium or by

Based on: G.S. Madretsma, A.P.M. van Dijk, C.J.A.M. Tak, J.H.P. Wilson, F.J. Zijlstra. *Submitted.*

pancuronium. The MNC had 2630 ± 350 nicotine receptors / cell (mean ± S.E.M.) with a K_d of 6.2 ± 1.4 nmol/l. The nicotine receptor present on the MNC had no affinity for any of the cholinergic agonists and antagonists tested.

These results indicate that nicotine inhibits the production of IL-2 and TNFα by peripheral blood MNC via a non-cholinergic nicotine receptor.

8.1 Introduction

Cigarette smoking has multiple effects on the immune system of animals as well as humans. Administration of nicotine to animals have been reported to affect their cellular and humoral immune system[1,2,3,4,5,6]. Nair *et al.* studied the *in vitro* effect of nicotine on natural killer cell activity in human lymphocytes and found a significant suppression[7]. Furthermore, nicotine modulates immunoglobulin production[8] and has a significant influence on the *in vitro* production of mediators of inflammation. Saareks and associates reported that nicotine causes a dose-dependent increase of prostaglandin E_2 production and a decrease of leukotriene B_4 synthesis by polymorphonuclear leukocytes. The same authors also observed a reduced thromboxane B_2 production in platelet rich plasma following incubation with nicotine[9]. Recently, prolonged neutrophil survival has been reported, after incubation with nicotine. This effect was subscribed to suppression of apoptosis by nicotine[10].

In a study on the effect of nicotine on *in vitro* cytokine production by human mononuclear cells we found a significant inhibition of the PHA-stimulated production of tumor necrosis factor α and interleukin 2, reaching its maximum effect at nicotine concentrations equivalent to nicotine levels measured in the plasma of smokers[11].

Leukocytes contain receptors for a number of compounds such as acetylcholine[12], corticosteroids[13], β_2-agonists[14], opiates[15] and insulin[16].

In this study ligand binding experiments were performed to demonstrate the presence of binding sites with a high affinity for nicotine on human non-adherent mononuclear cells. In order to test the hypothesis that nicotine inhibits cytokine production through a

cholinergic nicotine receptor we performed *in vitro* experiments with mononuclear cells and nicotine in the presence or absence of two cholinergic nicotine receptor antagonists, hexamethonium and pancuronium.

8.2 Materials and Methods

Approval for this study was obtained from the Medical Ethical Committee of the University Hospital of Rotterdam.

Isolation of mononuclear cells

Mononuclear cells were isolated from heparinized blood obtained from 5 healthy non-smokers. The isolation took place immediately after blood sampling and cell were characterized as described previously[11].

Cytokine production

Non-adherent MNC were cultured (2 x 10^6 cells / well) in 24 wells culture plates (Costar, Cambridge MA, USA) in the presence or absence of the cholinergic nicotine receptor antagonists hexamethonium (10^{-5} mol/l) (Sigma Chemie, Belgium) or pancuronium (10^{-5} mol/l) (Organon Teknika B.V., The Netherlands). After 60 minutes varying concentrations of nicotine-hydrogen-tartrate (BDH Ltd Poole, U.K.) were added to the wells. Following incubation for 24 hours, phytohemagglutinin (PHA: 10 μg/ml; Sigma Chemie, Belgium) was added, and the incubation continued for another 24 hours. At the end of the incubations, the supernatants were harvested and stored at - 80 °C until IL-2 and TNFα concentrations were assessed by ELISA (IL-2: Medgenix, Belgium, sensitivity: 70 pg/ml and TNFα: C.L.B., The Netherlands, sensitivity: 3 pg/ml). The concentration of the cytokines produced in the presence of PHA and the absence of nicotine, hexamethonium and pancuronium was used as control and set at 100 %. All incubations were performed in duplicate.

Nicotine receptor binding studies

Binding to nicotine receptors was measured using a binding assay with N-methyl-^{3}H-nicotine (Amersham, U.K.). All incubation were performed in triplicate. A stock cell solution of 10 x 10^6 cells per ml in Hanks' Buffer without calcium and magnesium (Life Technologies B.V., The Netherlands) was kept at 4 °C for 30 minutes. Aliquots of 100 μl of this solution were added to polypropylene tubes containing 50 μl Hank's buffer with or without an excess of nicotine, or 50 μl of the appropriate tritiated nicotine concentration. After an incubation of 15 minutes at 4 °C the assay was concluded by adding 4 ml ice cold Phosphate Buffered Saline (PBS; Oxoid, U.K.), followed by an incubation on ice for 30 minutes. The content of each tube was brought on glass microfibre filters (Whatman International LTD, U.K.) under vacuum. The filters were flushed 3 times with 4 ml ice cold PBS. After drying, filters were placed in econo glass vials (Canberra-Packard Benelux N.V., The Netherlands) and kept overnight to enable solubilization after addition of 5 ml Emulsifier Scintillator Plus (Canberra-Packard Benelux N.V., The Netherlands). The amount of radioactivity in each vial was measured using a Liquid Scintillation Analyzer (Packard Instrument B.V., The Netherlands).

Specific binding was determined by subtracting the amount of nonspecifically bound radioligand from the total amount bound. Data were analyzed by constructing a Scatchard plot[17].

Displacement of tritiated nicotine by various drugs

In order to further characterize the binding sites on the non-adherent MNC we performed displacement experiments with various compounds. These compounds included known cholinergic agonists and antagonists (table 8.2). Furthermore, we tested compounds known to inhibit *in vitro* cytokine production by human non-adherent MNC.

Displacement experiments were performed as described above for 'tritiated nicotine receptor binding studies', with the difference that a single concentration of tritiated nicotine and multiple concentrations of the drugs tested were used.

Statistics

IL-2 and TNFα production are expressed as mean ± S.E.M. (n = 5). Statistical comparison between control and drug-treated cell cultures were made by paired t-test. A P-value < 0.05 was considered significant. Plots of nicotine-treated cultures in the absence of antagonist were compared with plots in the presence of antagonists using Manova.

Cluster differentiation	Percentage
CD3	70.4 ±2.5
CD16/CD56	16.1 ±0.7
CD20	7.5 ±1.7
CD14	0.9 ±0.2
Not classified	5.1 ±1.3

Table 8.1 Non-adherent mononuclear cells subdivided in the different cell type by FACS. The antibodies used were Leu 14, Leu 11, Leu 19, B4 and My4.

8.3 Results

Effect on cytokine production

The cell fraction used consisted predominantly of T-lymphocytes (table 8.1). Phytohemagglutinin stimulation resulted in an IL-2 production ranging from 840 to 2470 pg/ml, with a mean ± S.E.M. of 1480 ± 255 pg/ml and a median of 1465 pg/ml. For TNFα the range was 3740 - 5570 pg/ml, mean ± S.E.M 4690 ± 340 and the median 4960 pg/ml. Nicotine caused a dose-dependent inhibition of both cytokines. Pre-incubation of the non-adherent MNC with the cholinergic nicotine receptor antagonists hexamethonium and pancuronium did not block the effect caused by nicotine (figure 8.1).

Assessment of nicotine receptor number and K_d in non-adherent MNC.

In figure 8.2, a representative Scatchard plot from one of the ligand binding experiments with tritiated nicotine is shown. The slope is equal to the negative reciprocal of the dissociation constant ($-1/K_d$) and the intercept on the abscissa equals the total concentration of receptors (B_{max}). The nicotine receptor content for the non-adherent MNC was 2630 ± 350 sites / cell (mean ± S.E.M.) with a K_d of 6.2 ± 1.4 nmol/l.

Displacement studies

Tritiated nicotine was most effectively displaced by unlabeled l-nicotine. None of the other compounds tested inhibited the binding of tritiated nicotine. Table 8.2 depicts the drugs tested and the percentage inhibition of tritiated nicotine binding.

Drugs	Inhibition (%)
l-Nicotine	100
Cotinine	< 10
Carbachol	< 10
Atropine	< 10
Dimethylphenylpiperazinium (DMPP)	< 10
Hexamethonium	< 10
Pancuronium	< 10
Prednisolone	< 10
Dexamethasone	< 10
Isoprenaline	< 10
Salbutamol	< 10

Table 8.2 Effect of cholinergic agonists, antagonist and compounds known to inhibit cytokine production by non-adherent MNC on the binding of tritiated nicotine to the receptor. All drugs, except cotinine, caused less than 10 % inhibition at 1000 fold excess.

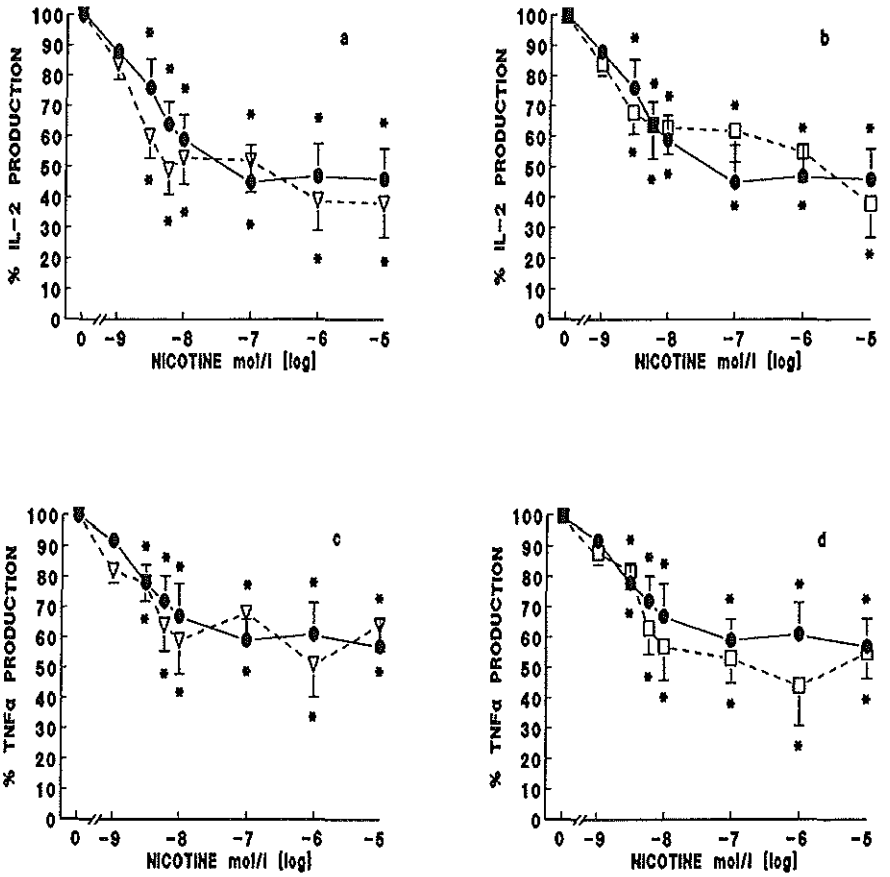

Figure 8.1 Effect of nicotine in the absence (solid dot) or presence of hexamethonium (open triangle) or pancuronium (open square) on the *in vitro* production of IL-2 and TNFα by human peripheral lymphocytes. The lymphocytes were stimulated with phytohemagglutinin (PHA: 10 μg/ml). Cytokine production (percentage of control) is presented as mean ± S.E.M. (vertical bars) and represent 5 consecutive experiments performed on cells obtained from 5 separate healthy volunteers. The paired t-test was used to compare control and nicotine treated cell cultures. Nicotine caused a significant inhibition of IL-2 and TNFα production, * $P < 0.05$ compared with control cultures incubated with medium and PHA. The effect nicotine is not blocked by the cholinergic nicotine receptor antagonists hexamethonium (a and c) and pancuronium (b and d).

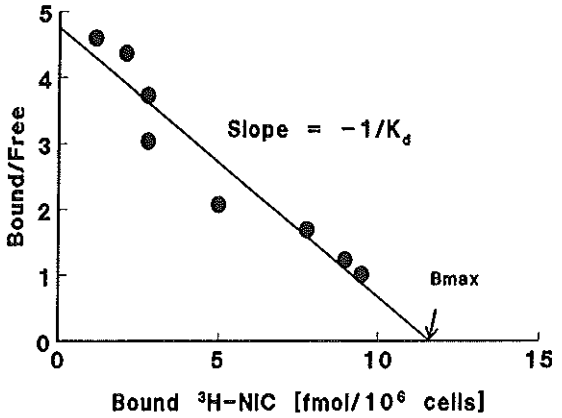

Figure 8.2 Scatchard plot of specifically bound N-methyl-^{3}H-Nicotine to non-adherent mononuclear cells. K_d and B_{max} in the case shown are 2.44 nmol/l and 2.33 fmol/10^6 cells respectively.

8.4 Discussion

Epidemiological studies have reported an inverse association between cigarette smoking and several inflammatory and immunological disorders[18,19,20,21,22,23,24]. Subsequently, nicotine administration has been shown to have beneficial effects in ulcerative colitis[25,26]. Cigarette smoking and nicotine have been reported to affect various actions of the immune system, including lymphocyte function and cytokine production[3,11,27].

The presence of receptors on human leukocytes with a high affinity for nicotine was first suggested almost two decades ago[28]. Data from our study confirm the presence of these receptors on human non-adherent MNC. However, little is known about the function of this type of receptor. In this study we set out to establish the plausibility of their immunological role by proving their involvement in the nicotine induced inhibition of cytokine production.

The fact that inhibition of cytokine production by nicotine could not be blocked by two types of cholinergic nicotine receptor antagonists suggests that these receptors are of a noncholinergic nature. Further evidence for this hypothesis is provided by the inability of multiple cholinergic agonists and antagonists to displace tritiated nicotine from the receptor.

Another possible explanation of a receptor mediated inhibition of cytokine production by nicotine is that nicotine exerts its anti-inflammatory effects through another type of known receptor. This assumption implies that receptors present on non-adherent MNC which mediate the anti-inflammatory effects of another compound would also have a high affinity for nicotine. A number of receptors present on human leukocytes are known to mediate anti-inflammatory effects, such as inhibition of *in vitro* cytokine production. Examples are the receptor for glucocorticoids[13] and β_2-adrenoceptor agonists[14]. The glucocorticoids as well as the β_2-adrenoceptor agonists tested in the displacement experiments failed to inhibit binding of tritiated nicotine to the receptor. This suggests that the receptor present on non-adherent MNC, to which tritiated nicotine binds, is neither the glucocorticoid receptor nor the β_2-adrenoceptor.

There are multiple methods to characterize receptors. In this study we used dose response curves in the presence or abcense of 2 types of known nicotine receptor antagonists. The fact that these antagonists could not block the effect of nicotine could also implie that the effect of nicotine is not receptor mediated. However, ligand binding studies established the presence of binding sites on MNC with a high affinity for nicotine. Although there was a considerable amount af non-specific binding in these assays, in all experiments we found a dose dependent displacement of tritiated nicotine by "cold" nicotine. In none of the experiments performed did we detect displacement of tritiated nicotine by any of the other compounds.

Transdermal nicotine administration has clearly been shown to have therapeutic effects in ulcerative colitis by two large placebo controlled double-blind trials[29,30]. However, the evident risks of cardiovascular and neoplastic diseases associated with smoking and nicotine use strongly hampers the therapeutic application of nicotine. A better understanding

of the mechanism through which nicotine exerts its anti-inflammatory effects might enable the development of compounds with the desired anti-inflammatory effects while lacking the cardiovascular and carcinogenic effects.

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Chapter 9

In vivo effect of nicotine on cytokine production by human non-adherent mononuclear cells*

Abstract - Ulcerative colitis (UC) is predominantly a disease of non-smokers and treatment with transdermal nicotine improves symptoms in UC patients, whereas smoking seems to have a deleterious effect in patients with Crohn's disease (CD). In CD the cytokine profile is of a dominant T-helper 1 (Th1) pattern whereas in UC the T-helper 2 (Th2) pattern predominates. To find an explanation for the beneficial effect of nicotine in UC and the deteriorative effect in CD we studied the *in vivo* effect of nicotine on the IL-2, IL-10 and TNFα production by human cells.

Eleven healthy male non-smokers were included in this study. The volunteers applied nicotine patches with a regulated release of 5 mg (day 1 and 2), 10 mg (day 3 and 4) and 15 mg (day 5, 6 and 7) nicotine per day.

Heart rate and blood pressure were recorded, nicotine and cotinine concentrations in plasma measured before and after 2, 4 and 7 days of treatment. Non-adherent mononuclear cells (NAC) were isolated from peripheral blood obtained from the subjects before and after 7 days of treatment. The NAC were cultured in the absence or presence of phytohemagglutinin for 48 hours. Total amount IL-2, IL-10 and TNFα formed were measured in the supernatants using specific ELISA's.

Treatment with nicotine caused a significant inhibition of IL-10 production by NAC. In contrast, nicotine patch treatment had no effect on the production of IL-2 and TNFα.

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Nicotine *in vivo* has an inhibitory effect on Th2 cell function as measured by inhibition of IL-10 production, but does not appear to have any effect on Th1 cell function.

9.1 Introduction

Inflammatory bowel disease (IBD) is a collective term for two distinct disease entities of unknown etiology, ulcerative colitis (UC) and Crohn's disease (CD). Both disorders are characterized by chronic inflammation of the gastrointestinal tract in which the T-helper (Th1 and Th2) cell may play an important role. One of the current views is that IBD might result from an imbalance between the Th1 and Th2 cell function, with Th1 cells being overactive in CD and Th2 cells in UC[1,2,3]. This idea is based on the cytokine profiles found in IBD. In CD elevated levels of the Th1 cell derived cytokines IL-2 and INFγ are reported, whereas in UC we find elevated levels of IL-10, produced by Th2 cells.

Epidemiological studies suggest that exogenous factors may play a role in the pathogenesis of IBD and it has become clear that environmental factors such as cigarette smoking may modulate the course of both forms of inflammatory bowel disease[4,5,6]. Smoking protects against ulcerative colitis and nicotine has a beneficial effect on the symptoms[7,8]. The mechanisms through which nicotine exerts its effect are still unknown, but there is evidence that smoking influences the cellular as well as the humoral immune system[9,10,11,12,13,14].

In order to find an explanation for the beneficial effect of nicotine in UC and the deteriorative effect in CD, we studied the *in vivo* effect of nicotine on the cytokine production by human non-adherent mononuclear cells.

9.2 Subjects and methods

Subjects

This study was approved by the Medical Ethical Committee of the University Hospital Rotterdam. Eleven healthy male non-smokers (5 ex-smokers and 6 lifelong non-smokers, mean age: 24 years) were included in the study after a detailed medical history to insure that none of the subjects were suffering from cardiovascular or other disorders. None of the subjects were taking medicines of any kind.

After explanation of the aim and scope of the study heart rate and blood pressure of the volunteers were recorded before drawing 60 ml of venous blood.

Treatment

Each subject received 2 patches with a regulated release of 5 mg nicotine over a period of 16 hours, 2 patches with a release of 10 mg and 3 patches with a release of 15 mg nicotine per 16 hours. On day 1 and 2 single 5 mg patches were worn, on day 3 and 4 single 10 mg patches, and on day 5, 6 and 7 single 15-mg patches. Patches were obtained from Pharmacia, Sweden. There was a stepwise increase of the nicotine dose and the subjects applied the patches before going to bed to limit the side effects.

Blood samples were obtained before and after 2, 4, and 7 days of treatment and 7 days after cessation of treatment for measurement of the nicotine and cotinine levels in plasma.

Isolation of mononuclear cells

Mononuclear cells (MNC) were isolated from 60 ml heparinized venous blood immediately after blood sampling before the patches were applied, after 7 days of treatment and 7 days after cessation of treatment. The method used was a modification of the technique described by Boyum[15]. Briefly, the blood was diluted 1:1 with Phosphate Buffered Saline (PBS; Oxoid, U.K.) before fractioning it by a one-step Ficoll-Paque

gradient (Pharmacia, Sweden) centrifugation at 1100 *g* for 15 minutes at 20 °C. The interphase was washed in PBS and resuspended in Dulbecco's Modified Eagles Medium (DMEM) containing HEPES and foetal calf serum (Gibco, U.K.), supplemented with penicillin and streptomycin (Flow Lab, U.K.).

Following isolation the MNC were incubated in culture medium for 90 minutes in a water saturated atmosphere of 37 °C containing 5 % CO_2. Non-adherent MNC (NAC) were harvested by repeated rinsing of the culture flasks (Costar, U.S.A.) with DMEM. The viability of the cells, assessed by Trypan blue exclusion, was over 95 %.

Characterization of non-adherent mononuclear cell fraction

The leucocyte subpopulations present in the non-adherent MNC fraction were analyzed by FACS using the monoclonal antibodies Leu 4, Leu11 and Leu19 (Becton Dickinson, Mountain View, CA) that recognize CD3, CD16 and CD19 respectively. CD14 and CD20 positive cells were characterized by My 4 and B4 (Coulter Cytometry, Florida)

Cytokine production

Non-adherent MNC were cultured (2 x 10^6 cells/ well) in 24 wells culture plates (Costar, Cambridge MA, USA) for 24 hours after which phytohemagglutinin (PHA: 10 μg/ml; Sigma Chemie, Belgium) was added to half of the wells followed by incubation for another 24 hours. The supernatants were harvested and stored at -80 °C until IL-2, IL-10 and TNFα concentrations were assessed by ELISA (IL-2: Medgenix, Belgium, IL-10: Pharmingen, U.S.A. and TNFα: Central Laboratory for Blood transfusions, The Netherlands). All incubations were performed in triplicate.

Side effects

Subjects were asked to grade possible side effects from 0 (none) to 5 (severe) at each visit.

Statistics

The IL-2, IL-10 and TNFα production and hemodynamic parameters are expressed as mean ± SD (n = 11). Statistical comparisons between control and nicotine-treated NAC were made by Wilcoxon paired t-test. A $P < 0.05$ was considered significant.

9.3 Results

Nicotine and cotinine levels in plasma

The plasma nicotine and cotinine concentrations rose significantly during treatment, reflecting the nicotine dose applied. By the seventh day the plasma levels reached the maximal level of 10.2 ng/ml and 148.4 ng/ml for nicotine and cotinine respectively.
One week after cessation of treatment the nicotine and cotinine concentrations were back to baseline values (table 9.1).

Day	Nicotine	Cotinine
0	0.2 ± 0.1	7.2 ± 12.8
2	3.5 ± 1.0	41.5 ± 9.9
4	6.9 ± 1.4	82.7 ± 16.4
7	10.2 ± 2.4	148.4 ± 44.8
14	0.3 ± 0.3	4.0 ± 5.3

Table 9.1 Plasma nicotine and cotinine concentrations (ng/ml), mean ± SD (n = 11), before (day 0), during (days 2, 4 and 7) and seven days after exposure to transdermal nicotine. Samples were drawn approximately 11 hours after application of the patch.

Characterization of NAC

The non-adherent MNC fraction consisted primarily of T-lymphocytes (table 9.2).

Cluster differentiation	Percentage
CD3	71.8 ± 2.1
CD16/CD56	14.7 ± 0.7
CD20	7.7 ± 1.7
CD14	0.8 ± 0.1
Not classified	5.1 ± 1.1

Table 9.2 Percentage of different cell types comprising the non-adherent mononuclear cells.Cells were characterized using the monoclonal antibodies Leu 14, Leu 11, Leu19, B4 and My 4.

Cytokine production

The effect of nicotine on the IL-2, IL-10 and TNFα production by stimulated and non-stimulated human NAC was studied. Treatment with nicotine caused a significant inhibition of IL-10 production by non-stimulated NAC (Pre: 275 ± 280 pg/ml) (Nicotine:

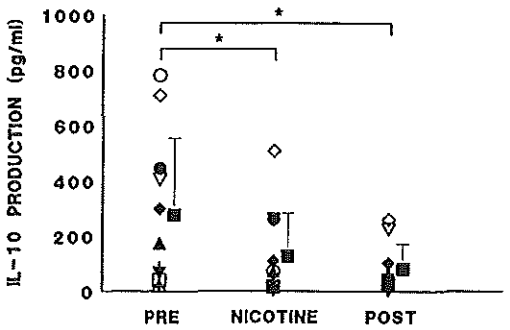

Figure 9.1 The effect of nicotine on IL-10 production by non-stimulated NAC (mean ± SD) in pg/ml, (n = 11) (* P < 0.05) . IL-10 production was measured before (Pre), after 7 days exposure to transdermal nicotine (Nicotine) and 7 days after cessation of treatment (Post).

130 ± 155 pg/ml). This inhibitory effect was still evident 7 days after cessation of treatment (Post: 85 ± 95 pg/ml) (figure 9.1). In contrast, treatment did not inhibit the PHA-stimulated IL-2, IL-10 and TNFα production. For IL-10 the PHA-stimulated production before, after 7 days exposure to transdermal nicotine and 7 days after cessation of treatment was 1085 ± 1430 pg/ml, 775 ± 1010 pg/ml and 640 ± 450 pg/ml, respectively. The PHA-stimulated IL-2 levels were 2120 ± 830 pg/ml, 2115 ± 1375 pg/ml and 1545 ± 2805 pg/ml. Stimulation of NAC resulted in TNFα concentrations of 4545 ± 935 pg/ml, 4055 ± 1440 pg/ml and 3760 ± 910 pg/ml respectively.

Side effects

Eight of the 11 subjects experienced some kind of side effects (5 out of 6 lifelong non-smokers and 3 out of 5 ex-smokers). In order of frequency the side effects were sleep disturbance or nightmares, itching, mild skin irritations, dizziness, nausea, chill, headache, tremor and vomiting. Although most subjects characterized the side effects as mild (score 1), two individuals experienced severe dizziness and nausea, leading to vomiting in one subject. Most side effects were experienced during the first night after raising the nicotine dose from 5 mg to 10 mg and subsided with continuation of the treatment. The severity of the side effects was not related to the subjects smoking history, although both individuals who experienced severe side effects were non-smokers and more lifelong non-smokers experienced side effects. Plasma nicotine and cotinine concentrations did not correlate with inhibition of cytokine production or side effects.

The treatment had no effect on heart rate, systolic and diastolic blood pressure, nor did any of the subjects experience palpitations.

9.4 Discussion

We chose to use a cell population consisting predominantly of T-cells in this study for two reasons. Recent studies have reported elevated levels of T-cell generated cytokines in inflammatory bowel disease, with Crohn's disease displaying a Th1 cell cytokine profile while ulcerative colitis is characterized by a Th2 cell cytokine profile. Another finding indicating an important role for the T-cell in the pathogenesis of UC is the observation that appendectomy has a protective effect against UC. The appendix is a helper T-cell organ[16].

Smoking has been indicated to influence the cellular as well as the humoral immune system[9,10,11,12,13,14] and recent studies have shown nicotine to have a beneficial effect in UC[8]. Binding studies have demonstrated receptors with a high affinity for nicotine on human lymphocytes[17,18].

The results of this study show that nicotine *in vivo* inhibits the IL-10 production by non-stimulated human NAC. This could indicate a functional inhibition of the Th2 cells, as this cytokine is primarily produced by Th2 cells. Recent reports have indicated an imbalance between Th1 and Th2 cell function in IBD, with the former being overactive in CD and the latter in UC.

The inhibition of IL-10 production by nicotine could also account for the lower immunoglobulin levels reported in smokers[11,12,13]. Interleukin 10 is a very potent inducer of B-cell proliferation and differentiation. In SAC-activated as well as in CD40 system activated B-lymphocytes IL-10 has proven to be the most potent cytokine in the induction of IgA-, IgG- and IgM- production[19]. Experiments with anti-IL-10 treated mice have also provided evidence for the important role of IL-10 in immunoglobulin production[20]. Although no significant inhibition of IL-10 production by PHA stimulated NAC was detected, there is clearly a trend visible.

We were not able to study the effect of nicotine *in vivo* on the IL-2 and TNFα production by non-stimulated human NAC due to the fact that in the majority of cases the production was beneath detection level. The PHA stimulated production of these cytokines

was not inhibited by treatment with nicotine patches. These findings might indicate lack of effect on Th1 cell function.

The inhibitory effect of nicotine was still present 7 days after cessation of treatment. The effect even seems to be greater. In a prospective study Miller *et al.* demonstrated that relative CD8 cell counts and the CD4 to CD8 ratio took three weeks to return to normal after smokers had stopped smoking[14]. This finding indicates that nicotine also induces T-cell differentiation and by doing so has a greater effect on younger cells. If this is the case the effect of nicotine would be more evident when the subjects would have been treated for a longer period of time. For ethical reasons this was only allowed to be performed in UC patients.

We conclude that nicotine *in vivo* significantly inhibits the IL-10 production by Non-stimulated human non-adherent mononuclear cells.

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PART 4

Synopsis

Chapter 10

Summary, general discussion and directions for further research

10.1 Summary

Chapter 1 is a review of basic and clinical aspects of idiopathic inflammatory bowel disease. Idiopathic inflammatory bowel disease is a chronic relapsing inflammation of the human gastrointestinal tract or part of it. There are two main clinical entities, Crohn's disease and ulcerative colitis, with distinct differences but also with many similarities.

Symptoms depend on the extent and seriousness of the inflammation and may vary from minor complaints to life threatening intestinal and extra-intestinal complications. Although the etiology remains obscure, epidemiological studies suggest a genetic susceptibility which causes inadequate immunological responses to environmental agents. Medical treatment of inflammatory bowel disease has progressed during the last decade. Current standard treatment consists of aminosalicylates and corticosteroids, either alone or in combination. A variety of alternative drugs are becoming available for patients with disease refractory to standard treatment. Of the newer drugs cyclosporin, azathioprine/6-mercaptopurine and methotrexate are the ones at present most often used.

Chapter 2 describes the synthesis, metabolism and anti-inflammatory effects of glucocorticoids. These are the most effective anti-inflammatory agents known. Glucocorticoids exert their actions after binding to specific receptors located in the cytoplasm. Binding of glucocorticoids to its receptor results in the down-regulation of the transcription of pro-inflammatory genes and up-regulation of the transcription of anti-inflammatory genes. Glucocorticoids affect the functions and the number of inflammatory cells, and reduce the vascular permeability by influencing endothelial cell shape and contractility and membrane composition.

Glucocorticoids were first used in the treatment of ulcerative colitis during the nineteen-forties. For mild to moderate exacerbations the optimal dose was found to be 40-60 mg of prednisolone daily. However, glucocorticoids do not reduce the rate of relapses. In a later stage it became clear that glucocorticoids are also effective in the treatment of acute exacerbations of Crohn's disease. The use of enemas has facilitated local treatment of inflammatory bowel disease, but it has not been able to abolish the systemic side effects entirely. Recently corticosteroids with a high first pass effect have been developed to decrease systemic effects without reducing local mucosal concentration. Glucocorticoids do not only affect the immune system but also many other systems, causing various side effects. In order to reduce these side effects, rapidly metabolized topical glucocorticoids have been developed and are being tried in the treatment of inflammatory bowel disease.

Chapter 3 is a report of *in vitro* experiments in order to asses whether the inhibition of the stimulated production of TNFα, IL-6, PGE_2 and LTB_4 by human mononuclear cells depends on the number or the affinity of the glucocorticoid receptors of these cells. Glucocorticoid receptor number and affinity were determined using a whole cell binding assay with tritiated dexamethasone.

A concentration dependent inhibition of TNFα, IL-6 and PGE_2 production was observed. Dexamethasone had no effect on LTB_4 production. A steroid receptor antagonist, RU486, significantly blocked the effect of dexamethasone, but no correlations were found between the inhibition of mediator release and affinity or receptor number.

In **Chapter 4** we investigated whether the variation in response to corticosteroids could be due to variation in number or affinity of glucocorticoid receptors (GR) on mononuclear cells (MNC). In order to test this hypothesis we assessed if GR-number and GR-affinity of MNC isolated from venous blood of IBD-patients not responding to corticosteroid therapy differ from those of healthy volunteers and IBD-patients who do respond to this type of therapy.

GR-content and K_d for the three groups were respectively, healthy volunteers: 4430 ± 340 sites per cell and 9.5 ± 0.7 nmol/l; responders: 3900 ± 210 sites/cell, 7.9 ± 0.6 nmol/l; non-responders: 2450 ± 310 sites/cell, 6.0 ± 1.4 nmol/l. Thus, mononuclear cells of IBD-patients not responding to glucocorticosteroids have a low glucocorticoid receptor number.

However, experiments with blood drawn from the same nonresponders 9 to 12 months later did not confirm this finding. A possible explanation for this discrepancy is that corticosteroid resistance in IBD is acquired and reversible, as seems to be the case in asthma.

Chapter 5 gives an overview of pharmacological and immunological actions of nicotine. The effects of nicotine on the immune system of animals depend on the species tested, the age of the animals, duration of exposure and the level of exposure. Short-term or acute exposure generally results in limited effects which soon fade away after cessation of exposure. Chronic exposure on the other hand usually has a suppressive effect on the immune system.

In humans smoking causes an increased leucocyte count in peripheral blood of which the extent depends on genetics, gender, age and the number of cigarettes smoked. On the other hand several studies have reported a decreased natural killer cell activity in leucocytes of smokers. The *in vitro* synthesis of mediators of inflammation and immunoglobulin production have been shown to be modulated by nicotine.

Epidemiological studies have shown nicotine use to be associated with an increased incidence of certain disorders and a decreased incidence of others. The availability of nicotine gum and patches has made it possible to study the efficacy of nicotine in the treatment of the disorders last mentioned. These studies have shown significant clinical improvement. However, the risk of addiction and the cardiovascular side effects have restrained clinician from applying nicotine as a standard treatment.

In chapter 6 we investigated the *in vivo* effect of nicotine on lipid mediators and cytokines produced by colonic mucosa in mice. Colonic mucosa was obtained from 15 BALB/c female mice. Six mice were treated with nicotine for 22 days and nine mice with placebo. The concentration of nicotine and cotinine in plasma reflected the dose of nicotine given. Colonic mucosa was homogenized in Krebs-Henseleit buffer and centrifuged. Mediators of inflammation were measured in the supernatant by radio immunoassay and ELISA.

The synthesis of the lipid mediators such as the prostanoids prostaglandin E_2, 6-keto-prostaglandin $F_{1\alpha}$ and thromboxane B_2, the 5-lipoxygenase products leukotriene B_4 en C_4 and platelet activating factor is not affected, whereas the synthesis of the pro-inflammatory cytokines interleukin 1ß and tumor necrosis factor α is significantly inhibited.

Chapter 7 describes the results of a study in which the *in vitro* effect of nicotine on IL-2 and TNFα production by human mononuclear cells, isolated from peripheral blood from healthy volunteers was investigated. Non-adherent MNC were preincubated with varying concentrations of nicotine or prednisolone for 24 hours followed by addition of phytohemagglutinin. The concentrations of IL-2 and TNFα in the supernatant were determined by ELISA.

Nicotine as well as prednisolone caused a significant inhibition of IL-2 and TNFα production. The maximum inhibition caused by nicotine was about 50 % of that caused by prednisolone and was reached at concentrations equivalent to nicotine levels measured in plasma of smokers.

Chapter 8 reports the results of experiments performed to assess whether the receptors for nicotine present on human MNC are involved in the nicotine induced inhibition of cytokine production. Non-adherent MNC were preincubated in the presence or absence of two cholinergic nicotine receptor antagonists, hexamethonium and pancuronium, followed by the addition of nicotine and phytohemagglutinin (10 μg/ml). The concentrations

of IL-2 and TNFα in the supernatant were determined by ELISA. Furthermore, ligand binding studies and displacement experiments were conducted to confirm the presence of receptors for nicotine on the MNC and to further characterize these receptors.

Interleukin 2 and TNFα production was significantly inhibited by nicotine. However, this inhibition could not be blocked by hexamethonium or pancuronium. The MNC had 2630 ± 350 nicotine receptors / cell (mean ± S.E.M.) with a K_d of 6.2 ± 1.4 nmol/l. The nicotine receptor present on the MNC had no affinity for any of the cholinergic agonists and antagonists tested. These results indicate that nicotine inhibits the production of IL-2 and TNFα by peripheral blood MNC via a non-cholinergic nicotine receptor.

Chapter 9 describes the *in vivo* effect of nicotine on the cytokine production by human non-adherent mononuclear cells. Ulcerative colitis is predominantly a disease of non-smokers and treatment with transdermal nicotine improves symptoms in UC patients, whereas smoking seems to have a deleterious effect in patients with Crohn's disease (CD). In CD the cytokine profile is of a dominant Th1 pattern whereas in UC the Th2 pattern predominates. To find an explanation for the beneficial effect of nicotine in UC and the deteriorative effect in CD, we studied the *in vivo* effect of nicotine on the IL-2, IL-10 and TNFα production by human cells.

Eleven healthy male non-smokers were included in this study. The volunteers applied nicotine patches with a regulated release of 5 mg (day 1 and 2), 10 mg (day 3 and 4) and 15 mg (day 5, 6 and 7) nicotine per day. Heart rate and blood pressure were recorded, nicotine and cotinine concentrations in plasma measured before and after 2, 4 and 7 days of treatment. Non-adherent mononuclear cells (NAC) were isolated from peripheral blood obtained from the subjects before and after 7 days of treatment. The NAC were cultured in the absence or presence of phytohemagglutinin for 48 hours. Total amount IL-2, IL-10 and TNFα formed were measured in the supernatant using specific ELISA's.

Treatment with nicotine caused a significant inhibition of IL-10 production by NAC. In contrast, nicotine patch treatment had no effect on the production of IL-2 and TNFα.

Nicotine *in vivo* has an inhibitory effect on Th2 cell function as measured by inhibition of IL-10 production, but does not appear to have any effect on Th1 cell function.

10.2 General Discussion

The etiology of inflammatory bowel disease remains unknown but recent studies suggest a dysfunctional immune response to environmental agents. Susceptibility to development of this dysfunctional immune response is probably genetically determined. Inflammation is the key pathological event in IBD and can be divided in three stages: initiation, perpetuation and development of tissue damage[1]. Mononuclear cells play an important role in all three stages.

The medical treatment of inflammatory bowel disease is based on suppression of the inflammation[2,3]. The substances mostly used are glucocorticoids. Although glucocorticoids have been effectively used in the treatment of IBD for over 30 years now[9], the precise mechanisms through which they exert their anti-inflammatory effects is far from elucidated. An example for this is the remaining puzzle why some patients fail to respond to treatment with glucocorticoids (corticosteroid resistance).

Failure of some patients to respond to treatment with corticosteroids[4,5] has lead to efforts to find other substances with anti-inflammatory effects useful in the treatment of inflammatory bowel disease. Epidemiological studies have identified multiple substances which influence the course of IBD[6]. One of these factors is cigarette smoking which is associated with a decreased incidence of ulcerative colitis[7,8]. This has lead to clinical trials with nicotine for active ulcerative colitis.

The aims of the experiments described in this thesis were to:

1. Study the anti-inflammatory properties of glucocorticoids (part 2) and nicotine (part 3) by evaluating their effects on inflammatory mediator production by (human) MNC.
2. Study the characteristics of the receptors for these substances in MNC.

The studies described in the second part of this thesis were focused on finding an explanation for corticosteroid resistance. First we set out to demonstrate the importance of the glucocorticoid receptor in the exertion of the effects of glucocorticoids. Chapter 3 clearly shows the inhibitory effect of dexamethasone on the production of inflammatory mediators to be glucocorticoid receptor mediated. Binding of the steroid receptor antagonist RU 486 to the glucocorticoid receptor blocks the effect of dexamethasone.

The next step was to correlate the clinical effectiveness of glucocorticoids with the glucocoticoid receptor characteristics such as receptor number in the MNC and the affinity of these receptors. The experiments conducted with MNC isolated from blood obtained from IBD patients shortly (4 - 7 weeks) after these patients had proven to be responders or nonresponders yielded promising results. The nonresponders had a significantly lower GR number. However, these results were not reproducible with blood drawn from the same nonresponders 9 - 12 months later. Possible explanation for this is that the reduced GR number is acquired. Sher and associates found a diminished GR binding affinity in MNC from patients with corticosteroid resistant asthma[10]. These authors reported the abnormal GR binding affinity to be acquired and cytokine-mediated, for it could be induced *in vitro* by incubation of MNC from healthy donors in the presence of both IL-2 and IL-4[11]. Furthermore, the diminished MNC GR binding affinity found in corticosteroid resistant asthma patients is reversible when the cells are incubated with medium alone, but sustainable by addition of IL-2 and IL-4 to the medium.

The studies presented in the third part of this thesis showed that nicotine affects the

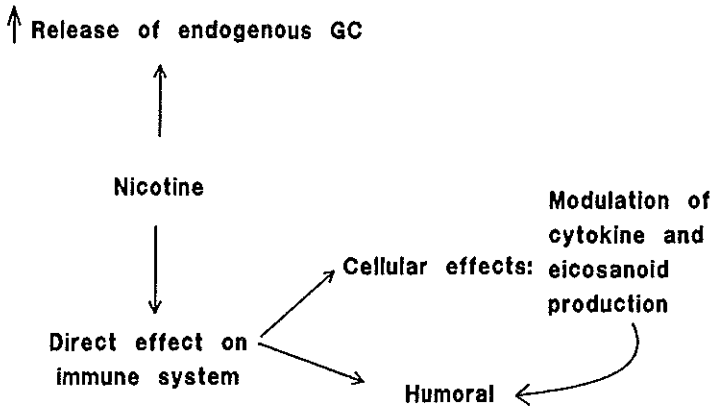
MNC *in vitro* and *in vivo*. Nicotine inhibits the IL-1ß and TNFα production by mouse colonic mucosa (chapter 6).

The effect of nicotine on human MNC was studied *in vitro* and *in vivo*. The *in vitro* production of the cytokines IL-2 and TNFα by human non adherent MNC was significantly inhibited by nicotine (chapter 7). Furthermore, these cells possess receptors for nicotine. Ligand binding studies showed these receptors to be of a non-cholinergic nature, which is in agreement with the finding that the effect of nicotine could not be blocked by hexamethonium and pancuronium, two cholinergic nicotine receptor antagonists (chapter 8).

The next step was to study the *in vivo* effect of nicotine on human MNC. Treatment of healthy volunteers with nicotine patches resulted in an inhibition of IL-10 production, whereas IL-2 and TNFα production was not affected (chapter 9). We concluded that this might indicate a differentiated effect on Th1 and Th2 cells, only inhibiting Th2 function. This would be in agreement with the epidemiological finding that cigarette smoking is associated with a decreased incidence for ulcerative colitis and an increased incidence for Crohn's disease. In ulcerative colitis the cytokine profile is predominantly of a Th2 cell type, whereas in Crohn's disease it is of a Th1 cell type. Experiments with genetically modified mice have contributed to the hypothesis that an imbalance between Th1 and Th2 function lays at the basis of the initiation phase of inflammatory bowel disease. IL-10 knock out and $CD45RB^{high}$ SCID mice who have a Th1 cytokine profile develop a "CD-like", transmural inflammation of the small bowel[12,13]. IL-2 knock out mice on the other hand exhibit a more "UC-like" inflammation of their colon[14].

All in all the exact mechanisms through which nicotine exerts its anti-inflammatory effects are still unknown. The presence of receptors for nicotine on MNC implies a direct effect on these cells. However, this cannot be proven without the availability of an antagonist for this receptor. On the other hand *in vivo*, nicotine also has an indirect effect on the immune system through the endocrine system. Nicotine induces increased plasma levels of GC in animals[15,16,17], as well in humans[18,19,20] in a dose-dependent manner[18,20]. This is achieved by enhancing the release of corticotropin releasing factor (CRF) and ACTH from

the hypothalamus and pituitary respectively[15,21,22].



10.3 Limitations of the applied methods

Mononuclear cells play a crucial role in inflammatory bowel disease. One could argue that the preceding statement is incomplete, in that in inflammatory bowel disease the MNC present in the gastrointestinal tract are the most important and not the MNC in the peripheral blood.

The experiments described in this thesis have been performed using MNC isolated from peripheral blood and not from colonic mucosa, which is an obvious limitation. In order to obtain a sufficient number of MNC from colonic mucosa one would need to many biopsies of patients undergoing colonoscopy. This problem could be overcome by isolating cells

from mucosa from patients undergoing surgery. Patients being operated for a malignancy could be used as controls. It is however, a subject of debate whether MNC from patients with a colonic malignancy should be considered normal. In the second place, the inflammatory bowel disease patients responding to corticosteroid treatment will probably not be operated on, so mucosal MNC could not be obtained from responders. Thirdly, although the nonresponders almost always undergo surgery, their mucosal MNC will not be useful for these experiments, as these patients are usually on high dose corticosteroids at the time of the operation.

10.4 Directions for future research

During the last decade considerable progress has been made in understanding the pathogenesis of IBD. This has led to the employment of clinical trials with substances which target specific effector mechanisms and mediators. These drugs have often shown little effectiveness, probably because inhibition of a specific pathway can be bypassed by parallel pathways.

Glucocorticoids still form the heart of the medical treatment of IBD. The finding that corticosteroid resistance is associated with altered receptor characteristics should be further investigated. Attempts should be made to test the hypothesis that these alterations are acquired and whether they are cytokine-mediated as seems to be the case in asthma.

One of the drugs which has demonstrated to be effective in active UC is nicotine[23,24,25]. However little is known about the mechanisms through which nicotine exerts it effect in UC. We have shown nicotine to modulate cytokine production *in vitro* and *in vivo* and demonstrated receptors for nicotine on human MNC. Binding experiments should be performed to further characterize these receptors in MNC. In addition research should be done to study the second messenger mechanisms involved and to reveal mRNA encoding these receptors. A more detailed knowledge of the receptor involved may facilitate the development of new therapeutic approaches more acceptable than nicotine itself.

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Chapter 11

Samenvatting in het Nederlands (Summary in Dutch):

In **hoofdstuk 1** wordt een overzicht gegeven van basaal wetenschappelijke- en klinische aspecten van chronische inflammatoire darmaandoeningen (IBD). De term IBD wordt gebruikt om twee ziekte entiteiten aan te duiden, namelijk de ziekte van Crohn en colitis ulcerosa, met duidelijke verschillen maar ook vele overeenkomsten. Bij beide aandoeningen gaat het om een chronische recidiverende ontsteking van het humane maagdarmstelsel of delen daarvan. Het klachtenpatroon is afhankelijk van de uitgebreidheid en ernst van de ontsteking en kan variëren van kleine ongemakken tot levensbedreigende intestinale en extra-intestinale complicaties. Hoewel de etiologie nog niet bekend is, zijn er aanwijzingen dat het gaat om een genetische predispositie met als gevolg een inadequate immunologische response op omgevingsfactoren.

Gedurende het laatste decennium is er sprake van een geleidelijke vooruitgang van de medicamenteuze behandeling van IBD. De behandeling bestaat thans uit corticosteroïden en aminosalicylaten, afzonderlijk of in combinatie. Er komen steeds meer nieuwe middelen beschikbaar voor de behandeling van patiënten met ontsteking niet-reagerend op standaard behandeling. Van deze nieuwe middelen worden cyclosporine, azathioprine/ 6-mercaptopurine en methrotrexaat het meest toegepast.

Hoofdstuk 2 beschrijft de synthese, het metabolisme en de anti-inflammatoire effecten van glucocorticosteroiden, de meest effectieve ontsteking remmers thans bekend. Glucocorticosteroiden werken via binding aan specifieke in het cytoplasma gelokaliseerde receptoren. Binding van GC aan de glucocorticoid receptor resulteert in "down-regulatie" van de transcriptie van pro-inflammatoire genen en "up-regulatie" van de transcriptie van anti-inflammatoire genen. Glucocorticosteroiden beïnvloeden tevens de functie en het aantal van ontstekingscellen, en reduceren de permeabiliteit van de vaten door beïnvloeding van de

vorm en contractiliteit van endotheelcellen.

Glucocorticosteroiden werden in de jaren veertig voor het eerst in de behandeling van colitis ulcerosa toegepast. De optimale dosis voor milde tot matige ernstige exacerbaties bleek 40-60 mg prednisolon per dag te zijn. De kans op recidiveren van de ontsteking wordt niet door GC beïnvloed. In een later stadium bleken GC ook effectief in de behandeling van exacerbaties van de ziekte van Crohn. Gebruik van GC-klysmata heeft lokale behandeling van de ontsteking mogelijk gemaakt, maar de systemische bijwerkingen worden met deze vorm van therapie niet geheel voorkomen. Glucocorticosteroiden beïnvloeden niet alleen het immuunsysteem, maar ook multipele andere systemen, met als gevolg niet aan het immuunsysteem gerelateerde bijwerkingen. Om deze systemische bijwerkingen te reduceren wordt steeds meer aandacht besteed aan het ontwikkelen van GC die lokaal toepasbaar zijn en snel worden gemetaboliseerd indien opgenomen.

Hoofdstuk 3 is een beschrijving van *in vitro* experimenten, uitgevoerd om vast te stellen of remming van de produktie van TNFα, IL-6, PGE_2 and LTB_4 door humane mononucleaire cellen afhankelijk is van het aantal of de affiniteit van de glucocorticoid receptoren in deze cellen. Glucocorticoid receptor aantal en affiniteit werden bepaald m.b.v. een receptor bindingsassays met getritiëerd dexamethason.

Dexamethason veroorzaakte een dosis-afhankelijke inhibitie van de produktie van TNFα, IL-6 en PGE_2, maar had geen effect op de LTB_4 produktie. De steroid receptor antagonist RU486 veroorzaakte een significante remming van het effect van dexamethason. Er werd echter geen correlatie gevonden tussen de inhibitie van mediator produktie door dexamethason en de affiniteit of receptor aantal.

In de experimenten beschreven in **hoofdstuk 4** werden de in hoofdstuk 3 beschreven technieken gebruikt om de hypothese te toetsen dat de variatie in respons op behandeling van IBD met glucocorticosteroiden het gevolg is van variatie in GR aantal of affiniteit. Onderzocht werd of de IBD patiënten waarbij behandeling met GC niet effectief is gebleken

(nonresponders), verschilden qua GR aantal en/of affiniteit van de GR van de patiënten waarbij deze vorm van therapie wel succesvol was ("responders"). Gemiddeld bezaten de MNC van de "nonresponders" een significant lager aantal GR in vergelijking tot de "responders" en de gezonde vrijwilligers.

Bij herhaling van de experimenten met bloed afgenomen van de zelfde "nonresponders" 9 tot 12 maanden later bleek er geen sprake meer te zijn van een verlaagd receptor aantal. Het voorgaande kan mogelijk betekenen dat corticosteroid resistentie in IBD niet aangeboren is, maar verworven en het gevolg is van factoren zoals cytokinen. Soortgelijke bevindingen zijn beschreven voor corticosteroid resistente astma patiënten.

Hoofdstuk 5 geeft een overzicht van de farmacologische en immunologische effecten van nicotine. De effecten van nicotine op het immuunsysteem van dieren is afhankelijk van het soort, de leeftijd van de dieren, duur van blootstelling en de concentratie. Blootstelling voor een korte periode heeft over het algemeen weinig invloed en de eventuele effecten zijn weer snel verdwenen na beëindiging van de blootstelling. Chronische blootstelling daarentegen heeft gewoonlijk een suppressief effect op het immuun systeem.

Bij de mens veroorzaakt het roken van sigaretten een stijging in leukocyten aantal in het perifere bloed, waarbij de mate van stijging afhankelijk is van genetische factoren, geslacht, leeftijd en het aantal gerookte sigaretten. Meerdere studies hebben daarentegen een afname van natural killer cel activiteit in de leukocyten van rokers beschreven. De *in vitro* produktie van ontstekings mediatoren en immunoglobulinen kan door nicotine gemoduleerd worden.

Epidemiologisch onderzoek heeft aangetoond dat nicotinegebruik geassocieerd is met een toegenomen incidentie van bepaalde aandoeningen en een afgenomen incidentie van andere. Het beschikbaar komen van nicotine in niet-invasieve toedieningsvormen (kauwgum en pleisters) heeft de mogelijkheid geschapen de effectiviteit van nicotine bij de behandeling van de laatstgenoemde groep aandoeningen te onderzoeken. Studies bij colitis ulcerosa

patienten met een actieve ontsteking laten een significante klinische verbetering zien. Het risico op verslaving en, de cardiovasculaire en carcinogene bijwerkingen beperken de toepasbaarheid van nicotine als standaard therapie.

In hoofdstuk 6 wordt het *in vivo* effect van nicotine op de produktie van lipide mediatoren en cytokinen door colon mucosa van muizen beschreven. Colon mucosa werd verkregen van 15 BALB/c vrouwelijke muizen. Zes muizen kregen gedurende 22 dagen nicotine en 9 muizen kregen NaCl toegediend. Colon mucosa werd gehomogeniseerd in Krebs-Henseleit buffer en gecentrifugeerd. De concentratie gevormde ontstekingsmediatoren werd in het supernatant bepaald d.m.v. R.I.A. en ELISA.

Nicotine had geen invloed op de vorming van de lipide mediatoren zoals de prostanoiden prostaglandine E_2, 6-keto-prostaglandine $F_{1\alpha}$ en thromboxaan B_2, de 5-lipoxygenase produkten leukotriene B_4 en C_4 en platelet activating factor. Daarentegen werd de produktie van de pro-inflammatoire cytokinen IL-1ß en TNFα significant geremd.

Hoofdstuk 7 beschrijft de resultaten van een studie waarin de *in vitro* effecten van nicotine op de produktie van IL-2 en TNFα door mononucleaire cellen, geïsoleerd uit perifeer bloed van gezonde vrijwilligers, werd onderzocht. Niet-adherente MNC werden gedurende 24 uur geïncubeerd met verschillende concentraties nicotine of prednisolon, gevolgd door toevoeging van phytohemagglutinine. De concentratie gevormde IL-2 en TNFα werd in het supernatant bepaald d.m.v. ELISA.

Zowel nicotine als prednisolon veroorzaakten een significante remming van de IL-2 en TNFα produktie. De maximale inhibitie door nicotine bedroeg ongeveer 50 % van die van prednisolon en werd bereikt bij concentraties equivalent aan nicotine concentraties gemeten in plasma van rokers.

Hoofdstuk 8 geeft de resultaten weer van experimenten uitgevoerd om te bepalen of de op humane MNC aanwezige receptoren voor nicotine betrokken zijn bij de door nicotine

geïnduceerde remming van de cytokine produktie. Niet-adherente MNC werden gepreïncubeerd in aan- of afwezigheid van 2 cholinergische nicotine antagonisten, hexamethonium en pancuronium, gevolgd door toevoeging van phytohemagglutinine (10 μg/ml). De concentratie gevormde IL-2 en TNFα werden in het supernatant bepaald d.m.v. ELISA.

Tevens werden ligand bindings- en verdringingsexperimenten uitgevoerd om de aanwezigheid van receptoren voor nicotine op humane MNC aan te tonen en nader te karakteriseren.

Zowel de produktie van IL-2 als die van TNFα werd significant geremd door nicotine. Deze inhibitie kon echter noch door hexamethonium noch door pancuronium worden geblokkeerd. De MNC bezaten 2630 ± 350 nicotine receptoren per cel (gemiddelde ± S.E.M.) met een K_d van 6,2 ± 1,4 nmol/l. De nicotine receptoren aanwezig op de MNC hadden geen affiniteit voor de geteste cholinergische agonisten en antagonisten. Deze resultaten suggereren dat het remmend effect van nicotine op de IL-2 en TNFα produktie door humane MNC via een niet-cholinergische nicotine receptor verloopt.

Hoofdstuk 9 beschrijft de *in vivo* effecten van nicotine op de cytokine produktie door humane niet-adherente mononucleaire cellen. Elf gezonde mannelijke vrijwilligers kregen nicotine pleisters opgeplakt met een gereguleerde afgifte van 5 mg (dag 1 en 2), 10 mg (dag 3 en 4) en 15 mg (dag 5, 6 en 7) nicotine per dag. Niet-adherente MNC (NAC) werden geïsoleerd uit perifeer bloed van de vrijwilligers vóór en na 7 dagen behandeling. De NAC werden gedurende 48 uur in aan dan wel afwezigheid van phytohemaglutinine geïncubeerd. De totale hoeveelheid gevormd IL-2, IL-10 en TNFα werd in het supernatant bepaald d.m.v. ELISA.

Behandeling met nicotine veroorzaakte een significante inhibitie van de IL-10 produktie door NAC, maar had geen effect op de produktie van IL-2 en TNFα.

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Curriculum Vitae

Guno Stanley Madretsma werd geboren op 7 november 1964 te Paramaribo, Suriname. Het basis onderwijs en de middelbare school werden in Rotterdam gevolgd en afgesloten met het behalen van het diploma "ongedeeld V.W.O." In september 1986 werd aangevangen met de studie geneeskunde aan de Erasmus Universiteit te Rotterdam. Al vroeg in de studie werd zijn belangstelling getrokken door research en begon hij reeds in het tweede studiejaar, onder leiding van Dr. R.A.A. van Zanten op de afdeling Inwendige Geneeskunde II van het AZR (hoofd: Prof. J.H.P. Wilson), aan een studie naar de prognostische factoren bij leverfalen. Vervolgens werden onder leiding van Dr. J.A. van Amsterdam de eerste schreden gezet op het gebied van immunofarmacologisch onderzoek op het instituut Farmacologie van de EUR (hoofd: Prof. Dr. I.L. Bonta). Gedurende deze periode was hij tevens werkzaam in het studententeam van de Stichting Trombosedienst en Artsenlaboratorium Rotterdam (Medisch Direkteur: Dr. J. Jonker, internist). In het vierde jaar werkte hij als student-assistent op de afdeling Cardiologie, sectie echocardiologie (hoofd: Dr. W.J. Gussenhoven). Het afstudeeronderzoek voor het doctoraalexamen werd op de afdeling Inwendige Geneeskunde II van het AZR verricht en betrof een studie naar de mogelijkheden tot vroegdetectie van coloncarcinomen d.m.v. biochemisch en immunologisch onderzoek van faeces. In juli 1993 werd het artsexamen behaald, waarna werd aangevangen met het onderzoek op het instituut Farmacologie van de EUR, onder leiding van Dr. F.J. Zijlstra (hoofd: Prof. Dr. P.R. Saxena), dat geresulteerd heeft in dit proefschrift. Tijdens deze periode was hij tevens arts-docent bij het Oranjekruis. Sinds 1 augustus 1996 is hij in opleiding tot internist in het Academisch Ziekenhuis Rotterdam-Dijkzigt (Opleider: Prof. J.H.P. Wilson).

List of publications

Full Papers

1. G.S. Madretsma, A.P.M. van Dijk, C.J.A.M. Tak, J.H.P. Wilson, F.J. Zijlstra: Nicotine induced inhibition of IL-2 and TNFα production by human mononnuclear cells is mediated via a nonocholinergic receptor.
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2. G.S. Madretsma, L. Wolters, A.P.M. van Dijk, C.J.A.M. Tak, C. Feyerabend, J.H.P. Wilson, F.J. Zijlstra: *In vivo* effect of nicotine on cytokine production by human non-adherent mononuclear cells.
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Abstracts

1. G.S. Madretsma, L.M.M. Wolters, A.P.M. van Dijk, C.J.A.M. Tak, J.H.P. Wilson, F.J. Zijlstra: *In vivo* effect of nicotine on cytokine production by human non-adherent mononuclear cells. Jaar vergadering van de Nederlandse Vereniging voor Gastroenterologie, oktober 1996.
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2. G.S. Madretsma, A.P.M. van Dijk, C.J.A.M. Tak, G.J. Donze, J.H.P. Wilson, F.J. Zijlstra: Nicotine Inhibits the Production of cytokines by peripheral blood and lamina propria mononuclear cells. 4th United European Gastroenterology Week, September 17-21, Berlin. (Oral presentation).
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List of abbreviations

5-ASA	5-aminosalicylic acid
ANCA	antineutrophil cytoplasmic antibodies
ACTH	adrenocorticotrophic hormone
ANOVA	analysis of variance
CD	Crohn's disease
CNS	central nervous system
CRH	corticotropin releasing hormone
DEX	dexamethasone
DMEM	Dulbecco's modified eagle's medium
GC	glucocorticoid
GM-CSF	granulocyte-macrophage colony-stimulating factor
GR	glucocorticoid receptor
HLA	human leukocyte antibody
IBD	inflammatory bowel disease
IC_{50}	molar concentration which produces 50 % of the maximum possible inhibition by agonist / antagonist
I.E.L.	intra-epithelial lymphocytes
Ig	immunoglobulin
IGF-1	insulin-like growth factor
IL	interleukin
IL-1RA	interleukin 1 receptor antagonist
INFγ	interferon γ
kD	kilodalton
K_d	dissociation constant
LPS	lipopolysaccharide
LTB_4	leukotriene B_4
M	molar

Manova	multiple analysis of variance
MNC	mononuclear cells
NAC	non-adherent mononuclear cells
PBS	phosphate buffered saline
PGE_2	prostaglandin E_2
SAC	staphylococcus A Cowan
TGFß	transforming growth factor ß
Th1	T-helper-1 lymphocyte
Th2	T-helper-2 lymphocyte
TNFα	tumor necrosis factor α
UC	ulcerative colitis