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New Developments in Asthma Research

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Contents

Preface

Asthma is a disease that affects the lungs. It causes repeated episodes of wheezing, breathlessness, chest tightness, and nighttime or early morning coughing. Tens of millions of people throughout the world suffer from asthma. Airways are the paths that carry air to the lungs. As the air moves through the lungs, the airways become smaller, like branches of a tree. During an attack, the sides of the airways in the lungs become inflamed and swollen. Muscles around the airways tighten, and less air passes in and out of the lungs. Excess mucus forms in the airways, clogging them even more. The attack, also called an episode, can include coughing, chest tightness, wheezing, and trouble breathing. Environmental exposures, such as house dust mites and environmental tobacco smoke, are important triggers of an attack. This new book presents important new research on the causes on asthma as well as its diagnosis and treatment.

Chapter 1 explores the link with magnesium status and chronobiology which are well established. The quality of magnesium status influences directly the Biological Clock (BC) function, represented by the suprachiasmatic nuclei. Reversely, BC dysrythmias influence the magnesium status. Two types of magnesium deficits must be clearly distinguished: deficiency corresponding to an insufficient intake which can be corrected through mere nutritional Mg supplementation and depletion due to a dysregulation of the magnesium status which cannot be corrected through nutritional supplementation only, but requests the more or less specific correction of the dysregulation mechanisms. Both in clinical and in animal experiments, the dysregulation mechanisms of magnesium depletion associate a reduced magnesium intake with various types of stress including biological clock dysrythmias. The differentiation between Mg depletion forms with hyperfunction of BC (HBC) and forms with hypofunction of BC (hBC) is seminal and the main biological marker is melatonin (MT) production. We hypothesize that, magnesium depletion with HBC or hBC may be involved in chronopathological forms of asthma. Nocturnal asthma would be linked to HBC, represented by an increase in MT levels. The corresponding clinical forms associate diverse expressions of nervous hypoexcitability: depression, nocturnal cephalalgia (*i.e.* cluster headaches), dyssomnia mainly advanced sleep phase syndrome, some clinical forms of chronic fatigue syndrome and fibromyalgia. The main comorbidities are depression and/or asthenia. They take place during the night or the “bad” seasons (autumn and winter) when the sunshine is minimum. The corresponding chronopathological therapy relies on phototherapies with

sometimes additional psychoanaleptics. Conversely, asthma forms linked to hBC are less frequently studied and present a decrease in MT levels. They associate various signs of nervous hyperexcitability: anxiety, diurnal cephalalgia (mainly migraine), dyssomnia, mainly delayed sleep phase syndrome and some clinical forms of chronic fatigue syndrome and fibromyalgia. The treatment relies on diverse forms of “darkness therapy”, possibly with the help of some psycholeptics. Finally, the treatment of asthma involves the maintenance of conventional dosing schedule of anti-asthma drugs, a balanced magnesium intake and the appropriate treatment of the chronopathological disorders.

It has been suggested that consumption of fish and polyunsaturated fatty acids could have a protective effect against inflammation in the airways and the development of asthma and other allergic diseases. Ecological and temporal data suggest that dietary factors may have a role in recent increases in the prevalence of asthma. A possible contributing factor to the increased incidence of asthma in western societies may the consumption of a pro-inflammatory diet. In the typical Western diet, 20-25-fold more omega-6 fatty acids than omega-3 fatty acids are consumed, which causes the release of proinflammatory arachidonic acid metabolites (leukotrienes and prostanoids). Fish oils are a rich source of omega-3 polyunsaturated fatty acids (n-3 PUFA). The specific fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are homologues of the n-6 fatty acid, arachidonic acid. This chemistry provides for antagonism by n-3 PUFA of arachidonic acid metabolism to pro-inflammatory eicosanoids (4-series leukotrienes and 2-series prostanoids) and cytokines (tumor necrosis factor-α and interleukin 1-β), as well as production of less active n-3 eicosanoids (5-series leukotrienes and 3-series prostanoids). In addition, n-3 PUFA can suppress production of pro-inflammatory eicosanoids and cytokines. Chapter 2 analyzes the evidence for the protective effects of omega-3 fatty acids on the development of asthma. Alternative therapies for treatment that reduce the dose requirements of pharmacological interventions would be beneficial, and could potentially reduce the public health burden of this disease. While clinical data evaluating the effect of omega-3 fatty acid supplementation in asthma has been equivocal, it has recently been shown that fish oil supplementation, rich in n-3 PUFA, reduces airway narrowing, medication use and pro-inflammatory mediator generation in non-atopic elite athletes with exercise-induced bronchoconstriction. These findings are provocative and suggest that dietary n-3 PUFA supplementation may be a viable treatment modality and/or adjunct therapy in airway inflammation and for the development of asthma.

In about 10% of adult patients with asthma, aspirin and other nonsteroidal anti-inflammatory drugs precipitate attacks of dyspnoea as discussed in chapter 3. Aspirin challenge causes a significant rise in bronchoalveolar lavage fluid (BALF) levels of total Cys-LTs in the aspirin – induced asthma (AIA) subjects, but not in the aspirin – tolerant asthma (ATA) group. The rise in BALF cysteinyl leukotrienes (Cys-LTs) correlates significantly with the counts of bronchial mucosal eosinophils, but not with mast cells counts, confirming eosinophils as the predominant source of the Cys-LTs response to aspirin. Aspirin challenge is followed in AIA subjects by a dramatic rise in eosinophil counts within the BALF, suggesting migration of activated eosinophils into airway lumen. PGE_2 production by eosinophils or other cells has been suggested to act as a brake on leukotriene synthesis in all subjects. Removal of this brake by cyclooxygenase inhibitors triggers significant Cys-LTs

production only in AIA subject because they alone have high levels of overexpression of LTC_4 synthase. The relative lack of LTC_4 synthase expression precludes a detectable response in normal and ATA subjects.

Availability of LTC_4 synthase may be augmented by genetic up-regulation. LTC_4 synthase is present in eosinophils and mast cells. Expression of LTC_4 is variable, even in the same cell line. Recently, the genetic polymorphism of the 5' untranslated region of LTC_4 synthase has been described. It consists of two common alleles corresponding to the A-C transversion of nucleotide 444 upstream of the translation start. The C444 allele is twice as common in aspirin asthmatics as in normal controls or asthmatics not sensitive to aspirin. Patients with AIA have up-regulated LTC synthase mRNA expression in blood eosinophils, and increased gene transcripts are most pronounced in carriers of the C444 allele.

Eosinophil infiltration of airway tissue appears to be a central feature of AIA. The airway expression of interleukin-5, known to be involved in recruitment, activation, maturation and perpetuation of survival of eosinophils is markedly increased in AIA patients.

To conclude, aspirin may remove PGE_2 – dependent suppression in all subjects, but only in patients with AIA does increased bronchial expression of LTC_4 synthase allow marked overproduction of Cys-LTs leading to bronchoconstriction.

It is the aim of chapter 4 to elucidate the close and direct bi-directional communication between inflammation and local nervous system which controls airway function and is therefore relevant for symptoms of allergic asthma including inflammation, airway hyperreactivity and airway obstruction.

Allergic bronchial asthma comprises reversible bronchus obstruction based on a pathological airway hyperreactivity. This airway hyperreactivity is under close control of the lung's autonomic nervous system which predominantly consists of the non-adrenergic non-cholinergic (NANC) and the cholinergic nervous system. There is growing evidence that mediators emerging from local allergic airway inflammation directly modulate the plasticity of the autonomic neuronal network. Neurotrophins – especially Nerve Growth Factor and Brain-Derived Neurotrophic Factor – deriving from immune cells and epithelia directly affect sensory nerves of the NANC-system. In addition, eosinophilic products like major basic protein (MBP) were shown to enhance cholinergic nerve function representing the dominant bronchoconstrictory pathway by blocking inhibitory presynaptic M2 receptors. In animals models, the release of acetylcholine from postganglionic parasympathetic nerve terminals is under control of presynaptic muscarinergic M2 receptor.

Allergic airway diseases such as asthma and rhinitis are increasing in the westernised countries, presumably explained by several simultaneously acting mechanisms where environmental factors may play a significant role. Chapter 5 reviews attempts a quantitative evaluation of the adjuvant effects from chemicals and environmental pollutants on the occurrence of sensitisation and allergic airway diseases. Laboratory animal studies showed that tobacco smoke possessed adjuvant effect. In the westernised countries, smoking together with a high exposure to allergens at workplaces had been a dominating factor for development of sensitisation, accounting for 0-71% of the sensitised cases; and accounting for about half of the asthma cases. Smoking among adults and exposures to environmental allergens, which are considered lower than exposures to occupational allergens, also accounted for a substantial number of asthma cases (0- ~23%). Environmental tobacco smoke

(ETS) exposures in adult account for less of the asthma cases (0- ~11%). Smoking during pregnancy may explain about 2-8 % of the asthma cases in children, but the mechanism need not be through an adjuvant effect. ETS exposures in children may account for about 5%. Laboratory animal studies suggested that indoor pollutants, including house dust, surfactants and quaternary ammonium disinfectants may possess adjuvant effect. However, the epidemiological knowledge is currently too sparse to allow conclusions about a possible risk of these exposures in humans in the westernised countries. Although ozone and nitrogen dioxide had adjuvant effect in animal studies, epidemiological studies showed little effect in humans. Exposure to motor vehicle emission may account for about 0 - <10 % of the asthma cases in the westernised countries. Development of animal models allowing risk assessment of adjuvant effect of chemicals and pollutants should be highly acknowledged, as results from these models may be able to compensate for the lack of relevant epidemiological studies.

Chapter 6 examines respiratory allergic diseases (rhinitis, rhinosinusitis, bronchial asthma and its equivalents) which appear to be increasing in most countries and subjects living in urban and industrialized areas are more likely to experience respiratory allergic symptoms than those living in rural areas. This increase has been linked, among various factors, to air pollution, which is now an important public health hazard. Laboratory studies confirm the epidemiologic evidence that inhalation of some pollutants, either individually or in combination, adversely affect lung function in asthmatics. The most abundant air pollutants in urban areas with high levels of vehicle traffic are respirable particulate matter, nitrogen dioxide and ozone.

In particular ozone, respirable particulate matter and allergens impair lung function and lead to increased airway responsiveness and bronchial obstruction in predisposed subjects. However, besides acting as irritants, airborne pollutants can modulate the allergenicity of antigens carried by airborne particles. By attaching to the surface of pollen grains and of plant-derived paucimicronic particles, pollutants can modify the morphology of these antigen-carrying agents and alter their allergenic potential. In addition, by inducing airway inflammation, which increases airway epithelial permeability, pollutans overcome the mucosal barrier and so facilitate the allergen-induced inflammatory responses. Experimental studies have shown that diesel exhaust particulate (DEP) can modify the immune response in predisposed animals and humans. Indeed, DEP increases in vivo IgE and cytokine production at the human respiratory mucosa thereby inducing allergic inflammation of the respiratory airway and the subsequent development of clinical respiratory symptoms. All these results implicate DEP in the enhanced allergic inflammatory response Pollen allergy is a useful model with which to study of the relationship between air pollution and respiratory allergic diseases. It has been suggested that air pollutants promote airway sensitisation by modulating the allergenicity of airborne allergens. Furthermore, airway mucosal damage induced by air pollution may facilitate the access of inhaled allergens to the cells of the immune system. Several factors can influence this interaction: type of air pollutant, plant species, climatic factors, degree of airway sensitisation and hyperresponsiveness of exposed subjects However, the role of climatic factors such as barometric pressure, temperature and humidity in triggering and/or exacerbating respiratory allergic symptoms is still poorly understood.

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Chapter I

Chronopathological Forms of Asthma due to Magnesium Depletion with Hypo- or Hyper-Function of the Biological Clock: Therapeutic Implications

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ABSTRACT

Asthma is a chronic, inflammatory disorder of the airways leading to airflow limitation. Its worldwide rise, mainly in developed countries, is a matter of worldwide concern. Inflammation of the bronchial mucosa and bronchial hyperresponsiveness are the hallmark features of asthma of all severities. Nocturnal asthma (NA) frequently occurs and would concern two thirds of asthmatics. But, it remains controversial whether NA is a distinct entity or is a manifestation of more severe asthma. Generally, it is considered as an exacerbation of the underlying pathology. The pathological mechanisms

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likely involve endogenous circadian rhythms with pathological consequences on both respiratory inflammation and hyperresponsiveness. A decrease in blood and tissue magnesium levels is frequently reported in asthma and often testifies to a true magnesium depletion.

The link with magnesium status and chronobiology are well established. The quality of magnesium status influences directly the Biological Clock (BC) function, represented by the suprachiasmatic nuclei. Reversely, BC dysrythmias influence the magnesium status. Two types of magnesium deficits must be clearly distinguished: deficiency corresponding to an insufficient intake which can be corrected through mere nutritional Mg supplementation and depletion due to a dysregulation of the magnesium status which cannot be corrected through nutritional supplementation only, but requests the more or less specific correction of the dysregulation mechanisms. Both in clinical and in animal experiments, the dysregulation mechanisms of magnesium depletion associate a reduced magnesium intake with various types of stress including biological clock dysrythmias. The differentiation between Mg depletion forms with hyperfunction of BC (HBC) and forms with hypofunction of BC (hBC) is seminal and the main biological marker is melatonin (MT) production. We hypothesize that, magnesium depletion with HBC or hBC may be involved in chronopathological forms of asthma. Nocturnal asthma would be linked to HBC, represented by an increase in MT levels. The corresponding clinical forms associate diverse expressions of nervous hypoexcitability: depression, nocturnal cephalalgia (*i.e.* cluster headaches), dyssomnia mainly advanced sleep phase syndrome, some clinical forms of chronic fatigue syndrome and fibromyalgia. The main comorbidities are depression and/or asthenia. They take place during the night or the "bad" seasons (autumn and winter) when the sunshine is minimum. The corresponding chronopathological therapy relies on phototherapies with sometimes additional psychoanaleptics. Conversely, asthma forms linked to hBC are less frequently studied and present a decrease in MT levels. They associate various signs of nervous hyperexcitability: anxiety, diurnal cephalalgia (mainly migraine), dyssomnia, mainly delayed sleep phase syndrome and some clinical forms of chronic fatigue syndrome and fibromyalgia. The treatment relies on diverse forms of "darkness therapy", possibly with the help of some psycholeptics. Finally, the treatment of asthma involves the maintenance of conventional dosing schedule of anti-asthma drugs, a balanced magnesium intake and the appropriate treatment of the chronopathological disorders.

Key words: Asthma, Magnesium deficiency, Magnesium depletion, Hyperfunction of the biological clock (HBC), Hypofunction of the biological clock (hBC), Melatonin, Phototherapy, Darkness Therapy

MAIN ABBREVIATIONS

NA = nocturnal asthma
NNA = non nocturnal asthma
COPD = Chronic obstructive pulmonary disease
FEV_1 = forced expiratory volume in one second
PEF = Peak expiratory flow
BC = biological clock
SCN = suprachiasmatic nuclei

MT = melatonin
HBC = hyperfunction of the biological clock
NhE = neural hypo excitability
hBC = hypofunction of the biological clock
NHE = nervous hyperexcitability

INTRODUCTION

Asthma is an important health priority worldwide because of its high and increasing prevalence, its high morbidity and mortality despite effective treatment and innovative research developments and its direct or indirect costs [166, 336]. It affects approximately 5-12% of the population and is a frequent cause of emergency hospital admission [34, 135]. It is the most common inflammatory chronic disease in childhood [48, 155, 166, 258, 268, 302]. A number of distinct mechanisms underlie the development of this disorder [54]. The National Heart, Lung and Blood Institute has stratified asthma severity and distinguished between mild intermittent asthma and three categories of persistent asthma, mild, moderate and severe, including in this characterization the frequency of nocturnal awakenings [318]. While many asthma attacks are relatively mild and can be treated and controlled at home, some are more severe and may even require hospitalization.

The striking increase in prevalence and severity of asthma over recent decades in affluent societies and the rarity of this disease in less affluent populations confirms the importance of environmental factors in the cause of asthma, although which environmental factors are responsible is still not clear [86, 226]. Accumulating evidence points towards an important role of diet, obesity and gastrooesophageal reflux in determining the expression of the disease [17, 18, 155, 360]. Family studies show that genetic factors are also important in determining individual susceptibility to asthma [86, 340]. Finally, the role of psychological factors in the development and exacerbation of asthma, as well as in the precipitation and provocation of asthma attacks, is known [206, 366].

Asthma is a clinical syndrome consisting of chronic airway inflammation, airway hyperresponsiveness, and expiratory airflow limitation with recurring episodes of wheezing, dyspnea, tightness in the chest, and cough that reverses after bronchodilator treatment [318, 332]. The prognosis for asthma depends on the levels of obstruction and bronchial hyperresponsiveness [41, 302].

It has long been recognized that asthma presents a diurnal rhythm in the occurrence and severity of symptoms with nocturnal worsening between 4 AM and 8 AM [67, 224]. Up to 74% of asthmatics awaken at night at least once a week due to wheezing, chest tightness or cough [350]. This nocturnal aggravation appears to be related to an exaggerated response to a circadian rhythm in lung function observed in healthy individuals [67]. It is generally admitted that it would result from both several circadian rhythms and fading effect of medication administered at bedtime [30]. Nocturnal asthma (NA) indicate severe asthma and asthma deaths generally occurs between midnight and 8 AM [39]. However, it remains controversial whether NA is a distinct entity or is a manifestation of more severe asthma [53].

We showed recently that different manifestations linked to chronopathological forms of magnesium depletion were regularly observed in various, rather common pathologies including migraine, sudden infant death, multiple sclerosis or affective disorders [95, 96, 98, 99, 100, 102] that greatly improved from treatments based upon these chronobiological data [94]. For instance, photic cephalalgia (mainly migraine) often related to magnesium depletion with hypofunction of the BC may greatly improved after treatment correcting both magnesium imbalance and the chronobiological disorder by darkness therapies in addition to conventional treatments. At the opposite, seasonal affective disorder (SAD) or winter depression may be related to magnesium depletion with hyperfunction of the BC. Its treatment relies on correcting both magnesium imbalance and chronobiological disorder by light therapies [100, 102].

Two types of magnesium deficits exist: Magnesium *deficiency* corresponds to an insufficient intake which can be corrected through mere nutritional Mg supplementation whereas Mg *depletion* due to a dysregulation of the magnesium status cannot be corrected through nutritional supplementation only, and requests the more or less specific correction of the dysregulation mechanisms. *Depletion* is frequently due to the association of a reduced magnesium intake with various types of stress including biological clock dysrhythmias. The differentiation between Mg depletion forms with hyperfunction and forms with hypofunction of the Biological Clock (BC) is seminal and the main biological marker is melatonin (MT).

We hypothesize hereafter that some forms of nocturnal asthma (NA) would be linked to a Hyperfunction of the Biological Clock (HBC) where magnesium status could be implicated. We would show that some other forms of asthma among non-nocturnal asthma patients (NNA) could be, on the contrary, linked to a magnesium depletion with hypofunction of the Biological Clock (hBC). It may be assumed that all these asthma patients must be treated with the same conventional asthma treatment, the same balanced magnesium intake but will benefit from either light or darkness therapies according to their asthma chronobiological phenotype.

The aim of the present study is to consider (i) the frequency of magnesium depletion in asthma (ii) the two opposite forms of associated chronopathological disorders *i.e* hyper-function (HBC) or hypo-function (hBC) of the biological clock (BC) (iii) the interaction between magnesium and the various treatments with light (phototherapies) or dark ("darkness therapies") therapies on the chronopathological forms of magnesium depletion.

I. Magnesium Deficit in Asthma

Magnesium has been implicated in respiratory diseases although conclusions were too often drawn from effects of parenteral high pharmacological $MgSO_4$ doses [202, 325]. Its well-known muscle relaxing effect induce a reduction of bronchospasm and an increase in airways diameter [34, 36, 59, 72, 88, 142, 144, 150, 151, 170, 218, 245, 254, 256, 282, 283, 303, 311, 319, 325]. It relaxes bronchial smooth muscle *in vitro* [325] and bronchodilate asthmatic airways *in vivo* thus improving lung function in human patients [254, 319]. Potential mechanisms of the smooth muscle relaxation induced by magnesium **pharmacological doses** may be linked to (i) direct relaxation of bronchial smooth muscle,

[325] (ii) calcium channels blocking properties together with an activation of adenylate cyclase, both leading to inhibition of myosine kinase resulting in myorelaxation [47, 74, 303], (iii) inhibition of cholinergic neuromuscular transmission with decreased sensibility to depolarizing action of acetylcholine [79], (iv) increased beta receptor affinity favoring the effects of beta-2 mimetics [106, 285], (v) stabilization of mast cells and T-lymphocytes (vi) stimulation of nitric oxide and prostacyclin generation. Some of these effects may be responsible for the anti-inflammatory properties of magnesium [26, 47, 85, 151, 161, 243, 319]. In addition, magnesium favors many pulmonary immunological defense mechanisms [26] and intervenes in melatonin regulation [98, 102] (cf I.4).

I.1. Hypomagnesemia in Asthma

Relevant epidemiologic studies showed that plasma magnesium concentrations in asthmatics from various countries are generally lower compared to healthy controls [11, 17, 18, 257]. Serum total magnesium level under 0.74 mmol/L is almost always associated with more severe asthma and more hospitalizations while patients with mild or moderate asthma may have normal magnesium levels (0.82 ± 0.08 mmol/L) [11, 373]. Multiple regression analysis showed that severe asthma is the only factor associated significantly with hypomagnaesemia [11]. No effect is observed in chronic asthma for inhaled beta-agonist, inhaled steroid or theophylline therapy on serum Mg level [11]. No alteration in serum Mg level was observed during asthmatic attacks [110, 175] or histamine and methacholine challenge [85, 373].

I.2. Other Magnesium Disturbances in Asthma

A urinary magnesium significant loss (6.81 ± 3.9 vs. 2.79 ± 1.39 mmol/day, $p = 0.01$) was observed in placebo-treated persistent moderate asthmatic children [25] or babies suffering from bronchial obstructive bronchitis [26].

Tissue magnesium may also decrease, as shown by reduced erythrocyte levels during asthmatic attacks or histamine and methacholine challenge [85, 104, 373] which normalized in the symptom-free period [111].

Stable asthmatics have a low skeletal magnesium content which reveals the Mg deficiency in asthmatics [146]. This deficit in body stores is revealed by the parenteral loading test in some patients with stable bronchial asthma: the ratio of magnesium retention to urinary excretion and bronchial reactivity to inhaled methacholine is significantly inversely correlated with the erythrocyte magnesium level [149].

I.3. The Two Forms of Magnesium Deficits in Asthma

Hypomagnesemia has been implicated in chronic asthma through mechanisms involving modulation of inflammatory processes [52, 104] and regulation of bronchomotor tone [254,

319]. The magnesium deficit can be characteristic of an insufficient magnesium intake and of alterations in magnesium retention mechanisms as well. In addition, beta-2 agonists which are the first line of asthma therapy and theophylline can stimulate magnesium efflux in peripheral tissues [165, 177] leading to an aggravated magnesium deficit of the cells [25].

The various biological markers of magnesium deficit may not be due to magnesium deficiency, but testify to a clinical form of magnesium **depletion**. We have highlighted the possible importance of several types of magnesium depletion in the aetiopathogenesis of diverse diseases, particularly of magnesium depletion caused by the association between an insufficient intake of magnesium and a chronopathological stress [95, 96, 98, 99, 102].

1.3.1. Magnesium Deficiency

Magnesium d*eficiency* is linked to an insufficient intake and may be corrected through a physiological nutritional oral magnesium supplementation, over a long period of time. It is noteworthy that chronic magnesium deficiency in human beings is frequent. On all the continents a large part of the population has a dietary intake lower than the recommended dietary allowances (RDAs) for magnesium. The RDA for magnesium intake is 350 mg/day for an adult male, 280 mg/day for a female and 10-13 mg/kg/day for growing children [250]. The magnesium requirement of almost all healthy adults is 6 mg/kg/day [97]. In France 23% of women and 18% of men have dietary magnesium intakes lower than the 2/3 of the RDAs for Mg [42, 94, 97, 158, 294, 374].

A pharmacological magnesium therapy involving in clinical practice mainly oral or intravenous high doses of magnesium sulfate are completely inappropriate in that indication. It is reserved to some indications generally in emergency situations. These two magnesium treatments are basically different in nature and action. The first one is devoid of any toxicity. The second, causes a iatrogenic magnesium load, whatever the magnesium status, and may induce magnesium toxicity. It is a real scientific fraud and an ethical misconduct to fail to differentiate between the safety of a nutritional physiological oral magnesium supplementation and the potentially dangerous effects of high pharmacological doses. But this basic distinction between the two types of magnesium treatments is too often overlooked in papers on magnesium therapy.

Magnesium deficiency in asthma: Poor magnesium intake is associated with impairment of pulmonary function, objectived by a decrease in forced expiratory volume in one second (FEV_1) and a higher risk of both wheezing and airway hyperreactivity. Consequently, individuals with a low Mg intake may be at increasing risk of developing asthma or a chronic airflow obstruction [322]. A suboptimal intake of dietary nutrients such as Mg was recently recognized to be a potential risk factor for asthma, especially in childhood [18, 155]. The decrease in dietary Mg intake could be, at least in part, the reason of the increasing allergic diseases.

Atoxic nutritional magnesium therapy may palliate the coexistent magnesium deficiency. A beneficial effect of magnesium on lung function, airway reactivity or wheeze was observed in two observational studies [42, 158, 322] but not confirmed in others [50, 320]. These conflicting results could be attributable to the fact that supplementation is only effective in magnesium deficiency whereas it would be without effect in magnesium depletion [98].

Pharmacological magnesium treatment for chronic obstructive pulmonary diseases or asthma is not very efficient and may be potentially hazardous in that indication [98].

To sum up: Magnesium deficiency may be considered as an adjuvant nutritional disorder in asthma but asthma *per se* does not only depends on the deficiency

1.3.2. Magnesium depletion

Magnesium d*epletion* is due to a dysregulation of the magnesium status which cannot be corrected through nutritional supplementation only, but requires the most specific correction of the dysregulation mechanism. There exists as many clinical forms of magnesium depletion as many possibilities of the dysregulation of the magnesium status. But both in clinical therapeutics and in animal experiment, the dysregulation mechanisms of magnesium depletion frequently associates reduced magnesium intake with various types of stress [94, 95, 98, 102]. Among these, dysrhythmias by dysregulation of the Biological Clock must be considered.

Magnesium depletion in asthma: Many disturbances of magnesium levels clearly indicate magnesium deficit in asthmatic patients. Serum (or plasma) and erythrocyte magnesium are usually normal, but in both **severe** or **acute asthma,** lower erythrocyte magnesium is often reported while magnesium plasma remains unchanged. Decreases in Mg levels in both polymorphonuclear cells and muscles can be observed. Magnesium depletion in asthma may result from the coexistence of major magnesium deficits together with a chronobiological dysrythmia of the biological clock [11, 78, 84, 88, 90, 91, 94, 95, 104, 110, 111, 271, 284, 374).

I.4. Links between Magnesium Status and Chronobiology

Circadian rhythms are endogenously generated by the biological clock (BC) represented by the suprachiasmatic nuclei (SCN) of the anterior hypothalamus. The links with magnesium status and chronobiology are well established [98]. The quality of magnesium status influences directly the Biological Clock function.. Reversely, BC dysrythmias influence the magnesium status. A close relationship exists between BC and the magnesium status.

1. Magnesium from physiological to pharmacological concentrations can directly enhance melatonin secretion by stimulating serotonin N-acetyltransferase, the magnesium key enzyme for synthesis of melatonin (MT) and to enhance indirectly the production of MT through an increased activity of the SCN [91, 240, 241].
2. Magnesium deficiency may decrease MT production and SCN function [91, 92].
3. MT can decrease magnesemia through its effects on Mg distribution [73, 91].

Consequently it obviously appears that chronobiology and nutritional magnesium intake interact with a possible central magnesium regulation. MT production is controlled both by photoperiod and magnesium status. Light, through SCN, decreases MT production, darkness having the opposite effect [244, 276-278, 346-348]. Mg deficiency decreases MT production whereas Mg overload stimulates it [91, 96]. It could be assumed that a balanced magnesium

status might be necessary for an optimal MT function and a darkness therapy effectiveness. Reversely MT might potentiate the effects of Mg therapy [3, 61, 96, 108, 112]. We will develop the clinical forms of magnesium depletion associating Mg deficiency with dysfunction of the Biological Clock (BC), either hyper- (HBC) or hypo-function (hBC) in asthma. Finally, the interaction between magnesium and the various treatments with light or darkness therapies on the chronopathological forms of magnesium depletion will be discussed.

II. CHRONOPATHOLOGICAL FORMS OF ASTHMA

II.1. Hyperfunction of the Biological Clock (HBC) in Asthma

HBC may be due to either **primary** disorders of BC (SCN and pineal gland) or **secondary** with an increased homeostatic response in the case of hyposensitivity to inducing light. This means that the biological clock behaves in homeostatic hyperfunction resulting in **Nervous hypoExcitability** (NhE). The corresponding clinical forms associate diverse expressions of NhE: depression, nocturnal cephalalgia without photophobia (*i.e.* cluster headaches), dyssomnia mainly advanced sleep phase syndrome (ASPS), some clinical forms of chronic fatigue syndrome and fibromyalgia. The main comorbidities are depression and/or asthenia. They take place during the night or the "bad" seasons (autumn and winter) when the sunshine is minimum. The therapy relies on classical asthma treatment, a balanced magnesium intake and sometimes additional psychoanaleptics and adequate chronopathological therapy [98-100, 102].

II.1.1. Clinical Form of Asthma with HBC: Nocturnal Asthma

Lung function in a healthy individual varies in a circadian rhythm, with peak lung function occurring near 4:00 PM (1600 hours) and minimal lung function occurring near 4:00 AM (0400 hours). An episode of NA is characterized by an exaggeration in this normal variation in lung function from daytime to nighttime, with diurnal changes in pulmonary function generally higher than 15%. There is also a circadian variation in bronchial hyperresponsiveness with an eightfold increase in bronchial reactivity overnight as opposed to a twofold increase in non nocturnal asthma (NNA) [227, 318]. Approximately, 75% of asthmatics suffering from nocturnal symptoms awakened one night per week, 64% three nights per week and 39% every night [350]. The occurrence of NA is associated with increased morbidity and inadequate asthma control, and has an important negative impact on quality of life [55, 225]. But, it remains controversial whether NA is a distinct entity or is a manifestation of more severe asthma [55]. According to the National Heart, Lung and Blood Institute, NA is a variable exacerbation of the underlying asthma condition associated with increases in symptoms, need for medication, airway responsiveness, and/or worsening of lung function [224]. The distal lung units, specifically the collateral channels, may be selectively altered at night in NA, possibly because of smooth muscle contraction, inflammation and/or oedema [192].

The mechanisms by which nocturnal asthma develop remain unclear and may vary from patient to patient [55]. Several factors may contribute to NA (allergen exposure in bed, supine position, interruption of the bronchodilator therapy, gastro-oesophageal reflux, tenseness of the airways and secretion accumulation) but they do not constitute a general concept for the explanation of nightly exacerbation [221].

In patients with NA, circadian variations in airflow limitation are seen, with decreases in peak expiratory flow rate (PEF) and forced expiratory volume in one second (FEV_1). According to Sutherland et al. [332, 333], in this asthma phenotype, the circadian worsening in NA is associated with increased airway inflammation [226] increased airways responsiveness [227], and worsened airflow limitation [355]. Aggravation of dyspnoea at night, reduction of PEF when awaking [197, 354], bronchostriction mainly during rapid eye movement [310] have been reported. Many hormonal, neural, cellular and humoral factors show diurnal fluctuations which favour a constrictive bronchial response in the night [221]. The better marker that varies in a circadian rhythm is melatonin which increases during the nighttime (cf. II.1.2.1.). *In vitro* studies have shown exogenous melatonin to be pro-inflammatory in asthma but it is unknown whether endogenous melatonin levels are a controller of airway inflammation in nocturnal asthma [332]. Under physiological conditions, the melatonin level increases when the anti-inflammatory cortisol level is decreased. This suggests that melatonin levels may influence the secretion of hypothalamic-pituitary-adrenal axis thus inducing inflammation [184]. Other markers also show circadian fluctuations and correlate with the overnight decrement in lung function. For instance, nadirs in epinephrine and cortisol levels that occur in the body around 10 PM to 4 PM and elevated histamine and other mediator levels that occur between midnight and 4 AM play a major role in the worsening of asthma during the night [188]. The other circadian variations include alveolar tissue inflammation [189, 190], intrinsic adrenergic hormonal milieu changes [22], hypothalamic-pituitary-adrenal axis dysfunction [334], alterations of both the affinity and activity of glucocorticoid receptors [191] and ß-adrenergic receptors [349], increase in alveolar tissue CD4+ lymphocytes which play a pivotal role in eosinophil recruitment, increase in peripheral blood and alveolar eosinophil and macrophage number and function [22, 53, 118, 189, 190, 352.] (i) T-lymphocytes are though to play a major role in the pathogenesis of asthma by producing inflammatory cytokines and possibly chemokines allowing eosinophils to migrate from the blood through the vascular endothelium [122, 123, 190, 214, 332] (ii) Mast cells release after antigen activation TNF-α, arachidonic acid metabolites, proteases, histamine, serotonin and nitric oxide that contribute to cellular influx into the lung [229]. Interestingly, in agreement with our hypothesis, recent findings indicate that melatonin is also important in the control of cell recruitment from the bone marrow and the migration of the inflammatory cells to the lungs since pinealectomy in an experimental model of allergic airway inflammation in rats reduced the total cell number count in the lung and at the same time bone marrow proliferation that both returned to control levels after treatment with melatonin [229]. This could be related to the facts that T lymphocytes have cell surface G protein–linked and nuclear melatonin receptors [190]and macrophages and CD4+ lymphocytes have been proposed as major sites of melatonin's actions [129]. As a whole, melatonin is pro-inflammatory [332]. But, it is also reported that melatonin may also exert protective effects in inflammation as well as in other pathologies by stimulating several

anti-oxidative enzymes and by inhibiting the inducible nitric oxide synthase (iNOS) activity in neutrophils and macrophages responsible for the overproduction of nitric oxide (NO) in µM range in inflammation [248]. However, a recent study on 5 nocturnal asthma subjects showed an increase in exhaled NO, with a circadian variation. The peak of NO was reached at 4 PM, the time of best pulmonary function [127] whereas exhaled NO decreased during the night. The authors suggest that the significant decrease in exhaled NO may reflect an important chronobiological defect in NO production which in view of its bronchodilator action could play a role in nocturnal exacerbations of asthma [127]. If this hypothesis is true, then melatonin would also act in a negative fashion by inhibiting iNOS. (iii) Cell influx includes eosinophils, neutrophils and lymphocytes capable of secreting various inflammatory mediators that lead to subsequent tissue damage, bronchoconstriction and airway hyperreactivity. Airway neutrophilia has been reported in severe persistent asthma which is often associated with increased nocturnal symptoms [172]. These human peripheral blood mononuclear cells may also synthesize melatonin [115]. Finally, matrix metalloproteinase-9 (MMP-9) expression increased in nocturnal sputum of severe asthma patients compared with patients with mild asthma or normal subjects. MMP-9 are responsible for remodelling of the extracellular matrix (ECM) and may facilitate leukocytes migration through the ECM and between endothelial cells [230]. Interestingly, we reported also an increase in MMP-9 in magnesium-deficient mice [262].

All these chronobiological events promote nocturnal worsening of asthma and increased nocturnal deaths [336].

II.1.2. Characteristics of HBC in Asthma

II.1.2.1. Biological Characteristic (increase in the Melatonin)

The major biological characteristic is represented by **an increase in the melatonin levels** in various fluids, corresponding to the elective marker of the biological clock [98, 333]. Plasma melatonin measurement has long been the reference, but salivary melatonin measurement was shown as a reliable, sensitive and easy method to monitor changes in the circadian rhythms of melatonin [358, 375].

A 1 hour delay of peak serum melatonin levels was reported in NA [333]. In this NA phenotype alone, melatonin levels are negatively correlated with overnight change in FEV_1 suggesting a chronopathological mechanism of asthma, melatonin being, in our opinion, only a biological marker. However, many recent data must be taken into account (i) melatonin is also synthetized by several other tissues of the body including the immune system [115], (ii) it may have a role in modulating airway function, since melatonin receptors have been shown to be present in the lungs of experimental animals [264], (iii) it exhibits immunoenhancing properties by regulating cytokine production of immunocompetent cells [56] (iv) it affects asthma severity because it enhances allergic airway inflammation [229] and airway smooth muscle tone in animal models [272, 359]. When melatonin was added *in vitro* to peripheral blood mononuclear call samples collected from patients with NA and healthy subjects at 4 P.M. and 4 A.M., an inflammatory response was observed with increased production of interleukin-1, intereukin-6 and tumor necrosis factor alpha at both times. In NA patients, the

cytokine response could not be further stimulated at 4 A.M., as observed in non nocturnal asthma, suggesting chronic overstimulation *in vivo* [332].

Consistent with the concept of HBC is the common observation that pediatric active asthma abates when puberty occurs [251, 372], while melatonin levels decrease physiologically. This physiological decrease in melatonin corresponds to a real improvement of both NA prevalence and severity. Similarly, specific immunotherapy in children sensitized to pollens led to a significant improvement of their symptoms at the end of treatment and to a decrease in both melatonin and beta endorphin levels. These results suggest the disappearance of the opioid-melatonin system stimulating influence on the immune system [130]. Asthma is one of the most common medical conditions that can complicate pregnancy [147]. Various stresses in pregnant women may convert a simple Mg deficiency into *Mg depletion* including environmental factors such as smoking, viruses, pollens but the role of chronopathological stress appears to be too often neglected [100]. The measurement of melatonin levels in pregnant women was never reported to our knowledge but aggravations were reported in 16-22% of mild asthmatics and in 83% of severe asthmatics, the majority of them improving after delivery [132, 327]. This suggest an increase in melatonin levels during pregnancy which would return to the previous situation after delivery.

A beneficial effect of light (or at least cessation of darkness) on lung function was observed in two observational studies. In an intractable nocturnal asthma woman, resistant to various bronchodilators and steroids, significant improvement of asthma was observed by awakening the patient quietly at 3 AM (thus interrupting darkness) before the melatonin peak with inhalation of 2.5 mg salbutamol [298]. Similarly, phototherapy and partial sleep deprivation lead to an improvement in 4 asthmatics [215, 216].

An immediate and long term efficacy of the high mountains climate (1560 m) was reported in various allergic diseases including asthma, involving insolation as an important factor of asthma improvement [105]. Conversely, clear and consistent seasonal patterns are observed for asthma hospitalizations with an autumn peak, when light decreases (and viral infections increase) and a summer trough when light is maximum in the northern hemisphere [70, 126, 238].

II.1.2.2. The Clinical Forms of Neurohypoexcitability (NhE) Resulting from HBC are Both Central and Peripheral

All the clinical forms **of NhE resulting from HBC** may coexist with the same chronobiological characteristics: nocturnal and hibernal pathologies, increase in melatonin levels, and clear improvement by light. The major comorbidity is represented by depressive states and asthenia. We called that type of persons “photophile” patients, in that they are clearly improved during the daylight and the “nice seasons” [98].

II.1.2.2.1. The Central Forms Associate Psychic, Algic and Hypnic Manifestations:

a) Depression whose main type corresponds to the seasonal affective disorder (SAD) or winter depression. Psychic asthenia (psychasthenia) would somewhat represent a minor form of SAD. An important comorbidity of depression with asthma (31-34 % of asthma patients) was shown in random and representative population samples, rather than in a clinical sample

[4, 135, 247]. Three specific symptoms, dyspnoea, wakening at night with asthma symptoms, and morning symptoms, are particularly strongly associated with depression. There was also a significant and clinically important impact on the quality of life of those who reported wakening at night, morning symptoms and dyspnoea. A link between asthma and respiratory disease and suicidal ideation and suicide attempts has been reported [139].

- **Sleep disturbances**. The changes which characterize NA have been reported not only to circadian events but also to sleep itself [224, 318]. The most **common sleep disturbances** among asthmatic patients were (i) obstructive sleep apnea representing a source of severe sleep fragmentation [28, 370] and (ii) advanced sleep phase syndrome with early morning awakening (51%), difficulty in maintaining sleep (44%) and daytime sleepiness (44%) [27, 35, 172].

In asthmatic shift workers, exacerbations took place during the daytime, when they slept. Circadian variation was intimately related to sleep (when melatonin increases) and virtually independent of solar time [66], but these results were repudiated later by the same group [154].

In a rat model of asthma, sleep (particularly rapid eye movement, REM) deprivation, reliably suppressed eosinophils in either the bronchoalveolar lavage fluid or the bronchial lamina propria, underlying the role of REM sleep in NA [167].

- **Cephalalgia** *without photophobia* (and even with photophilia) represents a nocturnal and hibernal disorder. It is the case of cephalalgia with obstructive sleep apnea periods and of cluster headaches [62]. The patient is healthier during the “nice” seasons [98, 202, 230].

II.1.2.2.2. The Peripheral Manifestations are Neuromuscular, Mainly Represented by Myalgia and Muscular Asthenia

Some clinical forms of the fibromyalgic syndrome with HBC associating to muscular troubles, depression, chronic fatigue syndrome, cephalalgia and dyssomnia may be a type of nervous hypoexcitability linked to HBC [152, 180, 183, 236, 274, 342].

In women with endometriosis, hypothyroidism, **fibromyalgia, chronic fatigue syndrome**, autoimmune diseases, allergies and **asthma** are significantly more common than in women in the general population [315]

II.2. Hypofunction of the Biological Clock in Asthma

II.2.1. Clinical Form of Asthma With hBC

Whereas nocturnal asthma gave rise to a great number of clinical and epidemiological studies, non nocturnal asthma (NNA) is rarely studied as a whole. We do not suggest that all the NNA are asthma with hBC but we hypothesize that among them, some forms may be related to hBC when they associate decreased melatonin levels and clinical symptoms of

nervous hyperexcitability (NHE) *i.e.* anxiety ranging from generalized anxiety to panic attacks, diurnal cephalalgia (mainly migraine), dyssomnia, such as delayed sleep phase syndrome, some clinical forms of chronic fatigue syndrome and of fibromyalgia [98, 102]. They may be due to either primary disorders of the BC or to a secondary homeostatic response to light hypersensitivity. The organism responds to the pathogenic effect of this light hypersensitivity by protective reactive *photophobia*, whose mechanism is still unclear [217]. The treatment relies on diverse forms of darkness therapies, possibly with the help of some psycholeptics [98, 102].

II.2.2. Characteristics of hBC in Asthma

II.2.2.1. Biological Characteristic

The main biological marker of hBC is *a decrease in melatonin* (or its metabolite) *levels* in various fluids [98, 102]. An important decrease in the 24-h mean level and amplitude of both plasma melatonin [184] and salivary melatonin [114] was observed in mild intermittent or persistent and moderate to severe asthma patients. Chronic glucocorticotherapy reduced activity of the pituitary adrenal axis and suppressed melatonin rhythm [184, 203].

The decrease in amplitude (difference between the low daytime melatonin and the higher level at night) observed in asthma patients might be related to the pathological state of asthma [114]. The underlying mechanism of the decrease in melatonin parameters is unknown. However, in stressed rats, increased corticosterone may have a direct effect on pinealocytes or melatonin is more rapidly metabolized during the stress [21, 224].

II.2.2. 2. Clinical Characteristics

The clinical characteristics of the secondary forms of chronobiological NHE are of circadian as well as of seasonal type: the symptomatology is mainly *diurnal* and observed *in spring and summer*, when *light hyperstimulation is obviously maximum* during daylight or during the fair seasons. The clinical forms of NHE are both central and peripheral [95, 98, 99, 102].

II.2.2.2.1. The Central Forms Associate Psychic, Algic and Hypnic Manifestations

a) Nervous hyperexcitability: Migraine and chronic respiratory inflammation like rhinitis, sinusitis, and asthma have been reported to be the most commonly seen disorders in chemical sensitivity patients [376]**.** A chemical odor intolerance was also reported that would indicate a phenomenon of dishabituation leading to generalization with both hypersensitivity to light and odors) [51]. Dishabituation is the contrary of habituation, a physiological phenomenon characterized by a more or less gradual decrease of the responses to repetitive stimuli of constant parameters [237]. Dishabituation, corresponds first to a decrease in habituation leading to a rapid recovery of the initial sensory reactivity and secondly may even lead to potentiation (or sensibilization) and sometimes to generalization involving other stimuli [237]. Dishabituation is often reported nowadays in pathological studies, such as photic cephalalgia (headaches with photophobia *i.e.* migraine).

A common background of these "dishabituated" patients is the presence of a magnesium depletion with hypofunction of the biological clock (hBC) [102]. Chemical odor intolerance and anxiety sensitivity in asthma patients were significant predictors of physical symptoms [51].

b) Cephalalgia mainly migraine: A frequent association between migraine and various allergic disorders have been reported [75, 213, 316]. Bronchial asthma is, like migraine, a paroxysmal disorder with attacks and symptom-free intervals which alter the quality of life [253]. In addition, both migraine and asthma are psychosomatic disorders [205, 366]. Finally, recent studies using anti-inflammatory drugs (montelukast, a leukotriene receptor antagonist or coxibs, inihibitors of cyclooxygenase) demonstrated consistent beneficial results in both asthma and migraine prevention [71, 77, 83, 117].

The prevalence of migraine is significantly higher in children with atopic disorders compared to those without [239]. Rhinitis in children was found to be associated with maternal migraine [145]. Among children whose mothers had neither migraine nor asthma/allergies, 3.2% had asthma while this incidence was found to be more than 6% for children whose mothers had migraine, but not asthma/allergies [60]. The risk of asthma among children born of women who had both migraine and asthma/allergies was greater than the risk associated with each maternal disease [316]. Headaches in adults were found to be more prevalent among those whose family members were reported to have allergy, asthma and migraine [160]. Genetic-epidemiological studies showed that migraine and asthma co-segregate in the family, indicating a possible common genetic background, involving some specific HLAs [60, 128, 222, 316]. The comorbidity asthma-migraine may rely on increased plasma levels of endothelin-1, a potent vasoconstrictor and a mediator in the inflammatory process (through matrix-metalloproteinase 9 (MMP-9) particularly [230, 323]. These disorders are inkeeping with the well-known similar disturbances due to magnesium deficit [99, 262].

c) Dyssomnia, mainly represented by delayed sleep phase syndrome. In asthma and COPD, the night sleep is delayed or shortened and deep sleep is often reduced or even absent [197]. A large study showed that asthma individuals are in addition at increased risk for complaints of difficulty with inducing sleep [224].

d) Anxiety. A strong and consistent link between asthma and anxiety disorders has been often reported. An important comorbidity of anxiety with asthma (40-53% of asthma patients) was shown in random and representative population samples and in clinical samples [4, 7, 135, 247, 260]. This relationships appear strongest among those with more severe disorders in terms of both asthma and anxiety disorders. The strongest links appear between lifetime severe asthma and generalized anxiety disorder (GAD), as well as panic attacks and panic disorder [5, 49, 58, 124, 133, 137-140, 247, 260, 269, 335, 357, 369]. An association between respiratory diseases and panic attacks was documented among adults [137, 260, 369] and youths [135, 260]. Several studies have also noted elevated rates of asthma among psychiatric inpatients and outpatients with anxiety disorders [44, 232].

II.2.2.2.2. Peripheral Manifestations

The ***central and peripheral manifestations*** are neuromuscular, mainly represented by photosensitive epilepsia, which may be either generalized or focal, authentified through EEG with intermittent light stimulation (ILS) with its corresponding form observed among TV viewers and video game players [99, 148, 265, 299]. Some migraine equivalents may be associated in this context.

Accessorily, the nervous form of chronopathological magnesium depletion with hBC may appear clinically as chronic fatigue syndrome (CFS) [93, 301] or as fibromyalgia [99, 363].

II.2.3. Indirect Evidences Suggesting the Possible Role of HBC in Asthma

Physiological and chronobiological factors for decrease in **melatonin production** are similarly deleterious factors for asthma with hBC.

a) For instance, in some mild or moderate asthma patients (about 12%) asthma improved during pregnancy. This results mirrors the worsening previously described in a majority of severe asthma with HBC and would indicate an increase in melatonin during pregnancy.

b) Aspirin sensitive asthma patients usually suffer from an active disease, despite the avoidance of aspirin and cross-reactive drugs, attributed to a decreased melatonin synthesis and an increased sensitivity of platelet to melatonin (and its metabolite) as compared to aspirin-tolerant asthma patients [109].

c) Diurnal, seasonal, climatic photostimulation must be at risk in those patients. A retrospective study, on a cohort of 108 cases of asthma death in 1-19-year-old in Denmark, showed that death occurred predominantly in summer in the 15-19-year age group [174]. The authors attributed the death to an insufficient medical survey. But, we suggest that the decrease in melatonin levels at puberty aggravated by light exposure in summer could be also involved. Increased visits to hospital were also reported during the wet season in Trinidad, i.e. during summer, when the sunlight is obviously important [168].

d) Finally both corticotherapy and hormone replacement therapy cause a decrease in daily melatonin secretion without disturbing circadian rhythm [185]. This data must be taken into account by clinicians in those forms of asthma with hBC.

To sum up: The frequency of asthma linked to Mg depletion with HBC or hBC is presently unknown. It may be assumed that a number of nocturnal asthma are probably related to magnesium depletion with HBC, and conversely that some forms of non nocturnal asthma are linked to magnesium depletion with hBC. The response would be brought by appropriate measurements of both magnesium and melatonin levels in various fluids or tissues.

III. TREATMENT OF ASTHMA WITH DYSFUNCTIONS OF BC

The two opposite chronobiological forms of asthma would benefit from the same pharmacologic asthma treatment, the same balanced magnesium intake but the right opposite treatments of the chronobiological disorders.

III.1. Conventional Pharmacological Treatment

Asthma management guidelines recommend the use of preventive medication in sufficient amounts to control asthma symptoms [353]. Nonadherence to treatment is often implicated in the aggravation of asthma. According to current US guidelines, nocturnal symptoms of asthma occurring more often than once weekly may indicate inadequate control of asthma [249].

III.1.1.General Considerations

The therapeutic agents used for the management of **chronic asthma** are mainly inhaled long-acting beta-2 agonists and steroids. **Acute exacerbations** can occur and are challenging to manage. Supplemental oxygen, repeated doses of inhaled beta-2 agonists and systemic corticosteroids (oral if tolerated or inhaled) [40, 121, 313] are the mainstay therapies used to relieve bronchospasm and airway obstruction. Because all patients do not respond to maximal therapy, other strategies either older (theophylline, magnesium) or more recent (heliox, leukotriene modifiers) are being evaluated [329].

Understanding the kinetics of the different drug preparations allowed most effective timing of dose [225, 318]. Chronopharmacology should optimize the desired effects of medications and minimize undesired ones in asthmatic patients [114, 321].

However, the treatment of asthma is not under the scope of the present paper and will not be developed hereafter, with exception of beta-2 agonists and magnesium. We would only point out some informations on current asthma agents which may be important in chronopathological asthma.

Briefly, **beta-2 agonists** which are detailed below (cf III.1.2) are the first line of asthma therapy, but their safety is debated [101]. The mortality rate in patients with acute severe asthma is still rising and has been partly attributed to their adverse effects [11].

The anti-inflammatory properties of **corticosteroids** make them reference for the treatment of **acute asthma**. All of them (including prednisone, methylprednisolone, hydrocortisone and dexamethasone) are efficient in acute asthma whatever the administration route (oral, intravenous or intramuscular) [14]. However, controlled trials found that a single dose of dexamethasone suppressed melatonin production in eleven healthy volunteers [80] or in asthma patients [185, 203]. These observations may be of clinical relevance in chronobiological asthma. Finally, *in vitro*, in peripheral blood mononuclear cell from patients with nocturnal asthma, or *in vivo*, in glucocorticoid-resistant asthma, a reduced responsiveness to corticosteroids at night, requiring an increase dosing has been reported. This resistance to the effects of steroids was attributed to an inhibition of glucocorticoid receptor (GR) linked to a circadian increased expression of GRbeta, an endogeneous inhibitor

of steroid action, mainly in macrophage [55, 120, 192, 208]. The dosing of corticosteroids in the morning optimally improve bronchial potency in asthma while the risks of adrenal suppression and of osteopenia observed with dosing at other times are significantly reduced or even suppressed [275]. A retrospective study showed that inhaled corticosteroid dispensing to adult asthmatics led to a reduced risk of intensive care unit admission for asthma, a surrogate for life threatening exacerbation (103). Finally, a large retrospective study showed that inhaled corticosteroids administered chronically and prudently within the recommended dose ranges do not endanger the functioning of the hypothalamic-pituitary-adrenal axis whereas the increasing tendency to use higher doses of inhaled corticosteroids is not supported by reliable published information [63].

Theophylline may have some interesting therapeutic effect, but given its toxicity profile, it is unclear whether it offers any advantage over maximal beta-2 agonist.

Ironically, different electrolyte disturbances are induced by acute asthma medications. Among them, hypomagnesemia which is attributed to an increased urinary magnesium excretion and/or to various indirect mechanisms including lipolysis and calcium redistribution [95] appears as a side effect of beta agonists, steroids and xanthines, used for the management of acute asthma [11, 37, 181, 284]. Hypomagnesemia, as the other electrolyte disturbances, may result in exacerbation of the overall condition. Consequently, nebulized beta-2 agonists and aminophylline which are the mainstay therapies for asthma exacerbation must be used carefully in subjects presenting abnormal electrolyte levels. In contrast to acute asthma, therapeutic agents used to treat patients with chronic asthma would not induce electrolyte disturbances [12]. In addition, the toxicity of theophylline, a phosphodiesterase inhibitor that lowers myocardial magnesium levels, is intensified by beta-adrenergic agonists and corticosteroids and may lead to severe nervous and cardiac effects with often fatal issue [309].

Newer therapies such as ventilation strategies with **heliox** (helium and oxygen) and intravenous **leukotriene modifiers** currently being evaluated may or may not prove to be beneficial in the future [329]. A substantial improvement has been observed with the combination of salbutamol and **ipratropium bromide** [280] and from the triple combination of salbutamol, ipratropium and flunosolide [47, 281].

The data evaluating the use of **magnesium** in asthmatic patients are scare and most are small trials or case reports. In addition the results are often conflicting (cf III.4.2.1.2.) In any case, our purpose is to focus mainly on beta-mimetics and magnesium, to take stoke on the possibility of magnesium therapies in asthma and to differentiate between cases where the therapeutic association of beta-2 mimetics and magnesium is beneficial and those where it is deleterious hence contraindicated.

III.1.2. Beta-2 Agonists

For acute asthma, repeated doses of nebulized beta-2 agonists and to a lesser extent IV aminophylline are the mainstays therapies used to relieve bronchospasm and airway obstruction [181, 365]. Beta-2 agonists are the first line of asthma therapy, but their safety is debated. Importantly, beta stimulation may have consequence on regulation of magnesium status. **Physiological** beta stimulation during magnesium deficiency may induce an homeostatic **increase** in magnesemia. In contrast, excessive beta stimulation, by use of

pharmacological high doses of beta-2 agonists, may induce a **decrease** in magnesemia which could be deleterious for asthmatic patients [101].

III.1.2.1. Nature and Action of Beta-2 Adrenergic Receptors

Adrenergic receptors are classified as alpha (α-1, α-2) and beta (β-1, β-2, β-3) according to their responses to diverse adrenergic stimulations. Generally, adrenergic stimulations have an excitatory effect on alpha receptors and an inhibitory effect on beta receptors [9]. Beta-1 receptors mainly concern the heart. Beta-2 receptors are implicated in smooth muscle relaxation in pulmonary, vascular and uterine apparatus particularly. Beta-3 receptors are the main beta adrenoreceptors in adipocytes with some distinctive links with magnesium status. Beta adrenergic receptors belong to the very large family of seven transmembrane domain-containing stimulatory G protein-coupled membrane receptors. They interact with guanine nucleotide regulatory proteins and magnesium dependent adenylate cyclase. Their activation increases the intra-cellular concentration of cyclic AMP (cAMP) which induces phosphorylation of many key proteins of muscle contraction through activation of a cAMP dependent-Protein Kinase A (PKA) [10, 87, 141, 164, 223, 304, 362]. cAMP induces myorelaxation directly by inhibiting myosine-kinase through phosphorylation by PKA and indirectly (i) by decreasing cellular free Ca^{2+} resulting from Ca^{2+} reuptake by the sarcoplasmic reticulum (ii) by activating K+ channels by phosphorylation thus provoking cell hyperpolarization and inhibition of calcium inflow [364].

A genetic variation in beta adrenergic receptors influencing both susceptibility for asthma and therapeutic response was reported recently [106]. Indeed, A/J inbred mice bound less dihydroalprenolol (beta-antagonist) than C57/BL/6J inbred mice in the absence but not in the presence of magnesium. The gene responsible for the Mg^{2+}-sensitive dihydroalprenolol binding was named “Badm” for beta-adrenergic magnesium effect.

Two main genetic variations in beta 2 receptor itself were reported in a group of asthma patients [273]. The more frequent polymorphism (arginine16→ glycine) identified a subset of asthmatic patients likely to be steroid-dependent and to require immunization therapy. This severe phenotype, frequent in nocturnal asthma patients, was found only in homozygous patients [349]. It corresponds to an increase in agonist-promoted down-regulation of beta-2 receptor expression [143, 273, 339] resulting in an inefficiency of beta-2 agonist treatment [187, 209, 228]. This mutation could have a role in NA [349]. Indeed, the beta2-adrenergic receptors in circulating white blood cells are down regulated at 4AM in patients with NA, which does not happens in normal subjects [337]. The other polyporphism (Glutamine 27→ glutamic acid) was resistant to agonist-promoted down-regulation of receptor expression [143] and was found to be associated with elevated levels of IgE in subjects from asthmatic families [82], supporting previous data relating increased levels of cAMP to increased IgE synthesis [113]. These studies allowed highly significant associations with a number of phenotypes related to asthma, including steroid dependence and bronchodilator responsiveness.

Beta adrenergic receptors play a major role in the regulation of the magnesium status since they can modify exchanges between intra-cellular and extra-cellular magnesium. Activation and modulation of beta-adrenergic receptors might intervene among the neurohormonal factors of the physiological regulation of magnesium status [37, 38, 89-91,

95, 102, 288-290, 177, 361]. Beta receptor physiological stimulation may induce hypermagnesemia through an efflux of magnesium out of the cell via a Na^+-dependent mechanism. But the regulating feedback mechanism of magnesium status through beta-adrenergic receptors may become ineffective when an excessive beta stimulation occurs. These last beta-adrenergic effects are coupled to **lipolysis which reduces magnesemia** mainly through (i) chelation of magnesium by non esterified fatty acids, (ii) increased magnesium uptake by adipocytes, and (iii) at least partly, enhanced urinary excretion of magnesium [37, 38, 89-91, 95, 102, 288-290, 177, 361].

To sum up, the **physiological beta stimulation** may be involved in the regulation of magnesium status by an homeostatic increase in magnesemia during magnesium deficiency. Reversely, **excessive beta stimulation by pharmacological high doses of beta 2 agonists** may induce a deleterious decrease in magnesemia. Reversely, magnesium homeostasis is required for beta receptor function .

III.1.2. 2. Beta-2 Agonists and Obstructive Disorders

The short acting beta-2 agonists (salbutamol, fenoterol, terbutaline, pirbuterol) are essential in emergency treatment of severe asthma and have an important prophylactic role in the prevention of exercise-induced bronchoconstriction. Different routes of administration may be used including inhalation, nebulisation, subcutaneous or intravenous injection. Inhaled beta-2 agonists are initially used. In absence of response, intravenous associated beta-2-agonists are generally useful. The therapeutic response should be evaluated mainly by using the peak expiratory flow (PEF) determination [199, 308].

Long acting beta-2-agonists (salmeterol, formeterol, bambuterol), used in inhalation or *per os*, have provided advantages on short acting beta-2 agonists such as prolonged bronchodilation, reduced diurnal and nocturnal symptoms, improved sleep quality and reduced requirement for short acting beta-2-agonists. When added to inhaled corticosteroids, they produce greater improvement in lung function than increased steroid dose alone [199, 308].

Their mechanism of action is pharmacodynamic. Through stimulation of beta-2 receptor, these drugs lead to bronchorelaxation either directly by enzymatic stimulation or indirectly through Ca^{2+} redistribution (cf above). These mechanisms agree with the beta-adrenergic theory of atopic abnormality in bronchial asthma [338] and with the « calcium hypothesis of asthma » [101, 136].

III.1.2. 3. Side Effects of Beta-2 Agonists

Little if any benefit seems to be derived from regular use of short acting beta-2 agonists. Regular or frequent use can increase the severity of the pathological status. There has been controversy about the possible relationship between use of beta-2 agonists and morbidity or mortality related to asthma and COPD. For instance, the relatively non beta-2 selective agonist, **fenoterol** doubled the risk of asthma [324]. However results from a cohort study including 12.301 patients suggested that increased asthma deaths and near-deaths would be better **a class effect of beta agonists** and would not be reduced to a specific molecule such as fenoterol [324]. Various authors pointed out the severity of asthma as a potential confounding factor. However, a stratified analysis utilizing markers of chronic asthma severity showed,

after adjustment by available markers of asthma severity, that the increased risk of death either persisted or disappeared. This discrepancy was attributed to differences in the populations studied [2, 23, 24, 107, 125, 207, 267, 324].

Beta-2 agonists used in COPD treatment can induce numerous side-effects including consequences on cardiac function. They increase heart rate, prolong the electrical action potential duration, induce abnormal myocardial repolarisation. They may cause hyperglycemia, hypokaliemia and hypomagnesaemia with low potassium and magnesium concentrations in skeletal muscles. These biochemical changes may induce at the cardiac level alterations of the conduction pathways, arrhythmias leading to an increased risk of cardiac death [29, 146, 207, 210, 331, 351].

To sum up, beta-2 adrenergic receptor agonists are first-line of asthma therapy but their safety is debated. Fixed combination seems particularly indicated for severe asthma. Free combination appears as first-line therapy for patients with mild to moderate asthma [2, 29, 146, 207, 210, 331].

III.2. Indirect Asthma Therapies

Eviction of allergens, psychotherapic and alternative therapies will be also considered.

III.2.1. Environmental Control Measures

Environmental control measures are essential and should focus on limiting the patient's exposure to allergens. Removal of pets from the bedroom, use of mattress and pillow covers, and carpet-free floors are some examples of helpful changes [318]. Food allergy and intolerance can have a major part to play . Identification and elimination of certain foods or additives can have a major benefit.

III.2.2. All the Pathological Entities Accompanying Asthma Should be Diagnosed and Treated Appropriately [318]

a) Allergic rhinitis should be treated with anti-inflammatory medications;
b) Obstructive sleep apnea syndrome and snoring may be improved by continuous positive airway pressure;
c) Classical pharmacotherapy using psychoanaleptics (HBC) or psycholeptics (hBC) may be successful.

III.2.3. Asthma Education Programs

Asthma education programs that teach about the nature of the disease, medications, and trigger avoidance tend to reduce asthma morbidity. Other promising psychological interventions as adjuncts to medical treatment include training in symptom perception, stress management, hypnose, yoga and several biofeedback-assisted relaxation and breathing exercises are beneficial for stress reduction in general and may be helful in further controlling asthma [169, 206]. The need for ongoing education of the patient's family, the patient and

doctors on long-term management and management of acute attacks has be underlined (Jorgensen et al, 2003).

III.2.4. Herbal Medicine

Herbal Medicine has been shown in a number of trials to be beneficial in the treatment of asthma [162]. Safe herbs such as Boswellia and Ginkgo may be used as adjuncts to comprehensive plan of care while staying alert for drug-herb interactions [32, 169].

III.2.5. Needle Acupuncture

Needle Acupuncture is also useful if used regularly. Initially weekly treatments are reduced to, possibly monthly, enabling reduction of conventional medication in many cases. [32, 169, 320].

III.2.6. Homeopathic Remedies

Homeopathic remedies based on extreme dilutions of the allergen may be beneficial in allergic rhinitis but requires collaboration with an experienced homeopath. But they have not been yet validated.

III.2.7. Diet Is Important.

Asthmatics may benefit from hydration and a diet low in sodium and in omega-6 fatty acids and transfatty acids, but high in omega-3 fatty acids, in antioxidant vitamins and magnesium [32, 169, 320].

III.3. Balanced Magnesium Intake

As previously reported, about half the asthma are accompanied by symptoms of latent tetany due to primary Mg deficiency. Reversely, the frequency of allergic antecedents is high in cases of neural forms of primary magnesium deficit (39%). Today the main form of magnesium therapy is oral physiological magnesium supplementation. These palliative nutritional magnesium doses needed to balance magnesium deficiency are obviously devoid of any toxicity since their purpose is to normalize the insufficient magnesium intake [90, 91, 95, 98-102, 134]. It evenly can cause mild side-effects like diarrhea and abdominal cramps [303].

A large epidemiological study carried out in 2633 subjects showed that a high dietary magnesium intake was associated with better lung function and reduced risk of airway hyperreactivity and wheezing [41, 42]. In another study on 20 asthmatics, it was associated with significant improvement of asthma symptom scores whereas FEV1, PEF variables or decrease in use of a bronchodilator was not improved. But, the duration of Mg supplementation may have been too short to detect any improvement in their pulmonary function [158]. A decrease in airway responsiveness was observed in hyperresponsive asthmatics after 6 weeks of nutritional Mg supplementation [24]. Long lasting Mg supplementation (200 mg/day to 7-year old and 290 mg/day to older children) was clearly of benefit in moderate asthma children and was recommended as a concomitant drug in stable

asthma [25]. As a whole, nutritional magnesium therapy for pulmonary obstructive diseases physiologically palliates the coexistent primary Mg deficiency. The atoxic adjuvant therapy is always beneficial without side effects [100, 101].

But, when different stress transform the Mg deficiency into Mg depletion related to a dysregulation of the control mechanisms of magnesium status, nutritional physiological magnesium supplementation alone is ineffective. Mg depletion needs not only a balanced Mg intake but also and mainly the correction of its causal dysregulation. In the case of chronobiological forms of asthma treatment must include either "phototherapies" or "darkness therapies".

III.4. Chronobiological Treatments

III.4.1. Asthma with HBC

The different forms of HBC may be treated by various **phototherapies**. It is obvious that in chronobiological asthma with HBC, supplemental over-the-counter melatonin must be carefully avoided since it is yet present in large excess .

III.4.1.1. Bright Light Phototherapy

As for the other diseases based upon Mg depletion with HBC, BLT may be beneficial in this clinical form of NA. Even though not yet evaluated in proper clinical trials, three studies reported on a small number of patients the beneficial effects of BLT in asthma [193, 215, 216]. Therapeutic effect of the method occurs because of correction of internal asynchronism, stimulation of endogeneous synthesis of corticosteroids and antidepressive action [216]. The aim of bright light phototherapy is to lengthen the photoperiod, the marker of its efficiency being the decrease in plasma MT. BLT protective effect may result not only from melatonin suppression but also from multiple other mechanisms *i.e.* depression of immune response with suppression of inflammatory leukotrienes and cytokines [98, 270].

Classically, bright light phototherapy used in seasonal affective disorder requires full spectrum light with an intensity higher than 2000 lux, the best timing being early morning, optimally about 8.5 hours after melatonin onset [341, 342]. Conventional therapy uses full spectrum light without infrared nor ultraviolet rays. The circadian resetting response in humans, as measured by the pineal melatonin rhythm, is wavelength dependent, the peak of sensitivity of the human circadian pacemaker to light being blue-shifted (460 nm) relative to the three cone visual photopic system, the sensitivity of which peaks at approximately 555 nm [211, 317]. Since the 1980s numerous studies have shown that light therapy has beneficial effects when applied in certain types of sleep and mood disorders [292, 343]. Early clinical studies exposed subjects to 2,500 lux for 2-6 hours daily. These lengthy daily treatments induced two serious difficulties: compliance to treatment and side effects (headaches or vision problems) in some users. Shorter exposure (30 min) to brighter 10,000 lux light therapy produced a 75% rate of improvement in SAD without increasing side effects [341, 343]. However the treatment must be applied for the whole winter duration, since SAD symptoms rapidly reappear after treatment has ceased [201]. If conventional BLT constitutes now the therapeutic tool considered as inseparably linked to SAD, it has been also used in non seasonal depressions, senile dementia and sleep disorders in the elderly. In those

indications, bright light therapy appears as a non specific antidepressant agent and constitutes a speedy and efficient adjuvant to antidepressant medication [194, 234]. Non migrainous headaches, without photophobia, may be also an indication for bright light therapy through this antidepressant action [102]. We consequently think that BLT must be efficient in asthma with HBC, by improving chronobiolocal dysfunction of the BC and consequently both biological and clinical consequences.

Bright light therapy is operative through various neural and perhaps humoral mechanisms. Today, the mean central neural mechanisms of phototherapy seem to be increased serotoninergy, hypoactivity of inhibitory modulators such as taurine and kappa opioid receptors, and finally stimulation of inflammatory and oxidative processes [98]. An evolutive perspective suggests that heme moieties and bile pigments in animals mediate some non visual influence of light upon neuroactive gases (including CO and NO) and upon biorythms [259] through humoral phototransduction. Bright light can break the carboxyhemoglobin (HbCO) bond releasing CO and stimulate nitric oxide synthase to produce NO. If one considers hemoglobin not only as a scavenger but also as a transporter, it may convey photic information to all tissues, notably the brain, through the neuroactive gases: CO and NO in blood [119, 198, 259, 356]. Bright light is also able to reduce circulating levels of bilirubin and biliverdin, thus removing their vasoconstrictive and sedative effects [259]. The fall in bile pigments may result *in vivo* from the absorption of photons by the photo-sensitizer riboflavin [186] and from an effect of light on plasma albumin [6, 255].

III.4.1.2. Chromatotherapy

According to Agrapart's theory, the physical energy brought by one wavelength could act like the energy brought by the corresponding oligoelement [7, 8]. For instance, purple for 4-8 minutes would bring the same energy as magnesium ions. Chromatotherapy uses a short exposure to a specific wavelength once a week and like other energetic therapies carefully takes into account the nocturnal or diurnal prevalence of clinical symptoms. In asthma with nocturnal prevalence, purple irradiation of the chest for 4 minutes followed by 20 min of darkness once a week would be beneficial. More specific treatment of asthma using chromatotherapy on acupuncture points would give better result but may be used only by specialists. Even though successfully used in clinical practice, this method has not yet been validated [98, 102]. However, we could show the neuroprotective effects of one wavelength used in chromatotherapia on a validated neuropharmacological nutritional model, in DBA2 mice [261, 263].

III.4.1.3. Low Power Laser Biostimulation

A prospective analysis including 50 asthmatics showed that daily laser irradiation of acupuncture points for 10 days lead to a significant improvement of bronchial asthma which was achieved in a short time and last for several weeks, even months [235].

III.4.1.4. Pharmacotherapy of the Clinical Manifestations of HBC

The many studies demonstrating the efficacy of pharmacotherapy in mood disorders have supported the use of conventional, first-line antidepressant pharmacotherapy (i.e.

amitryptilline, fluoxetin, d-fenfluramine) [297, 343]. Analeptics such as psychostimulants (including caffeine) have also been found effective in other diseases with HBC [98, 102].

III.4.2. Asthma with hBC

The best physiologic stimulation of the BC is induced by light deprivation.

III.4.2.1. Stimulating "Darkness Therapies":

They may be physiologic, psychotherapic, physiotherapic or pharmacologic.

III.4.2.1.1. Physiological Darkness Therapies:

Darkness therapy per se and chromatotherapy

III.4.2.1.1.1. Darkness Therapy Per Se

Light deprivation may be obtained by placing the patient in a closed room, in a totally dark environment, with an eye mask on. This **genuine darkness therapy** may be used in acute indications, but should be of short duration. It is not compatible with any activity and is frequently associated with induction of bed rest, inactivity and sleep [13, 98].

Relative darkness therapy may be obtained by wearing dark goggles or dark sun glasses but the number of lux passing through is not negligible. This relative darkness therapy may be used as an accessory treatment in the restoration of a light dark schedule: a transition before a totally dark environment [98, 259].

III.4.2.1.1.2. Chromatotherapy

The diurnal forms of asthma may be benefit from a 4-min exposure of the chest once a week to yellow wavelength, the complementary color of purple indicated in the treatment of asthma with HBC. It must be followed by 20 minutes of darkness. Chromatotherapy on acupuncture points would be even more efficient. This method, although successfully used in practice, has not been validated yet [7, 8, 98-102, 104].

III.4.2.1.2. Psychotherapeutic Darkness Therapies

Asthma education programs are important **(**cf. III.2.3.). Cognitive behavioral strategies have been efficient for the treatment of photosensitivity. The treatment was to gradually increase exposure to computer monitor and television screen photostimulation. This desensitization procedure resulted in a complete removal of the patient's phobic anxiety from photostimulation and of avoidant behavior. This behavioral therapy has been used in photo-sensitive epilepsy and in migraine [204, 252]. Psychological therapies of migraine in childhood, such as relaxation training and biofeedback, were potentially superior to pharmacological treatment [153, 316].

III.4.2.1.3. Physiotherapic Darkness Therapy

Magnetic fields may be used to stimulate the BC in a variety of ways to treatment using very weak (picotesla), extremely low frequency (2 to 7 Hz) electromagnetic fields.

Transcranial treatment with alternative currents pulsed electromagnetic fields of picotesla flux density may stimulate various brain areas (hypothalamus particularly) and the pineal gland (which functions as a magneto-receptor). Clinical studies showed an improvement in both FEV1, PEF and other variables of lung function by pulsatile electromagnetic fields in both asthma children and in adults with asthma or COPD [295, 296].

III.4.2.1.4. Pharmacological Darkness Therapy

Three agents may stimulate the BC: magnesium, L-tryptophan and taurine but their efficiency seems limited.

III.4.2.1.4.1. Magnesium Treatment for Obstructive Disorders: A Reappraisal

As previously reported in this paper, two different types of magnesium therapy must be distinguished: nutritional physiological oral magnesium supplementation (cf III.3) and pharmacological magnesium therapy. Their nature and action are basically different. It is a real scientific fraud and an ethical misconduct to fail to differentiate between the safety of a nutritional physiological oral magnesium supplementation and the potentially dangerous effects of high pharmacological doses [90, 91, 95]. But this basic distinction between the two types of magnesium treatments is too often overlooked in papers on magnesium therapy. To discriminate between the two types of magnesium therapy it is necessary to kept in mind that the only indication for nutritional magnesium therapy is the disorder related to **magnesium deficiency** *i.e* to an insufficient magnesium intake, whereas pharmacological magnesium therapy is indicated whatever the magnesium status.

III.4.2.1.4.1.1. Pharmacological Magnesium Therapy

Pharmacodynamic effects of pharmacological magnesium therapy in obstructive pulmonary disorders are mainly **bronchodilatation** and antiinflammatory properties. In order to use the pharmacological properties of magnesium, *whatever the magnesium status*, it is necessary to over pass the magnesium homeostasis mechanisms and to induce a therapeutic magnesium overload *i.e.* a genuine iatrogenic hypermagnesemia. The parenteral route is suitable for acute applications whereas large doses of magnesium orally given are advisable for chronic indications. Both types of pharmacological magnesium treatments may induce magnesium toxicity [90, 91, 95, 102]. Early signs of Mg toxicity during intravenous treatment include vomiting, nausea, feeling of warmth, flushing, hypotension, bradycardia and other cardiac arrythmias, somnolence, double vision, slurred speech and weakness [303]. These side effects usually occur at total plasma Mg of 3.5-5 mmol/l. Hyporeflexia (loss of patella reflex), muscular paralysis, respiratory or cardiac arrest develop only at extremely high plasma Mg concentration (5-15 mmol/l). Magnesium toxicity is exaggerated in presence of hypocalcemia, hyperkalemia and uremia [303].

Indications for pharmacological magnesium therapy are of 3 types including **purely pharmacodynamic**, **etiopathogenic** [in three particular situations *i.e*. emergency, necessity (when the oral form is impossible) and sometimes after failure of nutritional oral physiological therapy] and **mixed** when the pharmacological magnesium treatment combines

its useful *pharmacodynamic* effects and a *etiopathogenic* treatment for magnesium deficiency [90, 91, 95].

Obstructive pulmonary diseases *per se* constitute pure pharmacodynamic indications of pharmacological magnesium therapy, irrespective to the magnesium status. But their frequent association with concomitant primary magnesium deficiency [41, 90, 97, 102, 157, 294] constitutes a mixed indication of magnesium pharmacological treatment. The e**fficiency** of such pharmacological magnesium therapy **is dubious,** the results being conflicting and sometimes negative [34, 36, 45, 59, 72, 88, 142, 144, 151, 170, 218, 254, 256, 279, 282, 305, 325].

III.4.2.1.4.1.2. Use of Magnesium Sulfate in Acute Asthma

A. Intravenous administration

The initial clinical use of intravenous $MgSO_4$ in bronchial asthma, in 1936, by Rosello and Pla [291] relieved dyspnea and stridor in an asthmatic patient. Subsequent observations (cf above) led to progressive partial disinterest [303]. In the last decade, the potential role of IV Mg in acute asthma has gained renewed interest [34, 65, 81, 256, 312, 313, 314]. The main effects of magnesium sulfate include decrease in airway resistance, increase in FEV1, increased in forced vital capacity and decrease in dyspnea and respiratory frequency [81, 249, 254, 256, 311, 319, 302]. There have been a number of case reports and uncontrolled studies indicating its effectiveness in relieving bronchospasm [34, 45, 64, 65, 81, 131, 195, 256, 282, 306, 312, 313, 314, 319, 344]. Generally, magnesium sulfate is administered intravenously to patients either children or adults with severe exacerbations of asthma. An intravenous administration of 2 g magnesium sulphate, as an adjunct to standard therapy, led to a significant improvement in pulmonary function [47]. However, recent controlled clinical trials have nor agreed on its efficacy [34, 36, 142, 254, 344]. Nevertheless, it seems in FEV_1 did not significantly improved in the moderate-group (FEV_1 >25% on presentation) of patients receiving as an adjunct to standardized emergency procedure 2 g of $MgSO_4$. In contrast, in the severe group (FEV_1 <25% on admission), there was a significant improvement in FEV_1 at 120 and 204 min and a decrease admission rate as compared to the placebo-treated group [34, 293].

In any case, this mode of administration requires careful monitoring for prevention of local and mainly systemic symptoms of magnesium overload, since peripheral vasodilatation and systolic hypotension can occur and patients sometimes have unpleasant flushing, nausea, and venous phlebitis from the infusion [94, 96, 98, 11, 161].

Monitoring of pulse, arterial pressure, deep tendon reflexes, hourly diuresis, electrocardiogram and respiratory rhythm recording is necessary [34, 36, 45, 72, 94, 96, 98, 101, 142, 144, 150, 151, 170, 254, 257, 311, 319, 325].

The possible role of the **anion** SO_4^{--} as regards toxicity must be discussed. The selection of a particular magnesium salt among others should take into account reliable pharmacological and toxicological data. It seems necessary to determine **the therapeutic index** (LD50 / ED50) of the various available magnesium salts before any pharmacological use.

Finally, the combination of intravenous relatively high doses of magnesium (pharmacological magnesium therapy) and beta-2 mimetics may be toxic, more often in

obstetrical indications than in pulmonary diseases since the doses are clearly lower in pulmonary indications. Contra-indications of this latter form of pharmacological magnesium treatment combined with beta-2 mimetics for pulmonary indications are less imperative than for tocolysis [101, 199, 219, 285].

B. Nebulized administration

Eventhough intravenous $MgSO_4$ has been shown to increase the bronchodilating response and to improve lung functions in treatment of severe asthma, however, its effect by the nebulized route is uncertain [161] (table I).

Table I – Pharmacological effects of nebulized magnesium ($MgSO_4$) in obstructive pulmonary disorders [47, 161]

Patients	Response	Reference
Magnesium alone before challenge testing in provocation tests		
Direct stimuli using either histamine or methacholine.	Dose-dependent decrease in bronchial hyperresponsiveness	286, 287
Indirect stimulus using indirect bronchostrictor (sodium metabisulfite)	Dose-dependent decrease in bronchial hyperresponsiveness	246
Provocation test using histamine	No effect **on bronchial hyperresponsiveness**	157
Magnesium alone		
Acute exacerbation of asthma (adults)	Bronchodilatation (magnitude similar in to salbutamol)	218
Acute exacerbation of asthma (children)	Bronchodilatation (magnitude and duration of the effect were less than due to salbutamol)	233
Stable asthma	**No effect** on bronchodilatation	156
Magnesium as an adjunct to nebulized salbutamol or albuterol		
Severe Asthma	Improvement in PEF *vs* salbutamol in isotonic saline*	246
Severe asthma; 30 min after 2-5 mg nebulized salbutamol	Increase in bronchodilator response (twice the increase in FEV_1) *vs* salbutamol in isotonic saline*	161
Mild to Moderate asthma exacerbations	**No benefits** *vs* salbutamol therapy alone	31, 305

*according to Kurtaran et al. [196] nebulized saline is not a placebo since it may trigger asthma [15, 307]

Beta-2-mimetic and magnesium therapies have common pulmonary indications. The specific beta-2-agonists represent the priority treatment. Combination with corticosteroids is useful and efficient. Other associated treatments failed to demonstrate their efficiency as adjunctive treatments but magnesium use may be of interest. Indeed, the possible additive bronchodilating effects of magnesium and salbutamol has shown that a mild sustained increase in serum magnesium concentration caused a significant leftward shift of the dose response curve to inhaled salbutamol in asthmatic patients, with no change in the maximum response. This finding suggests that magnesium increased beta2-receptor affinity [47].

When isotonic magnesium sulfate was used as a vehicle for nebulized salbutamol for patients with acute asthma, it increases the peak flow response to treatment in comparison with salbutamol plus normal saline in severe asthma [161, 246] but failed in mild to moderate asthma exacerbations [31]. It seems that bronchodilator effectiveness of adjuvant Mg is seen in life-threatening, rather than less severe exacerbations of asthma [34]. The increase in peak expiratory flow (PEF) is inversely related to the baseline value, which supports the observation that magnesium is particularly effective in the most severe asthma exacerbations [314]. However, nebulized saline used as a vehicle for salbutamol is not a placebo since it may trigger asthma [15, 196, 307].

Nebulisation of three 2.5 mg doses of salbutamol at 30 min intervals is within the therapeutic range recommended for treatment of severe asthma in emergency department. They can be mixed with 2.5 mL isotonic magnesium sulphate [161].

Inhaled magnesium seems well tolerated [59, 218, 282]. However, according to Hughes et al. [282] most crucial in terms of use of magnesium as an adjuvant was its formulation as an isotonic solution (250 mmol/L, resulting in a tonicity of 289 mosmol). Both hypotonic and hypertonic nebulizer solutions cause bronchostriction in patients with asthma.

III.4.2.1.4.1.3. Oral Administration of Magnesium in Stable Asthma

Long term Mg repletion may be achieved by the daily administration of 300-600 mg of Mg orally [1, 25]. A randomized, double blind placebo-controlled, prospective study showed that long lasting Mg supplementation (200 mg in children < 7 years old and 290 mg magnesium citrate in older children) is clearly benefit (lower requirement of short term inhaled beta-2 mimetics, higher FIV_1) in mild-moderate asthmatic children and is recommended as a concomitant drug in stable asthma [25]. Similarly, a daily magnesium intake of 400 mg/day can improve clinical symptoms in adults [158].

Beta-2 mimetics and palliating **nutritional magnesium** therapies may be associated in the COPD treatment. This combination may be beneficial and remains atoxic. In asthmatic patients, the coexistence of other clinical manifestations of magnesium deficiency such as neuromuscular hyperexcitability must be investigated: Chvostek sign click, iterative EMG tracings, idiopathic mitral valve prolapse. But the dynamic oral physiological magnesium load test (5mg/kg/day) constitutes the best evidence of magnesium deficiency [41, 42, 90, 91, 94, 95, 97, 101, 157, 158, 294].

III.4.2.1.4.2. *L Tryptophan* or 5-OH Tryptophan

L tryptophan or 5-OH tryptophan may stimulate the tryptophan pathway but they are unspecific as they do not only concern melatonin production but also serotonin synthesis. They may induce toxicity: eosinophilia-myalgia syndrome particularly [1, 98-102, 231, 328].

III.4.2.1.4.3. *Taurine*

Taurine is a sulfonated aminoacid which is present in the whole body in high concentration, mainly in the brain. It is the most abundant free aminoacid in many tissues mainly in proinflammatory cells such as polymorphonuclear leukocytes and tissues exposed to elevated levels of oxidants [326]. It has multiple function in cell homeostasis such as membrane stabilization, buffering, osmoregulation and antioxidant activities together with effects on neurotransmitter release and receptor modulation. Taurine may act as a protective inhibitory neuromodulator which participates in the functional quality of the neural apparatus and in melatonin production and action. Taurine plays a role in the maintenance of homeostasis in the central nervous system, during central nervous hyperexcitability particularly. This volume-regulating aminoacid is released upon excitotoxicity induced cell swelling. It has an established function as an osmolyte in the central nervous system. In the course of Mg deficit, the organism appears to stimulate taurine mobilization to play a role of a “magnesium vicariant agent”. But this compensatory action is rather limited [94-100, 102, 163, 200, 212, 266]. In addition, taurine either intracellularly or released into the extracellular medium, may protect cells against attack by oxidants either directly or via the formation of its chlorinated derivatives taurine-chloramine [69, 326]. It decreases the release of inflammatory mediators by neutrophils and macrophages and modulate T-cell activation *in vitro* [220, 345]. It reduces *in vivo* lung oxidant damages induced by a number of chemicals including ozone, nitrogen dioxide, paraquat, amiodarone and bleomycin [69]. At a daily oral dose of 1-3 mmol/Kg for 7 days before challenge, taurine did not reduce the bronchospasm produced by antigen challenge in an experimental model of asthma using sensitized Brown-Norway rats, but it prevented airway hyperreactivity, reduced the number of eosinophils and of lipid hydroperoxides and prevented dye extravasation in bronchoalveolar fluid [69]. This means that taurine, in addition to reducing the number of inflammatory cells, may lessen the oxidant burden by diminishing the generation by these cells of superoxide anion and other cytotoxic mediators [69]. Finally, taurine levels in bronchoalveolar fluid from antigen-challenged rats were higher than control values but treatment with taurine fails to further increase this levels [69]. This is of importance, since taurine levels in bronchoalveolar lavage fluid and airway secretions were increased in asthma patients [159]. The same results were obtained while replacing taurine by a daily dose of the antioxidant N-acetylcysteine (1 mmol/Kg for 7 days) [33].

III.4.2.2. “Substitutive Darkness Therapy” or Darkness Mimicking Agents

Because of the limited efficiency of the previous chemical agents, palliative treatments of hBC may be necessary.

III.4.2.2.1. Mechanisms of the Action of Darkness

The mechanisms of action of the darkness appear as the reverse of those obtained with bright light where direct cellular and neural effects intervene. Increased melatonin production is the best marker of darkness but it is only an accessory mechanism in the darkness effect. The main central neural mechanisms of darkness therapy associate decreased serotoninergy together with stimulation of the inhibitory neuromodulators (GABA, taurine) and stimulation of anti-inflammatory and anti-oxidative processes, which may lead to neural hypoexcitability (sedative and anticonvulsant). Humoral transduction may reinforce these last effects by decreasing neuroactive gases (CO and NO) through binding of CO with haemoglobin and by increasing melatonin, bilirubin and biliverdin, three antioxidants which have the capacity to quench NO. Apart from the exception of decreased serotoninergy, these effects of darkness are similar to those of magnesium.

Substitutive darkness therapy should palliate all the mechanisms of the action of darkness. The only available darkness mimicking agents are melatonin (its analogous and its precursors, L-tryptophan, 5-hydroxytryptophan) [98, 102].

III.4.2.2.2. Melatonin, an Accessory Darkness Mimicking Agent

Melatonin is the prototype of darkness mimicking agents. But, although the production of melatonin is the best marker of photoperiod, it appears to be only an accessory factor among the mechanisms of photoperiod actions. Most of the other mechanisms of the effects of darkness have been overlooked, which may account for the controversy around the therapeutic efficiency of MT. Its dosage varies from physiological doses (around 0,3 mg) to pharmacological doses: usually 3 mg/per dose and per day and even up to 300 mg as a contraceptive (testifying to the weak toxicity of the hormone). In case of chronopathology, with decreased MT production, MT constitutes a substitutive treatment of its deficiency [43, 68, 98, 99, 102, 348, 367, 368, 371]. Melatonin (3 mg for 4 weeks) was shown to improve subjective sleep quality in asthma whereas it did not induce significant difference in asthma symptoms [57]. Further studies looking into long-term effects of melatonin on airway inflammation and bronchial hyperresponsiveness are needed before melatonin can be recommended in patients with asthma [57]. Our paper indicates that it would be at least avoided in asthma forms with HBC.

CONCLUSION

Asthma prevalence and severity are increasing over the world besides effective treatments and may led in some cases to fatal death. Many factors may interact in the physiopathology of the disease and contribute to the severity of asthma. Nocturnal asthma seem very frequent and represent a severe form of asthma leading to the idea that asthma is chronopathological.

We recently suggested that various pathologies, including asthma would be linked to a magnesium depletion with chronopathological dysfunction of the BC either HBC or hBC. These forms are characterized mainly by variations of their biological marker, melatonin and by well identified clinical symptoms of either hypo- or hyper-nervous excitability

respectively. This led us to suggest the measurement of plasma or salivary melatonin and plasma or erythrocyte magnesium levels in asthma patients. This would allow first to identify the possible presence of a chronopathological form of asthma thus justifying, in addition to conventional treatments, a complementary beneficial treatment including a balanced magnesium intake and either phototherapies or darkness therapies in asthma with either HBC or hBC.

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Chapter II

Role of Omega-3 Fatty Acids in the Treatment of Asthma

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ABSTRACT

It has been suggested that consumption of fish and polyunsaturated fatty acids could have a protective effect against inflammation in the airways and the development of asthma and other allergic diseases. Ecological and temporal data suggest that dietary factors may have a role in recent increases in the prevalence of asthma. A possible contributing factor to the increased incidence of asthma in western societies may the consumption of a pro-inflammatory diet. In the typical Western diet, 20-25-fold more omega-6 fatty acids than omega-3 fatty acids are consumed, which causes the release of proinflammatory arachidonic acid metabolites (leukotrienes and prostanoids). Fish oils are a rich source of omega-3 polyunsaturated fatty acids (n-3 PUFA). The specific fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are homologues of the n-6 fatty acid, arachidonic acid. This chemistry provides for antagonism by n-3 PUFA of arachidonic acid metabolism to pro-inflammatory eicosanoids (4-series leukotrienes and 2-series prostanoids) and cytokines (tumor necrosis factor-α and interleukin 1-β), as well as production of less active n-3 eicosanoids (5-series leukotrienes and 3-series prostanoids). In addition, n-3 PUFA can suppress production of pro-inflammatory eicosanoids and cytokines. This chapter will analyze the evidence for the protective effects of omega-3 fatty acids on the development of asthma. Alternative therapies for treatment that reduce the dose requirements of pharmacological interventions would be beneficial, and could potentially reduce the public health burden of this disease. While clinical data evaluating the effect of omega-3 fatty acid

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supplementation in asthma has been equivocal, it has recently been shown that fish oil supplementation, rich in n-3 PUFA, reduces airway narrowing, medication use and pro-inflammatory mediator generation in non-atopic elite athletes with exercise-induced bronchoconstriction. These findings are provocative and suggest that dietary n-3 PUFA supplementation may be a viable treatment modality and/or adjunct therapy in airway inflammation and for the development of asthma.

Keywords: Bronchial responsiveness, exercise-induced asthma, exercise-induced narrowing, fish oil, polyunsaturated fatty acids

INTRODUCTION

Asthma is a significant worldwide health problem, with high and increasing incidence in many countries; (1) morbidity – reflected in hospital admission rates (2), use of medical services, drug use, and trends in mortality rates are substantial (3, 4). The incidence of asthma varies by region and by age, but the global burden of asthma can be approximated from measured prevalence (reflecting incidence, duration, persistence, and recurrence of disease). Approximately 20.3 million Americans (6.3 million children) had asthma in 2001; 73.4 per 1000 population (5), while it is estimated that around 300 million people in the world currently have asthma (6). Despite the progress that has been made in the treatment of asthma, it remains a major illness in terms of morbidity, suffering, and cost (7).

Asthma is a chronic inflammatory disorder of the airways and causes recurrent episodes of wheezing, breathlessness, chest tightness, and cough (8). Long-term airway remodeling is characteristic of asthma and may be associated with an increase in airway hyperresponsiveness to a variety of stimuli (8). Airway responsiveness is the tendency for airways to constrict under the influence of non-sensitizing physical stimuli such as cold air and exercise, chemical substances such as methacholine, or sensitizing agents such as allergens. Airway hyperresponsiveness can be defined as the increase above normal in the degree to which the airways will constrict upon exposure to these stimuli (9) and it is closely related to the underlying mechanisms of asthma as we currently understand them (10).

Inhaled corticosteroids, long-acting β_2-agonists and short-acting β_2-agonists have proven highly effective as medications in relief of symptoms, and have facilitated the management of asthma. In addition, daily medications such as leukotriene receptor antagonists and leukotriene enzyme inhibitors have recently proven effective in asthma therapy (11). However, these medications are not without real and potential side effects. Prolonged use of some medications may result in reduced efficacy, or tachyphylaxis. For example, daily use of long- acting β_2-agonists in the management of exercise-induced asthma in children has recently been questioned (12), and reversal of an asthma attack, such as exercise-induced asthma, may be ineffective in a large portion of asthmatics when short-acting β_2-agonists are used daily (13). Therefore, alternative treatment approaches in obstructive lung disease which focus on manipulation of dietary factors are of real interest since they could potentially reduce the dose requirements of pharmacological medications (14-23), and reduce the public health burden of this disease.

The purpose of this chapter is to critically examine the existing information regarding the relationship between omega (n)-3 polyunsaturated fatty acids (PUFA) supplementation and airway hyperresponsiveness in asthma, and in particular to address the question as to whether supplementing the diet with n-3 PUFA represents a viable alternative treatment for asthma.

MECHANISMS OF ACTION

Although the impact of n-3 PUFA on lipid mediator generation has been greatly clarified, the understanding of sub-cellular effects is still limited. Omega-3 PUFA affects biophysical characteristics of cellular membranes by alteration of the membrane phospholipid composition and may modify the function of membrane-linked enzyme systems and signal transduction pathways. Many of the anti-inflammatory effects of n-3 PUFA appear to be exerted at the level of altered gene expression and have been demonstrated only a limited number of times *in vitro*, and thus the extent of these effects *in vivo* is not yet clear.

Eicosapentaenoic acid (EPA) and docosahexeanoic acid (DHA), derived from fish oil, competitively inhibit n-6 PUFA arachidonic (AA) metabolism, thus reducing the generation of pro-inflammatory 4-series leukotrienes (LTs) and 2-series prostanoids (prostaglandins (PGs) and thromboxanes), (24) and the production of cytokines from inflammatory cells (25). The EPA-derived metabolites (5-series LTs and 3-series prostanoids) have lower biological activity compared with the analogous AA derived 4-series LTs and 2-series PGs. (Figure 1). 4-series cysteinyl LTs increase vascular permeability and contract smooth muscle cells, causing bronchoconstriction and vasoconstriction (26). The bronchoconstrictive and chemotactic potency of LTB_5 is two orders of magnitude lower than the activity of LTB_4 (27) (Figure 1). Consuming fish oil results in partial replacement of AA in inflammatory cell membranes by EPA (24, 25) and thus demonstrates a potentially beneficial anti-inflammatory effect of n-3 PUFA. Supplementing the diet with n-3 PUFA has been shown to reduce AA concentrations in neutrophils and neutrophil chemotaxis, reduce LT generation (24, 28) and reduce airway late response to allergen exposure (29). These data are consistent with the proposed pathway by which dietary intake of n-3 PUFA modulates lung disease.

Mounting evidence now suggests that fatty acids are not only the precursors of eicosanoids and other lipid mediators, but also can modulate signaling molecules and transcription factors such as nuclear factor-kappaB (NF-κB) (30-32). Since macrophages of induced sputum and bronchial epithelial cells from stable asthmatics exhibit increased NF-κB activity compared with cells from healthy individuals (Hart et al., 1998), it has been suggested that NF-κB plays a pivotal role in the pathogenesis of asthma (33-38). Recently, Lee and coworkers (39, 40) demonstrated that activation of general proinflammatory pathways, such as NF-κB and cyclooxygenase-2 (COX-2) expression by saturated fatty acids and inhibition of this induction by n-3 PUFA, are mediated through a common signaling pathway derived from toll-like receptor 4 (Tlr-4). If activation of Tlr-4 is modulated by n-3 PUFA, then signaling pathways downstream, such as proinflammatory transcription factor NF-κB, and consequent cellular responses (e.g., inducible nitric oxide, proinflammatory cytokines, TNF-α, IL-1β and eicosanoids [prostanoids and LTs]) should also be modulated by n-3 PUFA (39, 40) (Figure 2).

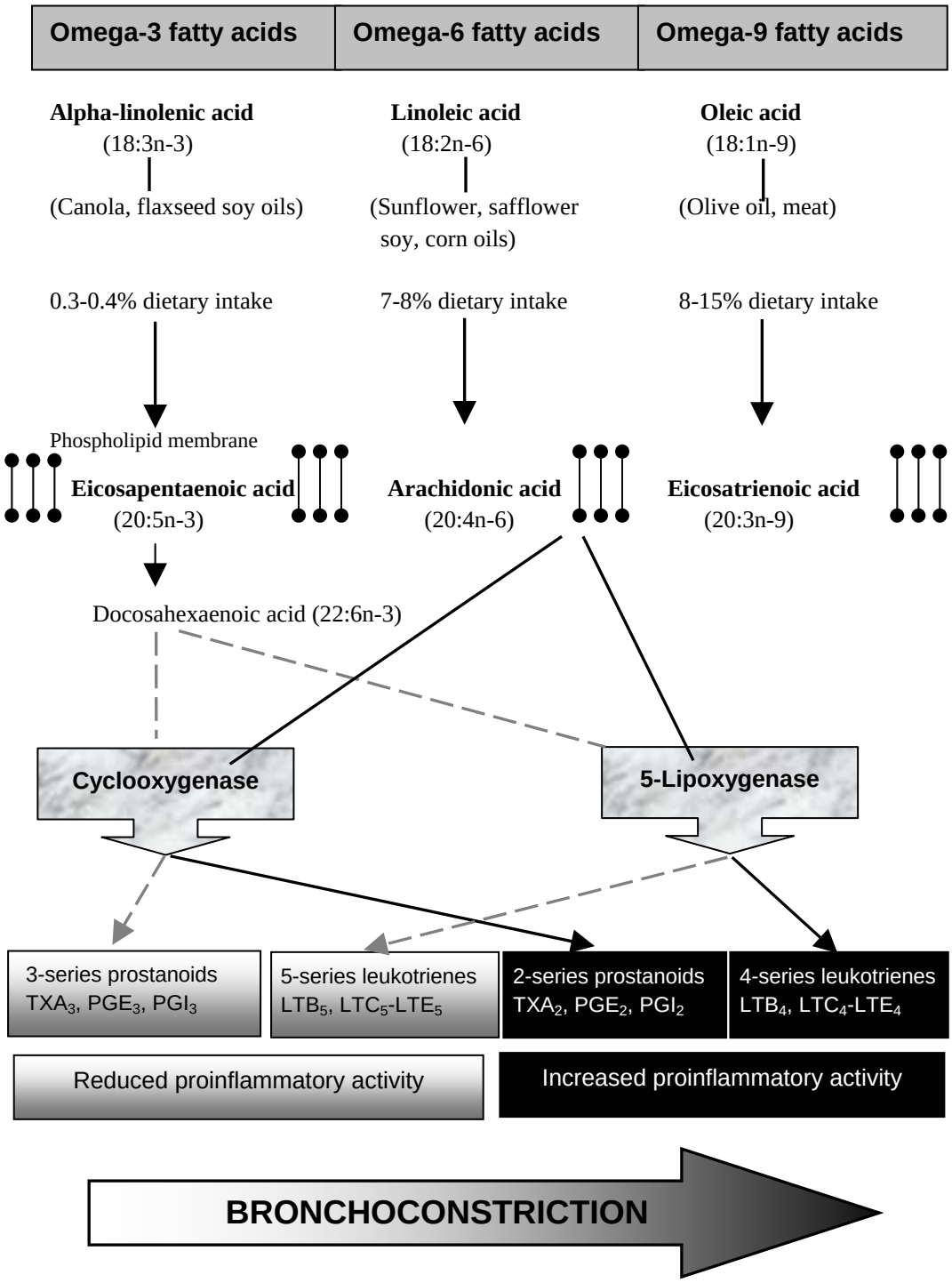

Figure 1**.** Metabolism of dietary fatty acids after ingestion via the cyclooxygenase and 5-lipoxygenase enzymatic pathways and subsequent effects on airway inflammation and bronchoconstriction. LT, leukotriene; PG, prostaglandin; TX, thromboxane)

Indeed, It has been demonstrated that proinflammatory cytokine inhibition in murine macrophages by n-3 PUFA is mediated, in part, through inactivation of NF-κB (41, 42) and inhibition of COX-2 and PGE_2 expression in blood monocytes with a Tlr-4 agonist (39). Therefore, since Tlr-4 conveys signals as a part of innate immunity from the endotoxin receptor (CD14) on the surface of macrophages to the inner cell, a downregulation of nuclear transcription factors, such as NF-κB formation of cytokines might be reduced after fish oil ingestion (43).

Cell culture studies have demonstrated that AA activates NF-κB in monocytic cells (44). It is possible that this is the mechanism by which AA induces COX-2 and inflammatory cytokines. In contrast, EPA has been shown not to activate NF-κB in a monocytic cell line (44), and to prevent NF-κB activation by TNF-α in cultured pancreatic cells (45). This reduced activation of NF-κB involved decreased degradation of IκB, perhaps through decreased phosphorylation (45).

Similarly, it has been shown that EPA decreased endotoxin-induced activation of NF-κB in human monocytes (42). This was associated with decreased IκB phosphorylation, perhaps due to decreased activation of mitogen-activated protein kinases. Collectively, these observations suggest direct effects of n-3 -PUFA on inflammatory gene expression via inhibition of the proinflammatory transcription factor NF-κB. *In vitro* experiments, in the investigation of the regulatory mechanisms of n-3 PUFA on NF-κB activity are warranted; specifically the interaction between n-3 PUFA, inflammatory eicosanoids and Tlr-4 receptor function in asthma needs investigation.

Omega-3-PUFA therefore seems to interfere with early inflammatory signal transduction processes and is thus capable of blunting hyper-inflammation. Elucidating the mechanism of this modulation could help us to understand how dietary n-3 PUFA achieve their specific effects on airway inflammation.

OMEGA-3 FATTY ACIDS AS A TREATMENT MODALITY IN ASTHMA

Over the past 3 decades there has been considerable interest in the therapeutic potential of n-3 PUFA for various inflammatory conditions such as rheumatoid arthritis, inflammatory bowel diseases, and asthma. Omega-3 PUFA, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in fish oils, compete with arachidonic acid (AA) as substrates for the formation of eicosanoids, such as leukotrienes (LTs) and prostaglandins (PGs) (24). Arachidonic acid-derived eicosanoids are pro-inflammatory, while EPA-derived eicosanoids are more immunoneutral. Moreover, n-3 PUFA appear to have additional anti-inflammatory effects mediated through direct action on neutrophil and monocyte production of inflammatory mediators (e.g., cytokines) and chemotactic responses (24, 25).

In the typical Western diet, 20–25-fold more n-6 PUFA than n-3 PUFA are consumed (46). This predominance of n-6 PUFA is due to the abundance of dietary linoleic acid (18:2n-6), which is present in high concentrations in soy, corn, safflower, and sunflower oils.

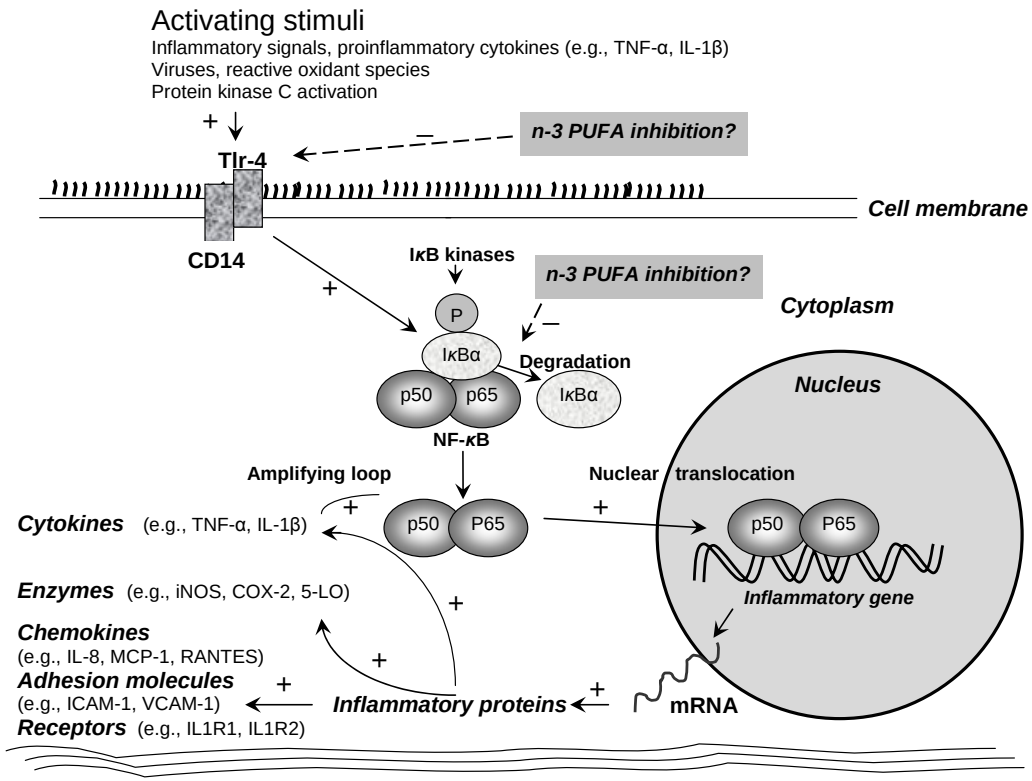

Figure 2. Selected pathways of toll-like receptor (TLr)-4 and nuclear factor (NF)-κ(beta)B by multiple inflammatory stimuli resulting in the coordinated expression of genes for several cytokines, enzymes, chemokines, adhesion molecules and receptors. A schematic representation of signaling cascades for various activating stimuli and activation of NF-κB (p50/p65). The activation of NF-κB begins with stimulation of specific receptor families at the cell surface (e.g., (TLr-4 and CD14), interleukin-1 receptor and tumor necrosis factor receptor 1) and recruitment of adaptor proteins that leads to specific pathways of transduction controlled by various kinases. This activation promotes the phosphorylation of IκB. This activation targets IκB for ubiquitination and degradation. As a consequence, the NF-κB inhibitory protein is removed and free NF-κB is rapidly translocated to the nucleus where is binds to specific promoter regions of various genes encoding inflammatory cytokines, enzymes, chemokines, adhesion molecules and receptors. The cytokines (tumor necrosis factor (TNF)-α and interleukin (IL)-1β are both activated and amplified by NF-κB. Potential sites of n-3 PUFA inhibition are shown. iNOS, inducible nitric oxide; mRNA, messenger RNA; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α; COX-2, cyclo-oxygenase-2; 5-LO, 5-lipoxygenase; IL-8, interleukin-8; MCP-1, monocyte chemoattractant protein-1; RANTES, regulated upon activation, normal T cell expressed and secreted.

By contrast, there is a low intake of the n-3 homologue of linoleic acid, α-linolenic acid (18:3n-3), which is present in leafy green vegetables and in flaxseed and canola oils. Once ingested, the 18-carbon fatty acids are desaturated and elongated to 20-carbon n-6 PUFA. Linoleic acid is converted to AA and α-linolenic acid is converted to EPA (20:5n-3) (Fig 1). Compared with linoleic acid there is little dietary intake of AA and EPA, which are present in meat and fish, respectively. Linoleic acid and α-linolenic are necessary for a complete diet and cannot be synthesized in vertebrates; therefore, they are essential fatty acids. As a consequence, the relative dietary amounts of n-6 and n-3 PUFA are determinants of the relative cellular amounts of linoleic acid and α-linolenic acid.

There has been increased emphasis on the beneficial effects for cardiovascular health of replacing lard and dairy fats rich in saturated fatty acids. This has led to increased consumption of vegetable oils rich in omega-6 PUFA and a simultaneous decrease in consumption of oily fish and leafy vegetables, the major sources of omega-3 PUFA (47). The anti-inflammatory properties of n-3 PUFA such as EPA and DHA and generally pro-inflammatory properties of dietary n-6 PUFA (48, 49), such as linoleic acid, suggest that these dietary trends may predispose some individuals to inflammatory disorders, including asthma.

Epidemiological Studies

The notion that consumption of dietary fatty acids can influence the development and activity of an inflammatory disease such as asthma is attractive in view of the complex metabolic role that fatty acids play in cell metabolism and structure. During the period of increasing asthma prevalence in England and Wales, dietary consumption of fatty acids also changed, with a marked increase in the intake of n-6 PUFA and a decrease in saturated fatty acids (50). Support for the hypothesis that fat intake may be important is available from a case-control study showing an association of higher fat intake with adult onset wheeze in Scotland (51), and a cohort study from Malmo in which men with asthma had a higher intake of dietary fat (52). Haby and colleagues (53) assessed the prevalence of asthma and risk factors for asthma in Australian pre-school children and found that a high level of PUFA (high n-6, low n-3) was associated with increased risk of recent asthma. Hodge et al. (54) found an inverse relationship between weekly oily fish intake over the course of 12 months and the prevalence of asthma in 574 school children in a cross-sectional study. These studies suggest that consumption of oily fish is associated with a reduced risk of asthma in childhood. Patal and coworkers (55) demonstrated an association between consumption of oily fish and symptomatic wheeze in individuals with and without physician diagnosed asthma. Takemura et al. (56) assessed the relationship between dietary fish intake and the prevalence of asthma among a Japanese childhood population and their results indicated that the frequency of fish intake was related to a decreased prevalence of asthma. Nafstad and coworkers (57) evaluated the relationship between the introduction of a fish diet during the first year of life and the risk of developing asthma and allergic rhinitis in a prospective 4-year cohort study of 2531 Norwegian children. This group of researchers found that the fish diet was negatively associated with the risk of developing allergic rhinitis and asthma.

Additionally, Oddy and colleagues (58) recently investigated whether childhood asthma was associated with the ratio of n-6 to n-3 fatty acids in the diet (n-6:n-3) using a cross-sectional study design. They found evidence for the promotion of a diet with increased n-3 PUFA (fresh or oily fish at least once a week, whole grain cereals, raw sunflower and flax seeds, and canola oil) and reduced n-6 PUFA (margarines, vegetable oils, processed foods) to protect children against symptoms of asthma. Woods and colleagues (59), in a community-based cross sectional study, sought to determine whether plasma levels of n-3 PUFA, as a measure of dietary intake, was protective against asthma and atopy in young adults. These authors did not find any evidence to suggest that n-3 PUFA are associated with a reduced risk of asthma or atopy. Interestingly, their results suggest that the n-6 PUFA gamma (γ)-linolenic acid has the strongest association with asthma. Due to the fact that this was a cross-sectional study, the authors were unable to establish a cause and affect relationship for the fatty acid/asthma associations found.

Clinical studies

Considering the role of LTs, PGs and cell–cytokine interactions in airway inflammation, remodeling, and hyperreactivity in asthma, the potential therapeutic effect of a diet rich in fish oil has been examined repeatedly**.** However, clinical data on the effect of fish oil supplementation in asthma has been equivocal. While no clinical improvement in asthmatic symptoms has been observed in some interventional studies (60-64), other studies have demonstrated an improvement in asthmatic status following n-3 PUFA supplementation (29, 48, 49, 65-69). Early short term trials (8 weeks) of up to 4 g/day of EPA in patients with severe asthma showed no clinical benefit, despite demonstrating profound suppression of neutrophil chemotaxis and LT mediator production (63). Six weeks of 3 g/day of EPA had a deleterious effect on patients with aspirin intolerant asthma (70), consistent with the known aspirin-like effect of cyclooxygenase inhibition by EPA. Further studies in milder asthmatics with 3.2 g/day for 10 weeks showed no benefit in either clinical symptoms or bronchial hyperresponsiveness (60), despite demonstrating attenuation of allergen-induced late-phase bronchoconstriction induced in the laboratory (29). A more prolonged trial for six months with 3.2 g/day of EPA also showed no clinical benefit in patients with pollen-induced asthma and seasonal hay fever (62). In addition, Stenius-Aarniala and coworkers (64) demonstrated no clinical benefit of 10 weeks of fish oil supplementation in relatively stable asthmatics. However, the method of assessing lung function is open to question since each subject used a Peak Flow Meter at home under no supervision. Recently Surette et al. (71) showed no change in baseline pulmonary function occurred in a population of atopic asthmatics, even though daily consumption of dietary γ-linolenic acid (GLA) and EPA inhibited LT biosynthesis. However, asthma severity and reliance on medication were not assessed. McDonald et al. (72) provided 2.7g EPA and 1.8g DHA for 10 weeks to 15 non-smoking asthmatics and found no change in peak expiratory flow rate following fish oil supplementation. Recently Broadfield and colleagues (73) evaluated whether a higher intake of n-6 PUFA or a lower intake of n-3 PUFA increased the risk of asthma, by measuring dietary fatty acid intake by a food frequency questionnaire (FFQ) and erythrocyte membrane

fatty acids, as an objective biomarker of intake. These authors compared individual fatty acid intake estimated by FFQ and by mass spectrometry of fasting erythrocyte cell membranes in 89 cases of asthma and 89 community-matched controls. These authors found that a higher erythrocyte membrane level of linoleic acid, an n-6 PUFA, was associated with a decreased risk of asthma. However, as the authors acknowledge, major limitations to their study design may include overmatching of the cases and controls, since social class is strongly associated with diet. This would have resulted in odds ratios that underestimated the true difference between asthmatics and controls. In addition, the limitations in FFQ in introducing measurement error have recently been highlighted (74).

In contrast, Dry and colleagues (66) have shown positive results using a small placebo-controlled trial of low-dose EPA (1 g/day) for 12 months in 12 adult asthmatics; after 9 months a small but significant improvement of 23% was found in FEV_1. However, no details were given of concurrent medication use or confirmation of compliance by leukocyte membrane phospholipid analysis. Hodge et al. (61) demonstrated that dietary supplementation with n-3 PUFAs over 6 months increased plasma levels of these fatty acids and reduced stimulated tumor necrosis factor (TNF)-α and circulating eosinophils, with a concurrent improvement in peak expiratory flow and reduced medication use in asthmatic children (61). Nagakura and colleagues (65) showed that dietary supplementation with fish oil (84 mg EPA and 36 mg DHA per day) over 10 months decreased asthma scores and reduced acetylcholine thresholds during an acetylcholine inhalation challenge in children with bronchial asthma. Okamoto et al. (49, 68) observed suppression of LTB_4 and LTC_4 generation by leukocytes and improvement in respiratory function following 4 weeks of perilla seed oil (n-3 PUFA rich) supplementation in asthmatic subjects. Payan and coworkers (28) found that high doses, compared to low doses, of EPA ethyl ester taken daily for 8 weeks increased LTB_5 generation, and reduced AA, LTB_4 and PGE_2 generation by polymorphonuclear (PMN) and mononuclear leukocytes in asthmatic patients. These authors did not report pulmonary function scores, medication use or asthma symptom scores. Villani et al. (67) observed a significant improvement in FEV_1 with a concomitant reduction in airway resistance after only 30 days supplementation with 3 g/day of n-3 PUFA in 7 atopic patients. Massuev (75) observed significant attenuation of the late allergic response in 13 asthmatic patients supplemented for 2 weeks with n-3 PUFA, and in another study showed that n-3 PUFA supplementation resulted in a significant decline of the late allergic response and reduced drug doses in 27 asthmatic patients (76). Provocative tests with allergen after 10 weeks of either n-3 PUFA or placebo showed a significant decline in the late allergic response and suppression of inflammatory mediators (50% reduction in the capacity of PMN to produce LTB_4) in the treatment group (29). Broughton et al. (48) demonstrated that supplementing the diet with 3.3 g/day of EPA and DHA daily in 27 asthmatic subjects ameliorated methacholine-induced respiratory distress, which may be predicted by LT metabolism. Emelyanov and colleagues (69) recently showed a decrease in daytime wheeze, concentration of exhaled hydrogen peroxide (a marker of airway inflammation) and an increase in morning peak expiratory flow rate in forty six atopic asthmatic patients receiving a lipid extract of New Zealand green-lipped mussel, rich in n-3 PUFA, for 8 weeks compared to placebo (olive oil).

The inconsistency among study results assessing the efficacy of fish oil supplementation in asthma may be attributable to the heterogeneity in definitions of the 1) Settings (e.g., hospital versus outpatient; countries); 2) Populations (e.g., age; gender; clinical picture of asthma, including its severity and concomitants, or triggers with the potential to impact asthma control); 3) Interventions and their contrasts with comparators (e.g., different types and amounts of oil and n-3 PUFA contents; controlled versus uncontrolled dosing); 4) the dosage (1 to 4 g/day) and duration (3 weeks to 12 months) of fish oil supplementation varies greatly among published studies; 5) Co-interventions (e.g., asthma medication with varying capacities to control asthma in the short- or long-term. For example, failing to assure that there is not an uneven distribution of corticosteroid users or doses across study arms/cohorts can restrict the ability to meaningfully attribute a significant or null effect to the actions of n-3 PUFA supplementation. Asthma medications' capacity to improve asthma symptoms can mask the benefits linked to the use of n-3 PUFA supplementation); and 6) The most commonly employed respiratory outcome measure in a large majority of the published studies evaluating the efficacy of n-3 PUFA supplementation in asthma is peak expiratory flow. However, peak expiratory flow may not accurately reflect changes in airway function as assessed by more reliable measurements such as forced expiratory volume in 1-second (FEV_1) or forced vital capacity (FVC)(77, 78).

In only in one study (61), was dietary manipulation performed as part of the treatment phase. It is noteworthy that this study demonstrated a significant improvement in peak expiratory flow and a reduction in asthma medication use on the n-3 PUFA diet (canola oil and canola based margarines and salad dressings), while a decrement in resting peak expiratory flow and increased medication use was observed on the n-6 PUFA diet (sunflower oil and sunflower oil-based margarines and salad dressings). Interestingly, Woods et al. (79) in The Cochrane Database of Systematic Reviews, assessing the efficacy of fish oil for asthma in adults and children, identified 22 studies for possible inclusion; however, the authors only included 9 studies. Reasons for non-inclusion were a) not a randomized controlled trial (4 studies), b) not using marine fatty acids in asthma (3 studies), c) no outcome measures reported (3 studies), and d) an inadequate intervention period (1 study). None of the studies reported asthma exacerbations, health status (quality of life) or hospital admissions. These authors stressed that further studies should address these issues. Woods et al. (79) concluded that they were unable to determine the effect of fish oil supplementation in asthma or answer the question whether increasing dietary marine n-3 PUFA by increased fish intake results in improved asthma control.

While consumption of supplements may be the most efficacious way to increase n-3 PUFA in the body, a food-based approach that is accepted and tolerated is a preferable way to ensure long-term nutrient intake. Because this dietary approach will enhance n-3 PUFA intake to a lesser extent than most capsule supplementation methods changes in mediator generation are likely to come about more slowly (80). Dietary approaches that allow an individual to meet their nutrient needs through multiple types of foods generally reduce an individual's risk of exposure to toxins, such as methyl mercury and PCB's, which are common contaminants of fish and other sea foods (Kris-Etherton et al., 2002). For those individuals who do not eat fish, have limited access to a variety of fish, or cannot afford to purchase fish, a fish oil supplement may be considered. On the other hand some individuals

may prefer to eat whole fish rather than taking fish oil capsules, since a common complaint from taking encapsulated fish oil are fishy aftertaste, gastrointestinal disturbances and nausea. (81).

Whether the type of n-3 PUFA (18-, 20- or 22-carbon fatty acid) exerts differences on plasma lipids, inflammatory mediator generation and airway hyperresponsiveness has not been adequately addressed under carefully controlled dietary conditions. Elongation and desaturation of α-linolenic acid to EPA and other n-3 PUFA occurs at low levels, this shorter fatty acid does get incorporated into tissues at the expense of n-6 PUFA, and could modestly contribute to changing the inflammatory mediator environment (James et al., 2000). It has been suggested that the extent of the conversion of α-linolenic acid to the longer-chain n-3 PUFA is modest (81). For example, Emken et al. (82) reported a 15% conversion, whereas Pawlosky et al. (83) found 0.2%; both reported that the conversion to DHA was much less than that to EPA. However, these studies were performed against a background diet high in n-6 PUFA. The desaturation and elongation enzymes that convert α-linolenic acid to EPA also convert linoleic acid (18:2n-6) to AA. Hence, these previous studies on EPA concentrations may have been performed under sub-optimal conditions for conversion of α-linolenic acid to EPA. Mantizioris and coworkers (84) demonstrated that dietary flaxseed oil, used in domestic food preparations, increased tissue EPA levels comparable with fish-oil supplementation against a background diet low in n-6 PUFA in healthy male volunteers. In a follow-up study using healthy volunteers with a diet low in n-6 PUFA consumption, Caughey et al. (85) confirmed that both flaxseed oil and fish oil supplementation resulted in a comparable increase in mononuclear cell EPA content with a concomitant inhibition of TNF-α and IL-1β synthesis. Flaxseed, but not flaxseed oil, is also rich in lignans which may have biological properties independent of n-3 PUFA. Lignans possess anti-platelet activating factor activity and are antioxidant in nature. However, their role in inflammatory conditions is presently undetermined.

OMEGA-3 FATTY ACIDS AS A TREATMENT MODALITY IN EXERCISE-INDUCED BRONCHOCONSTRICTION

Exercise is a powerful trigger of asthma symptoms and may result in asthmatic patients avoiding physical activity, resulting in detrimental consequences to their physical and social well-being. Approximately 90% of asthmatics and a high prevalence of non-atopic elite athletes are hyperresponsive to exercise and experience exercise-induced bronchoconstriction (EIB) (86). Exercise-induced bronchoconstriction (EIB), exercise-induced asthma or exercise-induced airway narrowing are synonymous terms that describe a condition in which vigorous physical activity triggers acute airway obstruction in individuals with heightened airway reactivity (87). EIB is not an isolated disorder or specific disease, but rather often part of the asthmatic diathesis where exercise is one of many stimuli that may induce airflow limitation and is a prominent and troublesome feature of asthma. A characteristic evident in individuals with EIB is a marked decrease in exercise capacity and breathlessness upon exertion. Significant numbers of healthy, non-asthmatic individuals demonstrate EIB and are often referred to as having solitary EIB. For the alert practitioner, EIB provides the ideal

opportunity to detect and better manage asthma, particularly since 29-51% of asthma is silent or undetected unless the subjects are exercised (88).

Arm et al. (60) was the first to evaluate the effect of fish oil (n-3 PUFA) supplementation on the airway response to exercise in patients with asthma (60). After 10 weeks of daily supplementation with 3.2 g EPA and 2.2 g DHA, subjects underwent a histamine challenge, exercise challenge and blood neutrophil studies. Although there was a significant increase in n-3 PUFA neutrophil content and a 50-percent inhibition of total LTB synthesis (LTB_4 and LTB_5), there was no detectable change in the clinical outcome (e.g., histamine response, exercise response, specific conductance of the airway or symptom scores).

Recently, Mickleborough and colleagues (89) demonstrated that 3 weeks of fish oil supplementation reduces the severity of EIB and resulted in a significant suppression of several pro-inflammatory mediators in non-atopic elite athletes who exhibited 'asthma-like symptoms' following exercise (89). The airway response to exercise was used to assess changes in non-specific bronchial responsiveness during dietary supplementation with n-3 PUFA. The exercise challenge test consisted of running to volitional exhaustion on a treadmill while breathing compressed dry air. The fish oil supplement had no effect on baseline pulmonary function in EIB (n=10) and control subjects (n=10) or following exercise in non-EIB control subjects. However, in the group of athletes who had a history of exercise-induced narrowing, the fish oil supplement reduced the fall in FEV_1 at 15 minutes post-exercise by almost 80% (Figure 3) in conjunction with a greater than 20% reduction in bronchodilator use. In addition, the increase in tissue phospholipid n-3 PUFA concentration in EIB subjects was coincident with a significant suppression of the proinflammatory eicosanoids LTE_4, PGD_2 metabolite 9α, 11β-PGF_2 and LTB_4 and proinflammatory cytokines IL-1β and TNF-α.

In a follow-up study Mickleborough and colleagues (90) recently examined the effect of fish oil supplementation in asthmatic patients who experienced EIB. This pilot study was conducted as a randomized, double-blind crossover trial over 8 weeks in a similar fashion to our previous work (89) and used a similar pharmaceutical-grade fish oil (3.2 EPA and 2.0g DHA) and placebo supplementation dosage and duration (3 weeks). Sixteen mild atopic asthmatic subjects with documented EIB volunteered for the study. There were no significant differences in baseline pulmonary function following exercise across diet. However, the n-3 PUFA diet reduced the post-exercise fall in FEV_1 by approximately 64% (Figure 4). In addition, there was a significant improvement in asthma symptoms scores and a reduction in bronchodilator use (total number of doses/puffs) on the n-3 PUFA diet. As per our previous study (89) proinflammatory mediator concentration was significantly suppressed on the n-3 PUFA diet. Using the relatively non-invasive technique of sputum induction we demonstrated that a diet supplemented with fish oil reduced airway inflammation in mild atopic asthmatics with EIB. Specifically, sputum differential eosinophil, neutrophil, lymphocyte and macrophage cell counts and sputum supernatant concentrations of proinflammatory eicosanoids LTC_4-E_4, LTB_4, PGD_2 and cytokines IL-1β and TNF-α were significantly reduced on the fish oil diet. These results strongly suggest that dietary supplementation with n-3 PUFA could decrease exercise-induced airway narrowing in asthmatics.

The divergent findings between the studies conducted by Mickleborough et al. study (89, 90) and that of Arm and colleagues (60) are difficult to reconcile, especially since the Arm et al. study had a longer duration supplementation period with an identical fish oil dosage**.** The negative findings observed by Arm et al. (60) may be due to methodological and statistical limitations.

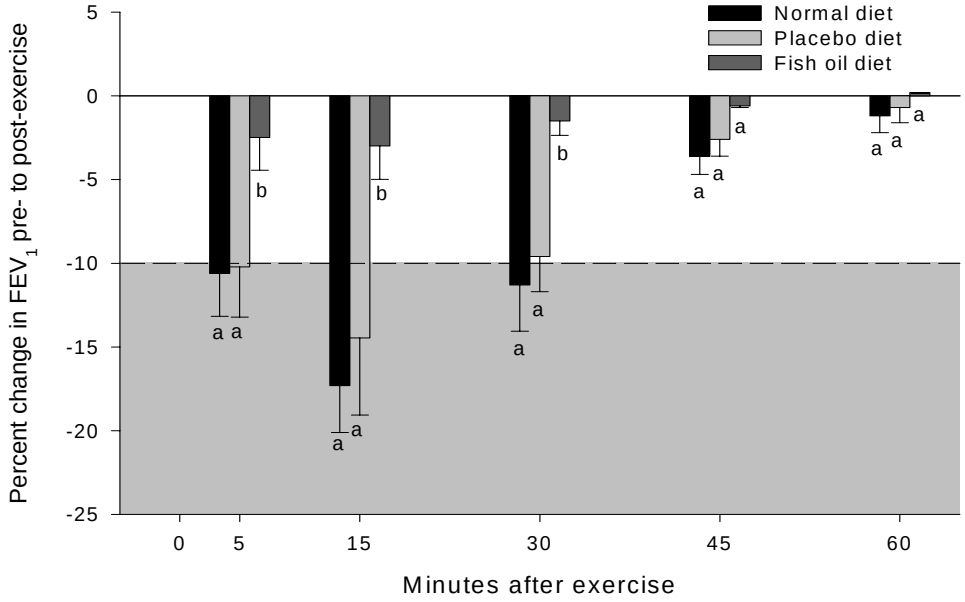

Figure 3. Percent change in FEV_1 pre- to post-exercise in elite athletes with exercise-induced bronchoconstriction. A reduction in FEV_1 in excess of 10% represents abnormal pulmonary function and is diagnostic of EIB. Letters (a, b) refer to comparisons by diet within respective time period. Different letters designate significant difference ($p < 0.017$). (89).

These authors exercised a cohort of mild asthmatics at very low exercise intensity (80% predicted maximal oxygen consumption for 8 min at ambient temperature and humidity. It is generally accepted that inhaling cold-dry air at high ventilation rates initiates EIB. Rundell and coworkers (Rundell et al., 2000) have shown that out of 23 subjects who tested positive for EIB in cold-dry air, 18 (78%) subjects tested negative in ambient conditions (21^{o}C and 50% relative humidity). This suggests that the exercise protocol performed in ambient conditions in the Arm et al. study (Arm et al., 1988) may have been less sensitive to identifying changes in airway hyperresponsiveness following exercise due to inadequate environmental stress. In addition, an assessment of the numbers used in the airway response to exercise of Arm and coworkers' study (60) (5 subjects receiving placebo and 6 subjects receiving fish oil supplementation) suggests an insufficient patients to detect a statistical difference and avoid a type I error**.**

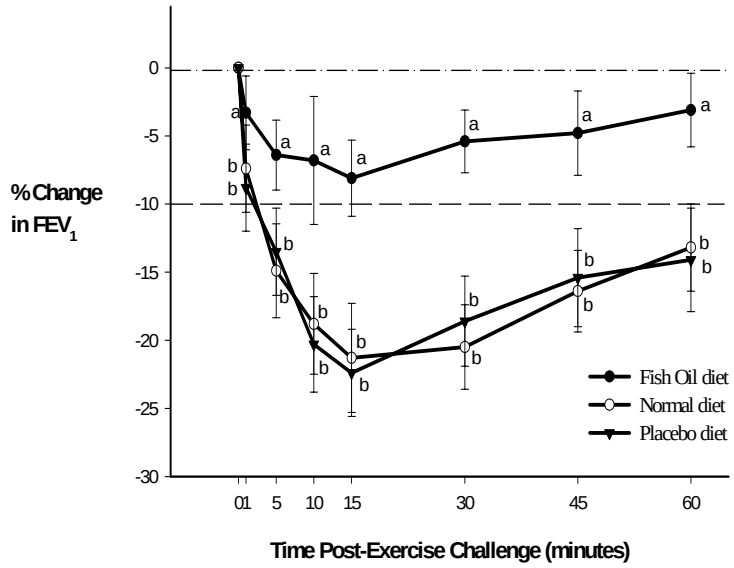

Figure 4. The percent change in FEV1 from pre- to post-exercise in asthmatic subjects across the three diets. Letters (a,b,c) refer to comparisons by diet within respective time period. Different letters designate significant difference ($p<0.05$). (90)

CONCLUSION

In view of the clinical consequences, these findings point towards prophylactic and acute therapeutic effects of fish oil supplementation in inflammatory diseases such as asthma, which seem to be attainable by simple rearrangement of nutritional components. It is possible that antiinflammatory drug use could be decreased in some patients with asthma in concert with increased fish-oil ingestion if both the drug and n-3 PUFA are exerting their therapeutic effects through the same molecular actions. There may be an opportunity for beneficial additive effects with fish oil supplementation or other dietary approaches to increasing intake of n-3 PUFA. Thus, the possibility exists for drug-diet interactions that confer greater antiinflammatory benefits than either intervention alone or at least similar antiinflammatory effects with less toxicity.

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Chapter III

Aspirin – Induced Asthma

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ABSTRACT

In about 10% of adult patients with asthma, aspirin and other nonsteroidal anti-inflammatory drugs precipitate attacks of dyspnoea. Aspirin challenge causea a significant rise in bronchoalveolar lavage fluid (BALF) levels of total Cys-LTs in the aspirin – induced asthma (AIA) subjects, but not in the aspirin – tolerant asthma (ATA) group. The rise in BALF cysteinyl leukotrienes (Cys-LTs) correlates significantly with the counts of bronchial mucosal eosinophils, but not with mast cells counts, confirming eosinophils as the predominant source of the Cys-LTs response to aspirin. Aspirin challenge is followed in AIA subjects by a dramatic rise in eosinophil counts within the BALF, suggesting migration of activated eosinophils into airway lumen. PGE_2 production by eosinophils or other cells has been suggested to act as a brake on leukotriene synthesis in all subjects. Removal of this brake by cyclooxygenase inhibitors triggers significant Cys-LTs production only in AIA subject because they alone have high levels of overexpression of LTC_4 synthase. The relative lack of LTC_4 synthase expression precludes a detectable response in normal and ATA subjects.

Availability of LTC_4 synthase may be augmented by genetic up-regulation. LTC_4 synthase is present in eosinophils and mast cells. Expression of LTC_4 is variable, even in the same cell line. Recently, the genetic polymorphism of the 5' untranslated region of LTC_4 synthase has been described. It consists of two common alleles corresponding to the A-C transversion of nucleotide 444 upstream of the translation start. The C444 allele is twice as common in aspirin asthmatics as in normal controls or asthmatics not sensitive to aspirin. Patients with AIA have up-regulated LTC synthase mRNA expression in blood eosinophils, and increased gene transcripts are most pronounced in carriers of the C444 allele.

Eosinophil infiltration of airway tissue appears to be a central feature of AIA. The airway expression of interleukin-5, known to be involved in recruitment, activation,

maturation and perpetuation of survival of eosinophils is markedly increased in AIA patients.

To conclude, aspirin may remove PGE_2 – dependent suppression in all subjects, but only in AIA patients does increased bronchial expression of LTC_4 synthase allow marked overproduction of Cys-LTs leading to bronchoconstriction.

MECHANISM OF ACTION OF ANTI-INFLAMMATORY DRUGS

Almost any type of chemical or mechanical stimulus releases prostaglandins. The key enzyme in their synthesis is prostaglandin endoperoxide synthase (PGHS) or cyclooxygenase (COX), which possesses two catalytic sites. The first, a cyclooxygenase active site, converts arachidonic acid to the endoperoxide PGG_2. The second, a peroxidase active site, then converts the PGG2 to another endoperoxide PGH_2. Prostaglandin H_2 is further processed by specific isomerases to form prostaglandins, prostacyclin, and thromboxane A_2. Cyclooxygenase activity has long been studied in preparations from sheep seminal vesicles and a purified, enzymatically active COX was isolated in 1976 [1]. We now know that cyclooxygenase exists in at least two isoforms, COX-1 and COX-2.

In 1971 Vane proposed that the mechanism of action of the aspirin-like drugs (nonsteroidal anti-inflammatory drugs; NSAIDs) was through the inhibition of prostaglandin biosynthesis [2], and there is now a general acceptance of the theory. The inhibition by aspirin is due to the irreversible acetylation of the cyclooxygenase site of PGHS, leaving the peroxidase activity of the enzyme unaffected. In contrast to this unique irreversible action of aspirin, other NSAIDs such as ibuprofen or indomethacin produce reversible or irreversible COX inhibition by competing with the substrate, arachidonic acid, for the active site of the enzyme.

Gravito et al. [3] have determined the three – dimensional structure of COX-1, providing a new understanding for the actions of COX inhibitors. Each dimmer of COX-1 comprises three independent folding units: an epidermal growth factor – like domain, a membrane – binding motif, and an enzymatic domain. The sites for peroxidase and cyclooxygenase activity are adjacent but spatially distinct. The conformation of the membrane – binding motif strongly suggests that the enzyme integrates into only a single leaflet of the lipid bilayer and is thus a monotopic membrane protein. Three of the helices of the structure form the entrance to the cyclooxygenase channel and their insertion into the membrane could allow arachidonic acid to gain access to the active site from the interior of the bilayer. The cyclooxygenase – active site is a long, hydrophobic channel, and Picot et al. [3] provided evidence to suggest that some of the aspirin – like drugs, such as flurbiprofen, inhibit COX-1 by excluding arachidonate from the upper portion of the channel. Tyrosine 385 and serine 530 are at the apex of the long active site. Aspirin irreversibly inhibits COX-1 by acetylation of the serine 530, thereby excluding access of arachidonic acid [3]. The S (-) stereoisomer of flurbiprofen interacts via its carboxylate with arginine 120, thereby placing the second phenyl ring within van der Waals' contact of tyrosine 385. There may be a number of other subsites for drug binding in this narrow channel.

The three – dimensional structure of COX-2 has also now been published [4]. It closely resembles the structure of COX-1, except that the COX-2 active site is slightly larger and can accommodate bigger structures than those that are able to reach the active site of COX-1. A secondary internal pocket of COX-2 contributes significantly to the larger volume of the active site in this enzyme, although the central channel is also bigger by 17%. Selectivity for COX-2 inhibitors is dependent on the presence of Val at position 523 instead of the Ile found on COX-1 [5].

The constitutive isoform, COX-1, has clear physiological functions. Its activation leads, for instance, to the production of prostacyclin, which is antithrombogenic when released by the endothelium [6] and cytoprotective when released by the gastric mucosa [7]. It is also COX-1 in platelets that leads to thromboxane A_2 production, causing aggregation of the platelets to prevent inappropriate bleeding [8]. The existence of the inducible isoform, COX-2, was first suspected when Needleman and his group showed that cytokines induced the expression of COX protein [9] and that bacterial lipopolysaccharide increased the synthesis of prostaglandins in human monocytes *in vitro* and in mouse peritoneal macrophages *in vivo* [10]. The increase was inhibited by dexamethasone and associated with *de novo* synthesis of new COX protein. A year or so later, COX-2 was identified as a distinct isoform encoded by a different gene from COX-1 [11]. The human COX-2 gene at 8.3 kb is similar to the COX-2 gene of mouse and chicken, but smaller than the 22-kb human COX-1 gene. The amino acid sequence of its cDNA shows a 60% homology with the sequence of the noninducible enzyme, with the mRNA for the inducible enzyme being approximately 4.5 kb and that of the constitutive enzyme being 2.8 kb. However, both enzymes have a molecular weight of 71 kDa and similar active sites for the natural substrate and for blockade by NSAIDs. The inhibition by the glucocorticoids of the expression of COX-2 is an additional aspect of the anti-inflammatory action of the corticostroids. The levels of COX-2, normally very low in cells, are tightly controlled by a number of factors including cytokines, intracellular messengers, and availability of substrate.

COX-2 is induced by inflammatory stimuli and by cytokines in migratory and other cells, suggesting that the anti-inflammatory actions of NSAIDs are due to the inhibition of COX-2, whereas the unwanted side effects such as irritation of the stomach lining and toxic effects on the kidney are due to inhibition of the constitutive enzyme, COX-1 [12].

COX-1 performs a “housekeeping” function to synthesize prostaglandins that regulate normal cell activity. The concentration of the enzyme largely remains stable, but small (two- to fourfold) increases in expression can occur in response to stimulation with hormones or growth factors [13, 14]. Normally, little or no COX-2 is found in resting cells but its expression can be increased dramatically after exposure of cells to bacterial lipipolysaccharides, phorbol esters, cytokines, or growth factors. However, “constitutive” levels of COX-2 have been detected in some organs such as the brain and in uterine tissues during gestation.

In the gastrointestinal tract, the so-called “cytoprotective” action of prostaglandins in preventing gastric erosions and ulceration is mainly brought about by endogenously produced prostacyclin and PGE_2, which reduce gastric acid secretion and exert a direct vasodilator action on the vessels of the gastric mucosa. In addition to these major actions, prostanoids

stimulate the secretion of viscous mucus as a protective barrier and gastric fluid as well as duodenal bicarbonate [15].

In the kidneys the cortex produces both PGE_2 and PGI_2 whereas the renal medulla synthesizes mostly PGE_2. Both of these prostanoids are potent vasodilators, while PGE_2 in addition inhibits reabsorption of sodium and chloride from the ascending limb of the loop of Henle [16]. Prostaglandins also attenuate the reabsorption of sodium by vasopressin in the collecting ducts, thus further increasing urine flow [17].

In the lungs, prostaglandins have important actions on the tone of the bronchial tree and on the diameter of the pulmonary blood vessels. The airways of most species, including humans, are constricted by $PGF_{2\alpha}$, TXA_2, PGD_2, and PGI_2 whereas PGE_2 is a weak bronchodilator. Asthmatics are 8000 times more sensitive to the bronchoconstrictor action of inhaled $PGF_{2\alpha}$ than healthy subjects. Mediators of inflammation such as bradykinin, histamine, and 5-hydroxytryptamine all release prostaglandins from lung tissue. Histamine releases $PGF_{2\alpha}$ from human lung fragments by stimulating H_1 receptors. Lungs of asthmatics produce more histamine than normal lungs, which correlates with the greater number of mast cells found in asthmatic lungs [18].

PATHWAYS OF ARACHIDONATE METABOLISM

Three enzyme systems – cyclooxygenases (COX), lipooxygenases (LOX), and cytochrome P450 – dependent monooxygenases (CYP450) – generate lipid mediators (eicosanoids) from arachidonic acid (AA) via oxygenase reactions that are regiospecific and stereospecific [19]. Mammalian LOXs are designated by positional specificity relative to oxygenation of AA; three principle forms are recognized: 5-, 12-, and 15-LOX. The synthesis of leukotrienes (LTs) is initiated by 5-LOX, which converts AA to 5-hydroperoxy-eicosatrienoic acid (5-HPETE) followed by dehydration of 5-HPETE to form LTA_4 [20].

There are two principal pathways of LTA_4 metabolism either via LTA_4 hydrolase to form LTB_4 [21] or via LTC_4 synthase to form LTC_4 [22]. LTA_4 hydrolase is found in many tissues in which 5-LOX is absent [23]. For example, the small intestine has the highest activity of LTA_4 hydrolase but lacks 5-LOX. However, LTA_4 hydrolase is a dual – function enzyme, possessing aminopeptidase activity; the activity in some tissues may be directed toward metabolism of peptides, cleaving the N-terminal amino acid of the peptide [24]. Activated leukocytes, capable of producing an abundance of LTA_4, can serve as sources of LTA_4 to cells that lack 5-LOX, resulting in generation of LTC_4 by these cells [17]. This type of cell-cell interaction is apparently essential to the progression of some forms of inflammatory disease. LTA_4 hydrolase is inactivated by its product, LTB_4, which influences enzyme activity by covalent modification of the hydrolase.

PROSTAGLANDIN RECEPTORS

The conventional prostaglandin receptor is a cell surface receptor that is specific for each of the several classes of prostanoids. These have been designated FP, IP, TP, EP, and DP for

$PGF_{2\alpha}$, PGI_2, TXA_2, PGE_2, and PGD_2, respectively [25]. They had been characterized pharmacologically according to specificity as well as the rank order of their response to agonists and inhibition by antagonists. This approach has distinguished several subtypes of EP receptors, which differ in their coupling to G-proteins and linkage to signaling systems.

PGE (EP) receptors, unlike those of the other prostanoids, have a widespread distribution and multiple subtypes and possess varied and even opposing actions because of coupling via G-proteins to a number of signal transduction pathways that effect Ca^{2+} mobilization and either stimulation or inhibition of adenylate cyclase [26]. The recognition of the several EP receptor subtypes within a tissue or even a cell explains seemingly paradoxical actions of PGE_2. The EP_2 and EP_3 receptors are responsible for PGE_2 stimulation and inhibition of camp formation, respectively, and, thereby, promotion of sodium excretion and inhibition of the action of AVP in the mTAL and collecting tubules. EP1 receptors, via a Gs-protein linkage, stimulate PLC and result in Ca^{2+} mobilization and PKC activation, the latter acting as a negative regulator of EP_1 receptor activity by decreasing PGE_2 binding affinity to the EP receptor and dissociation of the G-protein from the receptor [26].

PGE_2 is known to inhibit activation – initiated responses of a number of bone marrow – derived inflammatory cells including the neutrophils, peritoneal and alveolar macrophages, some T lymphocytes, and mast cells. These inhibitory effects are associated with an elevation of intracellular cyclic AMP. The actions of PGE_2 on neutrophils and mast cells appear to be mediated through the EP_2 subtype of the PGE_2 receptors.

EICOSANOIDS IN THE PATHOGENESIS OF EXERCISE- AND ALLERGEN – INDUCED ASTHMA

Asthma is a condition characterized by symptoms of wheezing, chest tightness, coughing, and dyspnea, as well as characteristic physiological abnormalities of variable airflow obstruction and airway hyperresponsiveness to bronchoconstrictor stimuli [27]. Airway responsiveness is a term that describes the ability of the airways to narrow after exposure to constrictor agonists. Thus, airway hyperresponsiveness is an increased ability to develop this response. Airway hyperresponsiveness consists both of an increased sensitivity of the airways to constrictor agonists, as indicated by a smaller concentration of a constrictor agonist needed to initiate the bronchoconstrictor response [28], and a greater maximal response to the agonist [29].

Asthmatics have airway hyperresponsiveness to a wide variety of bronchoconstricotr agonists. These include inhaled pharmacological agonists, such as histamine, acetycholine, metacholine, cysteinyl leukotrienes, and stimulatory prostaglandins [30, 31]. In addition, airway hyperresponsiveness is also present to a number of physical stimuli as exercise, and to inhaled allergens. The cysteinyl leukotrienes are, overall, the most important mediators causing bronchoconstriction in asthmatics. The bronchoconstriction that occurs during the early and late responses is in large part caused by allergen – induced release of cysteinyl leukotrienes. This was initially suggested by Brocklehurst [32], who demonstrated the production of slow – reacting substance of anaphylaxis (SRS-A) after sensitized lung fragments were challenged by specific allergens. SRS-A is now known to consist of the

cysteinyl leukotrienes (LT) C_4, D_4, and their stable excretory metabolite LTE_4 [33]. Several investigators have demonstrated increases in urinary LTE_4 after allergen – induced bronchoconstriction [34, 35]. The increases in urinary LTE_4 were significantly correlated, in one study, with the magnitude of the bronchoconstriction [34]. Interestingly, no significant increases in urinary LTE_4 could be demonstrated during the allergen – induced late asthmatic response, even though the magnitude of the bronchoconstriction was similar to the early response [34]. These data suggested that the cysteinyl leukotrienes were released during allergen inhalation and were important causes of bronchoconstriction during the allergen – induced early phase, but not the late – phase bronchoconstrictor responses. The best evidence for a central role for the cysteinyl leukotrienes in causing allergen – induced bronchoconstriction is suggested by the observations that a number of different LTD_4 – receptor antagonists and leukotriene synthesis inhibitors have been demonstrated to markedly attenuate the bronchoconstrictor responses after inhaled allergen [36]. These studies have indicated that LTD_4 antagonists and leukotriene synthesis inhibitors attenuate allergen – induced early responses by up to 80% [36, 37], and surprisingly considering the data on urinary LTE_4 excretion during the late response, also attenuate the late response by up to 50% [38], suggesting that, as inhaled leukotriene D_4 does not itself cause the development of late responses [38], newly generated cysteinyl leukotrienes, possible from inflammatory cells, such as eosinophils recruited into the airways during the late asthmatic response [39, 40], are partially responsible for the bronchoconstriction during this response. The component of allergen – induced early responses not influenced by antileukotrienes is caused by thromboxane A_2 [41] and histamine release, while the combination of cysteinyl leukotriene and histamine is mainly responsible for bronchoconstriction during the late response.

In asthma, prostaglandins are most easily considered in two classes. These are stimulatory prostaglandins, such as PGD_2 and $PGF_{2\alpha}$, which are potent bronchoconstrictors, and inhibitory prostaglandins, such as PGE_2, which can reduce bronchoconstrictor responses and can attenuate the release of acetylcholine from airway nerves.

ASPIRIN – INDUCED ASTHMA

Aspirin is not only one of the best – documented medicines in the world, but also one of the most frequently – used drugs of all times [42]. The history of use of salicylic acid derivates dates back over 23 centuries. Since the time of Hippocrates, the extracts of myrtle leaves, willow and poplar bark as well as wintergreen oil were used as natural painkillers. Salicylic acid was first synthesized by Herman Kolbe in 1859, then in 1899 Hoffman synthesized acetylsalicylic acid, subsequently introduced into the market by Bayer under the generic name aspirin (ASA) – the most frequently used drug nowadays [43].

Shortly after its introduction into therapy, aspirin was implicated as the cause of violent bronchospasm [44]. The association of aspirin sensitivity, asthma and nasal polyps was described by Widal *et al.* in 1922. This clinical entity, subsequently named ‘aspirin triad’, was brought to physician’s attention by Samter and Beers [45], who, in the late 1960s, presented a perceptive description of the clinical course of this syndrome.

In some asthmatic patients, aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) precipitate asthma attacks [46, 47]. This distinct clinical syndrome, now called aspirin – induced asthma (AIA) (or aspirin – sensitive or aspirin – intolerant asthma) affects about 10% of adults with asthma, more often women than men [48].

Not only aspirin, but several other NSAID precipitate attacks. Major offenders include: indomethacin, fenamic acid, ibuprofen, fenoprofen, ketoprofen, naproxen, diclofenac, piroxicam, tiaprofenic acid, noramidopyrine, sulfinpyrazone, and phenylbutazone. Not all of these drugs produce adverse symptoms with the same frequency. This depends on the drug's anticyclooxygenase potency, dosage and on individual sensitivity. If necessary, patients with AIA can take safely sodium salicylate, choline magnesium trisalicylate, dextropropoxyphene, azapropazone, and benzydamine. Most patients also tolerate paracetamol well at dose not exceeding 1000 mg daily.

In most patients, the first symptoms occur during the third decade as intense rhinitis. Over a period of months, chronic nasal congestion with rhinorrhea develops; physical examination often reveals nasal polyps and anosmia. Bronchial asthma and intolerance to aspirin develop subsequently, on average within 4 years. The intolerance presents as a unique picture: within 1 h of ingestion of aspirin, an acute asthma attack occurs, often accompanied by rhinorrhea, conjunctival irritation and scarlet flushing of head and neck [49]. Aspirin is a common precipitating factor of life – threatening attacks of asthma [50]; in a recent large survey, 25% of asthmatic patients requiring emergency mechanical ventilation were found to be aspirin – intolerant [51]. Attacks may be precipitated not only by aspirin, but also by several other NSAID that inhibit cyclooxygenase enzymes (cyclooxygenase-1 and –2).

In patients with AIA, their asthma runs a protracted course, despite the avoidance of aspirin and cross-reactive drugs. Blood eosinophil count is raised, and eosinophils are present in airways. Skin tests with aspirin are negative. Atopy traits are not rare, and, in fact are more common than in general population [52].

CHRONIC INFLAMMATION OF THE AIRWAYS

The airways of patients with AIA show signs of persistent inflammation, with marked eosinophilia, epithelial disruption, cytokine production, and upregulation of adhesion molecules [53, 54]. On bronchial biopsy specimens, eosinophils are 4-fold more numerous than in subjects with ATA and 15-fold more numerous than in biopsy specimens of normal mucosa [55]. There is a tendency for higher counts of CD68 (+) macrophages, whereas counts of T lymphocytes do not differ from other types of asthma or control subjects. Both increase [53] and suppression [55] of mast cell numbers, as compared with normal control subjects, have been reported, with the discrepancies probably reflecting mast cell activation and disruption.

Eosinophil infiltration of airway tissue appears to be a central feature of AIA. Eosinophil cationic protein is found in BAL fluid and increases after ASA-lysine segmental challenge in patients with AIA [56]. The airway expression of IL-5, known to be involved in the recruitment, activation, maturation, and perpetuation of survival of eosinophils, is markedly increased in patients with AIA [55]. Immunohistochemical staining of bronchial biopsy

specimens shows that eosinophils are the predominant cells containing LTC_4 synthase. Thus sheer numbers of eosinophils, loaded with normally expressed or increased Cys-LT enzymes, may be responsible for overproduction of total Cys-LTs, a differentiating feature of AIA. Two chemokines, RANTES and eotaxin, are present in nasal mucosa and polyp tissues, and their local expression is upregulated in individuals with chronic sinusitis [57]. Their role in the pathogenesis of eosinophilic inflammation in AIA remains to be established.

Persistent airway inflammation in AIA could result from non-IgE – mediated reactions to an endogenous or exogenous antigen or virus. These possibilities are supported by the finding of elevated markers of autoimmunity [58-60], enhanced IgG_4 synthesis [61], and HLA association with AIA [62]. A latent virus infection could [63]: (1) modify the genetic message for COX molecules, altering response to NSAIDs by synthesizing unknown metabolites that stimulate LT production; (2) evoke an immunologic response, perhaps dominated by specific cytotoxic lymphocytes and eosinophils, previously suppressed by PGE2; or (3) leave DNA fragments in native DNA, which might direct synthesis of altered enzymes.

DIAGNOSIS OF AIA

While a patient's clinical history might raise a suspicion of aspirin – induced asthma, the diagnosis can be established with certainty only by aspirin challenge [49]. There are three types of provocation tests, depending on the route of administration: oral, inhaled, and nasal [64]. Oral challenge tests are most commonly performed; in most patients threshold doses evoke positive respiratory reactions after ingesting 30-150 mg of ASA (average, 60 mg) [65]. In inhalation tests, aerosols of L-lysine acetylsalicylic acid are administered [66]. Inhalation challenge can be completed in 4 h, but reactions are usually restricted to the lower respiratory tract. Nasal provocation testing [67] is an attractive research model and can be also used as a diagnostic procedure in outpatients; its value, however, is limited by reduced sensitivity when compared with oral or inhalational provocation testing.

PATHOGENESIS OF ASPIRIN – INDUCED ASTHMA

Although clinical reactions precipitated by aspirin in asthmatic patients are reminiscent of immediate – type hypersensitivity reactions, numerous attempts to demonstrate specific antibodies against aspirin or its derivatives have been unsuccessful [68]. Neither differences in bioavailability of ASA nor the formation of salicylic acid seem to contribute to ASA – induced respiratory reactions [69]. Furthermore, in patients with AIA, asthmatic attacks can be precipitated not only by aspirin but also by other NSAIDs with distinct chemical structures, a fact that makes immunological crossreactivity most unlikely [70]. Over the years, the importance of arachidonic acid metabolism in pathogenesis of AIA has become apparent.

THE CYCLOOXYGENASE THEORY

The cyclooxygenase (COX) theory [71] proposes that precipitation of asthma attacks by ASA and NSAIDs is not based on antigen – antibody reactions but stems from the pharmacological action of the drugs [2], namely specific inhibition in respiratory cell of intracellular COX enzymes.

The original observations by Szczeklik *et al.* in 1975 [46, 70], that drug cross-reactivity can be predicted on the basis of each NSAIDs *in vitro* inhibition of COX, have been consistently reaffirmed over the years [72, 73]. After aspirin desensitization, cross – desensitization to other NSAIDs that inhibit COX also occurs [72]. These constitute the major facts supporting the COX theory [71].

The COX enzyme, which appears to be central to the mechanism of aspirin intolerance, exists in at least two isoforms, COX-1 and COX-2, encoded by distinct genes. Both isoforms are expressed in normal human respiratory epithelium [74] and are not quantitatively up-regulated in the main bronchi in stable asthma and chronic bronchitis. The overall expressions of COX-1 and COX-2 are similar in bronchial biopsies obtained from aspirin tolerant and aspirin – intolerant asthmatic patients [55, 75, 76]. Low doses of AIA, indomethacin, and piroxicam precipitate asthmatic attacks in sensitive patients and inhibit both enzymes, but are much more potent inhibitors of COX-1 than COX-2.

Nimesulide and meloxicam, drugs known to inhibit COX-2 substantially more than COX-1, are well tolerated by ASA – sensitive asthmatic subjects when given average therapeutic doses, but cause rhinorrhea and mild asthma attacks when ingested in higher doses [77, 78]. Introduction into the clinic of highly specific COX-2 inhibitors – celecoxib and rofecoxib [79] – will provide an interesting tool for further determining the role of COX isoforms in AIA [80-83].

When segmental bronchial challenge with aspirin was performed in AIA and aspirin – tolerant asthmatic (ATA) patients, and cyclooxygenase products were measured in BAL fluid 15 min after aspirin instillation, a marked difference became apparent [84]. Aspirin significantly depressed PGE_2 and thromboxane B_2 in both groups; however, PGD_2, $PGF_{2\alpha}$ and $9\alpha,11\alpha$-PGF_2 decreased only in ATA patients. In individual AIA subjects, PGD_2 levels showed a variability in response to aspirin from marked increase to depression [84]. Activated mast cells could be the source of PGD_2. The role of these cells in AIA has been suggested by Fisher *et al.* [85] and Warren *et al.* [86]. The latter reported that segmental bronchial challenge with indomethacin led to a rise in PGD_2 and histamine in the BAL fluid of threee AIA patients, in contrast to three ATA patients. A modest increase is seen even in the urinary levels of $9\alpha,11\alpha$-PGF_2 [87] and PGD metabolite [88], in AIA patients after aspirin bronchoprovocation.

Such an alteration of cyclooxygenase response to aspirin may result from an acquired change in the COX gene(s), perhaps as a consequence of a latent viral infection [63]. In AIA, the signal for 5-lipooxygenase activation stems from the removal by aspirin of PGE2, a prostaglandin that inhibits leukotriene biosynthesis [89] and appears to play a special role in this type of asthma [90].

THE LIPOXYGENASE PATHWAYS

The cysteinyl leukotrienes (Cys-LTs) are a family of bioactive fatty acid mediators that were originally described in 1940 as the slow – reacting substance of anaphylaxis (SRS-A) [91]. In 1979 the structure of SRS-A was elucidated, and the parent molecule, leukotriene C_4, was described [92], followed by its two biologically receptor – active metabolites, LTD_4 and LTE_4. The cysteinyl leukotrienes are synthesized during the process of the conjugation of leukotriene A_4 to reduced glutathione [93]. It is mediated by the enzymatic action of leukotriene C_4 synthase (LTC4S) [94], the perinuclear membrane enzyme, to form the intracellular parent compound, LTC_4. On its formation, LTC_4 is exported from the cell by a carrier – mediated mechanism and is processed extracellularly by a sequential cleavage of glutamic acid and glycine residues. These reactions are catalyzed by γ-glutamyl transpeptidase [95] and dipeptases [96], respectively, and yield LTD_4 and LTE_4, the receptor – active derivates. Thus the generation of the pathophysiologically important cysteinyl LTs is dependent on the pivotal enzyme – LTC4S.

Over the last few years, Cys-LTs have emerged as major mediators in AIA. Most patients with AIA excrete 2- to 10 – fold higher amounts of LTE_4 in urine than asthmatic subjects who tolerate aspirin [97]. Cys-LTs are also released into the nasal cavity after nasal challenge with aspirin [98] and into bronchi after challenge with lysine – aspirin [84].

Therefore, a clue to the underlying anomaly in aspirin intolerance is provided by the overproduction of Cys-LTs.

OVEREXPRESSION OF LEUKOTRIENE C_4 SYNTHASE IN AIA

Recently, a mechanism was described [55, 76] that resolves the question of why only patients with aspirin – induced asthma (AIA), but not those with aspirin tolerant asthma (ATA) or normal subjects, produce large amounts of Cys-LTs when the PGE_2 'brake' is removed by NSAIDs.

In bronchial biopsies from AIA, ATA patients and normal subjects, LTC_4 synthase was immunostained. Counts of cells expressing this terminal enzyme for Cys-LTs synthesis were fivefold higher in AIA biopsies than in ATA biopsies and 18 – fold higher than in normal biopsies. LTC_4 synthase (+) cells were predominantly eosinophils.

Aspirin challenge caused a significant rise in bronchoalveolar lavage fluid (BALF) levels of total Cys-LTs in the AIA subjects, but not in the ATA group. The rise in BALF Cys-LTs correlated significantly with the counts of bronchial mucosal eosinophils, but not with mast cells counts, confirming eosinophils as the predominant source of the Cys-LTs response to aspirin. Aspirin challenge was followed in AIA subjects by a dramatic rise in eosinophil counts within the BALF, suggesting migration of activated eosinophils into airway lumen.

PGE_2 production by eosinophils or other cells has been suggested to act as a brake on leukotriene synthesis in all subjects [99]. Removal of this brake by cyclooxygenase inhibitors triggers significant Cys-LTs production only in AIA subject because they alone havehigh levels of overexpression of LTC_4 synthase. The relative lack of LTC_4 synthase expression precludes a detectable response in normal and ATA subjects.

To conclude, aspirin may remove PGE_2 – dependent suppression in all subjects, but only in AIA patients does increased bronchial expression of LTC_4 synthase allow marked overproduction of Cys-LTs leading to bronchoconstriction.

GENETIC PREDISPOSITION TO LTC4S OVER-REPRESENTATION

Availability of LTC_4 synthase may be augmented by genetic up-regulation. The promoter region of the enzyme is complex and predicts interaction with many transcription enhancers. LTC_4 synthase is present in eosinophils and mast cells. Expression of LTC4 is variable, even in the same cell line. Recently, the genetic polymorphism of the 5' untranslated region of LTC_4 synthase has been described [100]. It consists of two common alleles corresponding to the A-C transversion of nucleotide 444 upstream of the translation start. The C444 allele is twice as common in aspirin asthmatics as in normal controls or asthmatics not sensitive to aspirin. Patients with AIA have up-regulated LTC_4 synthase mRNA expression in blood eosinophils, and increased gene transcripts are most pronounced in carriers of the C444 allele [101].

THE ROLE OF EOSINOPHILS

Eosinophil infiltration of airway tissue appears to be a central feature of AIA. The airway expression of interleuikin-5, known to be involved in recruitment, activation, maturation and perpetuation of survival of eosinophils is markedly increased in AIA patients [102].

Asthma is a chronic eosinophilic inflammatory disease. In aspirin – induced asthma eosinophils are the predominant source of Cys-LTs in the airways. Eosinophilia is a prominent feature in the blood, airways, and nasal polyps of aspirin – sensitive patients [103].

OTHER HYPOTHESIS

In 1995 Serhan *et al.* discovered a new pathway for lipoxin biosynthesis: COX-2 acetylated by aspirin, converts arachidonic acid into 15-epi-lipoxins [104, 105]. Based on these interesting findings, others suggested that aspirin – induced adverse respiratory reactions may be caused by excessive formation of 15-HETE and 15-epi-lipoxins [106].

The persistent airway inflammation could result from a non – IgE mediated reaction to an endogenous or exogenous antigen, possibly a chronic viral infection [63]. A latent virus infection could modify the genetic message for the COX molecule, making it prone to produce unknown metabolites that stimulate leukotrienes production in response to NSAID.

NASAL POLYPS

Nasal polyps are protrusions of an edematous mucous membrane, originating in the upper part of the nose around the openings to the ethmoidal sinuses. The polyps protrude into the nasal cavity from the middle (and superior) meatus, resulting in nasal blockage and abolished airflow to the olfactory region. Nasal polyposis, consisting of recurrent, multiple polyps, is part of an inflammatory reaction involving the mucous membrane of the nose, paranasal sinuses, and often the lower airways. Most patients with aspirin – induced asthma develop a characteristic pattern of chronic tissue inflammation associated with marked sinus mucosal thickening and nasal polyposis. Using the modern techniques of exploration of the nose and sinuses, such as endoscopy and computerized – tomography (CT) scan, the presence of a sinus disease is detected in almost all patients with aspirin – induced asthma. In several recent series on patients with chronic sinusitis undergoing functional endoscopic surgery, the prevalence of aspirin sensitivity was 11-20% [107]. Although many theories on the pathogenesis of polyposis have been presented, at present the origin of polyps is unknown. The most common disease associated with nasal polyps is asthma with aspirin intolerance. Other diseases, such as cystic fibrosis, primary ciliary dyskinesia, and Young's syndrome, also have a high frequency of nasal polyps. Inflammation, obstruction of sinus drainage, and infection are the factors that may contribute to the development of chronic rhinosinusitis. However, histopathological studies reveal similar inflammatory changes in both the sinus tissues and the nasal polyps. This finding suggest that inflammation appears to be the most important contributing factor to chronic rhinosinusitis.

Macroscopically, nasal polyps of aspirin – sensitive asthma (ASA) patients are similar to other types of polyposis. They are usually bilateral and multiple evaginations of the nasal mucosa attached by a pedicle and arising from the ethmoid sinus, the middle turbinate, and maxillary sinus. Nasal polyps have a characteristic appearance. They are smooth, soft, light gray, semitranslucent, movable structures. Nasal polyps in aspirin – sensitive asthma subjects most commonly follow an aggressive course filling the nasal cavity and often protruding from the anterior nares and/or projecting posteriorly into the nasopharynx. Facial deformation is not uncommon and it is due to midfacial expansion, which occurs as a consequence of the increased pressure on bones caused by nasal polyps and inflamed sinus.

The general histological appearance of nasal polyps and chronic rhinosinusitis associated with aspirin intolerance bears many similarities to that of bronchial asthma. Epithelial damage (desquamation) is frequently found in nasal polyps. Small areas of epithelial metaplasia without atypia can also be seen. As in the typical findings in bronchial asthma, a thickened basement membrane is a prominent characteristic of nasal polyps. Nasal polyps contain myofibroblast – like cells [108], which have also been described in asthma. The stroma of polyps and sinus mucosa is very edematous and contains a mixed inflammatory reaction with neutrophils, activated lymphocytes, mast cells, and eosinophils [108]. Eosinophils are the most abundant inflammatory cell type.

Recent studies indicate that inflammatory and structural cells within nasal polyps are an important source of cytokines [109-112]. Activated eosinophils produce a variety of cytokines including IL-3, IL-5, and GM-CSF. Nasal fibrblast and nasal epithelial cells derived from nasal polyps release GM-CSF, TNFα, IL-8, and RANTES. Epithelial cells from

nasal mucosa and polyps enhance eosinophil survival, suggesting that this can be a mechanism for eosinophil accumulation in the nose [110-112]. Lymphocytes are also present in nasal polyps and sinusitis. Recent studies have shown that TH2-type T lymphocytes in sites of allergic inflammation produce GM-CSF, IL-3, IL-5, and IL-4 [113]. All these cytokines contribute to eosinophil accumulation.

Patients with nasal polyps, chronic rhinosinusitis, and aspirin sensitivity should avoid all products that inhibit COX. Most patients will tolerate paracetamol at a dose not exceeding 1000 mg daily. They can safely take codeine, dextropoxyphene, opiates, and their derivatives (tramadol, pentazocin). However, the avoidance of NSAID is not a specific therapy because even a strict control of NSAID intake does not alter the course of bronchial and nasal disease.

PREVENTION AND TREATMENT

The general rules concerning treatment of AIA do not differ from the published guidelines on the management of asthma. Most patients with AIA have moderate or severe persistent asthma. However, patients with AIA whose sinusitis is in remission may have mild asthma. These observations were recently confirmed by a multicenter study performed by aprticipants of the European Network on Aspirin – Induced Asthma. Among 365 patients with AIA in the European Network on Aspirin – Induced Asthma study [114], 79% were receiving long –term oral or inhaled corticosteroid therapy. Half of the subjects were treated with systemic corticosteroids, and 32% were treated with inhaled corticosteroids alone, although in relatively high doses (800 to 2000 μg per day). Nearly 20% of patients were treated with intravenous corticosteroids during the year preceding registration in the database. In sporadic cases, cyclosporin may help reduce the need for higher doses of corticosteroids. However, its clinical effects are negligible, and side effects are of concern [115].

There are, however, some important differences in the care of patients with AIA. First, to prevent life-threatening reactions, patients with AIA should avoid ASA, all products containing ASA, and other analgesics that inhibit COX. Thus the education of physicians, pharmacists, and patients is extremely important. The patient should receive a list of drugs that are contraindicated, preferentially with both generic and trade names.

If necessary, patients can usually take acetaminophen (paracetamol). A cross-reaction prevalence of 34% after ingestion of 1000 and then 1500 mg of acetaminophen was reported in patients with AIA [116]. It is generally safer not to exceed a dose of 1000 mg of acetaminophen at the beginning of therapy but to administer small doses and monitor the patient for 3 hours. Patients with AIA can also safely receive sodium salicylate, salicylamide, choline magnesium trisalicylate, benzydamine, chloroquine, azapropazone, and dextropropoxyphen. These drugs are devoid of anti-COX activity or are weak COX-2 inhibitors. Unfortunately, they are poor analgesics and have only minimal anti-inflammatory effects. Nimesulide and meloxicam, drugs known to inhibit COX-2 more than COX-1, induced mild bronchial obstruction, although only at high doses [117-119]. Selective inhibitors of COX-2 are safe for patients with AIA because constitutive COX-1 will continue to synthesize the protective prostanoid, PGE_2.

There exists, however, another strategy to administer ASA or NSAIDs to patients with AIA. A state of ASA tolerance can be induced and maintained by aspirin desensitization. Small incremental oral doses of ASA are ingested over the course of 2 to 3 days until 400 to 650 mg of ASA is tolerated. ASA can then be administered daily, with doses of 80 to 325 mg used to maintain desensitization. After each dose of ASA, there is a refractory period of 2 to 5 days, during which ASA and other COX inhibitors can be taken with impunity. This is important for patients with AIA who have degenerative joint diseases, rheumatic diseases, and headaches and as a preventive measure for treatment of vascular diseases.

During the state of ASA desensitization, if ASA doses are increased to 650 mg and taken twice daily continuously, the patients usually experience improvement in their chronic respiratory symptoms and signs, especially in the nose [120, 121]. There are no clear – cut indications for ASA desensitization treatment, but nasal inflammatory disease appears to respond best. The ideal candidate for this treatment is a patient with AIA who just completed sinus / polyp surgery. ASA desensitization treatment was shown to delay recurrence of nasal polyp formation by an average of 6 years [121]. The mechanism of ASA desensitization in patients with AIA is only partially understood. It may lead to reduction of airway responsiveness to LTE_4 because of downregulation of Cys-LT receptors [122], which reduces receptor responsiveness to the same burden of Cys-LTs. At acute desensitization, urinary LT levels are the same as baseline levels and are therefore clearly available for stimulation of Cys-LT receptors. Patients maintained for months in a state of ASA desensitization still respond to oral ASA challenge with a rise in LTE4 urinary excretion, although the responses were blunted when compared with the original ASA challenges, and the patients were all asymptomatic [123].

A drug and a dye should be mentioned when discussing drug cross – reactivity in AIA. Intravenous hydrocortisone hemisuccinate may sporadically provoke bronchoconstriction in patients with AIA [124]. In a study by Feigenbaum *et al.* [125], only 1 of 44 ASA – sensitive asthmatic subjects reacted to both hydrocortisone succinate and methylprednisolone succinate. Coincidental IgE – mediated reactions to the succinate molecule, rather than cross-sensitivity, was supported by the fact that readministration of hydrocortisone succinate during the ASA – desensitized state continued to induce a respiratory reaction in this patient. Tartrazine, a yellow azo dye used for coloring foods, drink, drugs, and cosmetics was frequently cited in older reports as a cause of bronchospasm [126]. As early as 1979, Weber *et al.* [127] reported that in their ASA-sensitive asthmatic patients, reduced lung function values after tartrazine challenges resulted from withholding essential anti – asthmatic controller medications, rather than reactions to tartrazine. More recently, extensive and controlled studies indicate that tartrazine intolerance is extremely rare among ASA – sensitive asthmatic subjects [128, 129]. Because tartrazine does not inhibit COX [130], it is likely that the rare tartrazine – induced reactions in patients with AIA are IgE mediated and coincidental rather than cross-reacting.

The anti-LT drugs have been introduced for treatment of AIA [131]. Currently available drugs either inhibit LT synthesis by blocking 5-LO (i.e., zileuton) or by blocking specific Cys-LT receptors (i.e., zafirlukast, montelukast, and pranlukast). Pretreatment with LT-modifying drugs attenuated ASA – provoked nasal and bronchial reactions in most, but not all, patients [132-135]. However, all studies were performed with prior established provoking

doses of ASA. A recent study by Pauls *et al.* [136] reported that higher therapeutic doses of ASA overcame protection from pretreatment with zileuton. In these 10 patients who were theoretically protected by zileuton, after challenge with the same or larger provoking doses of ASA, 4 of 10 experienced significant asthmatic reactions, and 10 of 10 experienced naso – ocular reactions.

Bronchodilatation has also been observed after treatment with anti-LT drugs, indicating that Cys-LTs have an effect on intrinsic airway tone in AIA. Two clinical trials with anti-LT drugs in patients with AIA have been concluded. In a Swedish – Polish double – blind, placebo – controlled, cross – over study, 40 patients with AIA received 6-week add-on treatment with zileuton (600 mg given 4 times daily) [137]. While continuing baseline inhaled and systemic corticosteroids, zileuton provided acute and chronic improvement in pulmonary function measurements expressed both as increased FEV_1 values from baseline and higher morning and evening peak expiratory flow values compared with placebo – treated patients, despite a lowered requirement for rescue bronchodilators. Zileuton also diminished nasal dysfunction (remarkable return of smell, less nasal congestion, and a trend for less stuffiness and higher nasal inspiratory flow) and caused a small but distinct reduction in histamine – induced bronchoconstriction.

The Cys-LT receptor antagonist montelukast has been used in a double – blind, placebo – controlled, parallel – group, 4–week study in 80 patients with AIA whose symptoms were not fully controlled by treatment with inhaled or oral corticosteroids [138]. The patients received either oral montelukast 10 mg or placebo each night. Montelukast treatment significantly improved parameters of asthma control (weekly FEV_1, daily peak expiratory flow rate, and reduction in β-agonist use). Patients receiving montelukast had less nocturnal asthma and more asthma – free days and reported significant improvement in asthma – specific quality of life and global assessments. In rare cases of asthma, including patients with AIA, treatment with zafirlukast was associated with Churg – Strauss syndrome [139, 140]. It has been assumed that these patients already had eosinophilic vasculitis and systemic steroid withdrawal during zafirlukast therapy, which led to reappearance of their disease. However, Churg – Strauss syndrome during zafirlukast treatment was recently reported in 2 patients who were not receiving systemic corticosteroids [141].

The β_2-adrenergic agonist salbutamol [142, 143] has been reported to protect against ASA – induced attacks of asthma through a mechanism that seems unrelated to its bronchodilator properties. In a recently concluded study by Szczeklik *et al.* [142], salmeterol effectively prevented bronchial reactions to inhalation of lysine – ASA in patients with AIA. It also attenuated the expected ASA – provoked rise in urinary excretion of LTE_4 and the stable PGD_2 metabolite. Thus salmeterol appears to directly interfere with eicosanoid metabolism in AIA, a finding which might to be of clinical relevance.

Chronic eosinophilic rhinosinusitis frequently leads to development of nasal polyps in patients with AIA. Treatment is difficult. Intranasal fluticasone is an effective treatment for rhinitis in many patients with AIA [144], particularly when nasal passages are patent. Sinus/polyp operations are often performed, but recurrence of nasal polyps usually occurs. ASA desensitization followed by daily treatment with ASA 650 mg administered twice daily is also an option [124] and should be initiated when other measures fail or frequent sinus/polyp operations are required.

DESENSITIZATION IN ASPIRIN – INDUCED ASTHMA

We typically think of desensitization as an alteration in the immune response, engineered by repeated exposure to antigens, thus reducing IgE – mediated reactions. Since IgE – mediated mechanisms have not been established as being responsible for aspirin – induced respiratory reactions and are considered by most investigators to be unlikely perpetrators of this disease [145], desensitization is used here in its broadest sense and refers to reducing the reactions to ASA by repeated and increasing exposure to ASA until all reactions cease [146].

In 1922, Widal *et al.* published the first report of successful ASA desensitization [147]. By administering small and then increasing daily doses of ASA, they were able to induce tolerance to full therapeutic doses of ASA. They were able to induce tolerance to full therapeutic doses of ASA in one individual. Who had previously been shown to react to ASA with a documented respiratory reaction. In 1976, Zeiss and Lockey [148] reported a 72-hr refractory period to ASA after an indomethacin – induced respiratory reaction in a known ASA-sensitive asthmatic patient. In 1977, Bianco *et al.* [149] reported tolerance to ASA in a known ASA – sensitive athmatic patient, after repeated bronchial inhalation challenges with ASA – lysine. In 1980, Stevenson *et al.* [150] reported two ASA – sensitive asthmatic patients who became refractory to ASA after oral ASA challenges had induced typical bronchospastic reactions. Both patients, after achieving the ASA – desensitized state, were then treated with daily ASA over the ensuing months and experienced improvement in their respiratory disease.

All ASA – sensitive patients can be successfully desensitized to ASA [106, 146]. Desensitization is accomplished by reintroducing the dose of ASA that initiated the ASA reaction on the previous day. As soon as a reaction no longer occurs, after repeat exposure to the same dose of ASA, the next highest dose of ASA is given and repeated until further reactions cease. Once a reaction occurs, ASA – desensitizing doses are suspended for that day. The process of escalating ASA doses continues on successive days until the patient can tolerate 650 mg without any reactions. At this point, the patient can take any dose of ASA or nonsteroidal anti-inflammatory drug without any adverse respiratory reaction [150] and at the same time experiences an opening of nasal passages. After ASA desensitization, in the absence of further exposure to ASA, the desensitized state persists for 2-5 days with full sensitivity returning after 7 days [146].

During ASA – induced respiratory reactions, peripheral monocytes from ASA-sensitive asthmatic patients undergoing induced respiratory reactions synthesize increased amounts of leukotrienes. By contrast, after acute ASA desensitization, defined as 3 hr after first ingestion of ASA 650 mg without any adverse effect, peripheral blood monocytes synthesized significantly less thromboxane B_2 but only slightly less LTB_4, the preferential 5-LO product of peripheral monocytes [151]. Additionally, nasal LTC_4 and histamine, which had been increased in nasal secretions during ASA – induced respiratory reactions, disappeared at the point of acute desensitization. Similarly, serum histamine and tryptase levels, which were elevated during ASA – induced respiratory reactions, returned to low baseline levels following acute desensitization. During respiratory reactions induced by either oral or inhalation ASA challenges, LTE_4 urine levels were found to be significantly elevated when compared to baseline concentrations [152-154]. However, after acute desensitization, urinary

LTE_4 concentrations returned to baseline levels [155]. Arm *et al.* [156], on the first day following ASA desensitization, demonstrated a 20 – fold decrease in bronchial airway responsiveness to LTE_4, compared to predesensitization responses to inhalation of LTE_4 in ASA – sensitive asthmatics.

By contrast, after treatment with ASA 650 mg for 2 or more weeks, during chronic desensitization, Juergens *et al.* [151] showed that peripheral monocyte synthesis of LTB_4 declined substantially, to the same level found in normal controls. Nasser *et al.* [155] reported that during chronic ASA desensitization for 6 months, urine LTE_4 levels declined to lower values, but not to values found in normals. It was shown that treatment with ASA 650 mg was associated with significant declines in urinary LTE_4 levels in the majority of patients with the mean decrease statistically significant. However, urinary LTE_4 levels from some patients did not change during long – term ASA desensitization treatment. These experiments suggested that ASA desensitization, particularly long – term treatment with higher doses of ASA in some patients, probably inhibits both COX and other enzymes above LTE_4 and probably at the PLA_2 level, leading to diminished synthesis of both prostanoids and LTs. Simultaneously, LTE_4 bronchial receptors remain down-regulated [156], further blunting the effects of any available terminal LTs.

EUROPEAN NETWORK ON ASPIRIN – INDUCED ASTHMA

In 1993, 21 university departments from 14 European countries jointly set up the European Network on Aspirin – Induced Asthma (AIANE). The Department of Medicine of Jagiellonian University School of Medicine in Krakow, Poland has been the coordinating center. The whole project was financially aided by the Commission of the European Communities.

One of the chief aims of the AIANE has been to provide a good insight into clinical course of AIA. For this purpose a relational database has been developed. Recent data indicate, that all over Europe, aspirin – induced asthma develops in a similar characteristic way [157, 158]. Its course is influenced by sex and the presence of atopy. In the half of the patients, asthma is severe, and steroid-dependent. The uniform natural history of aspirin – induced asthma might suggest a common underlying principle.

CONCLUSION

In summary, diagnosis of aspirin – induced asthma should not pose difficulties to a perceptive physician. Once the diagnosis is made, the patient should be given a detailed list of drugs known to be major offenders, as well as a list of safe alternatives.

Several hypotheses presented here are now being tested. New hypotheses might be expected to emerge. Though aspirin – induced asthma guards its secrets well, it attracts more and more scientists and clinicians, convinced that unraveling the mysteries of this syndrome will give new insights into pathogenesis of asthma [159].

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Chapter IV

Autonomic Neuronal Plasticity in Chronic Allergic Asthma: Interactions between Nervous and Immune System

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ABSTRACT

Allergic bronchial asthma comprises reversible bronchus obstruction based on a pathological airway hyperreactivity. This airway hyperreactivity is under close control of the lung's autonomic nervous system which predominantly consists of the non-adrenergic non-cholinergic (NANC) and the cholinergic nervous system. There is growing evidence that mediators emerging from local allergic airway inflammation directly modulate the plasticity of the autonomic neuronal network. Neurotrophins – especially Nerve Growth Factor and Brain-Derived Neurotrophic Factor – deriving from immune cells and epithelia directly affect sensory nerves of the NANC-system. In addition, eosinophilic products like major basic protein (MBP) were shown to enhance cholinergic nerve function representing the dominant bronchoconstrictory pathway by blocking inhibitory presynaptic M2 receptors. In animals models, the release of acetylcholine from postganglionic parasympathetic nerve terminals is under control of presynaptic muscarinergic M2 receptor.

It is the aim of this chapter to elucidate the close and direct bi-directional communication between inflammation and local nervous system which controls airway

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function and is therefore relevant for symptoms of allergic asthma including inflammation, airway hyperreactivity and airway obstruction.

1. AIRWAY INFLAMMATION IN ALLERGIC ASTHMA

Allergic bronchial asthma (BA) is characterized by chronic airway inflammation that has been shown to play an important role in the development of airway hyperreactivity (AHR) and recurrent reversible airway obstruction. There is established great evidence that T cells play a central role in the pathophysiology of allergic BA (Kay 1996). Strong evidence supports the notion that Th2 cells orchestrate allergic inflammation driven by effector functions of B cells, mast cells and eosinophils. Differentiation of naive $CD4^+$ T cells (Th0) into Th1 or Th2 cells determines whether an antigen will induce a cellular or a humoral immune response. Delivery of foreign antigens to mucosal surfaces, such as pulmonary airways, has been shown to preferentially induce a Th2- mediated response. Both the expansion of Th2 memory cell clones during the secondary exposure to allergen as well as the commitment of naive T cells to Th2 cells, is required for the development of a Th2 immune response during allergic asthma. The availability of IL-12 and IFN-γ opposite to IL-4 is decisive for the maturation of Th0 cells into Th1 or Th2 cells (Constant and Bottomly 1997). Sources of IL-12 and IFN-γ are mainly macrophages and dendritic cells (DCs), while cells responsible for the initial IL-4 production are less well defined and apparently include naive T cells themselves (Rincon *et al.* 1997). The presentation of inhaled antigen to T cells by local antigen presenting cells (APC) is a critical step in triggering the local immune response towards a Th1 or Th2 type immunity. Increasing evidence suggests that an already deviated Th1 or Th2 cell response can be reversed or further enhanced depending on the type of APC`s responsible for restimulation and the subsequent secondary immune response (Desmedt *et al.* 1998). Recent data demonstrate, that DCs in the respiratory tracts are specialized for initiating a Th2 immunity at the mucosal sites (Stumbles *et al.* 1998). By contrast, within the airways B cells do not appear to be essential as antigen presenting cells, even though they support the induction and expansion of Th2 cells (Korsgren *et al.* 1997). Finally, the resident lung tissue macrophages play a pivotal role in initiating and development of a lung allergic immunity. They attenuate allergic inflammation and AHR by mounting Th1 responses in the bronchial mucosa that antagonizes Th2 responses to inhaled antigen (Tang *et al.* 2001a). This Th1-promoting activity is an inherent property of lung macrophages and regulated via priming these cells with IFN-γ. The importance of tissue macrophages during airway inflammation in allergic asthma has been further demonstrated by altered macrophage phenotyp pattern. Alveolar macrophages from atopic asthmatics demonstrate changes in surface expression of coregulatory molecules and show CD83 expression, a marker of mature DCs (Lensmar *et al.* 1999; Tang *et al.* 2001b). They also produce lower amounts of IL-12, which decreases Th2 type inflammation by stimulating Th1 cell differentiation and inhibition of IgE-synthesis. The result is a profound shift to Th2 inflammation. Additionally, an increased number of DCs further supports the Th2 immune response in allergic asthma.

Th2 cells produce a cytokine profile that predominantly includes IL-4, IL-5 and IL-13. These cytokines induce B-cells to undergo class-switching to IgE production. Two signals

have been described to be essential. The first one is provided by the Th2 cytokines IL-4 or IL-13, which interact with receptors on the surface of B-cells and cause a phosphorylation of the transcriptional regulator STAT6 via activation of the Janus family tyrosine kinases JAK1 and JAK3 (Hoey and Grusby 1999). The second signal for IgE class switching is a co-stimulatory interaction between CD40 ligand on the T-cell surface with CD40 on the B-cell surface (Bacharier and Geha 2000). Overall, Th2 cells, present in respiratory mucosa and regional lymphoid tissues of individuals with allergic asthma, can switch the antibody isotype from IgM to IgE, or they can cause switching to IgG2 and IgG4 (human) or IgG1 and IgG3 (mouse). IL-5 has pro-inflammatory properties by its function on development, differentiation, recruitment and survival of eosinophils. The importance of T cells or T cell-driven processes is further underlined by the effectiveness of anti-inflammatory therapies including glucocorticoids and several other drugs (Wong and Koh 2000). This concept is currently leading to the development of novel drugs including IL-5 and IgE inhibitors which allow to test this concept under in-vivo conditions (Kay 2001b; Kay 2001a).

1.1. Early Phase Response

Subsequent re-exposure to the same agent causes a biphasic, reversible airflow obstruction referred to as the early and late phase response. The early phase response (within minutes) is characterized by a rapid onset of mucosal edema, and an increase in airway smooth muscle tone which is associated with mast cell activation and degranulation following allergen cross-linking of the IgE molecules bound to the constitutively expressed high–affinity IgE receptor FcεRI on the surface of mast cells (Pauwels 1989; Turner and Kinet 1999). Mast cells are predominantly located in subendothelial and mucosal tissues, as well as in epithelial tissues in the vicinity of small blood vessels and postcapillary venules. The secretion of preformed factors (histamine, enzymes and toxic proteins) and newly generated mediators (chemokines, lipid mediators and cytokines) from mast cells is thought to be the initial step for the allergic late phase reaction (Wasserman 1984).

1.2. Late Phase Response

The allergic late phase response occurs hours after exposure and may persist for several days without therapy. It is characterized by airway narrowing and an influx of neutrophils, eosinophils and lymphocytes from the blood into the lung parenchyma and airway epithelium (Bousquet *et al.* 1990; Cockcroft 1988; De Monchy *et al.* 1985; Pauwels 1989). The mast cell-derived mediators have multiple functions in this respect: Enzymes such as mast cell chymase, tryptase and serine protease can activate matrix metalloproteinases causing tissue destruction. The synthesis and release of cytokines (mainly TNF-α) activate endothelial cells causing increased expression of adhesion molecules, which promotes the influx of inflammatory cells into tissues and thereby further enhances the chemoattractive feature of chemokines, cytokines and lipid mediators over several hours (Bingham, and Austen 2000; Mekori and Metcalfe 1999; Metcalfe *et al.* 1997; Williams and Galli 2000). Eosinophils, as well as neutrophils, basophils, and lymphocytes, are increased in airway tissue, including the

submucosa, epithelium and airway lumen (Bachert *et al.* 1990; Bascom *et al.* 1988; Calderon *et al.* 1994; Horwitz and Busse 1995). Eosinophils, mast cells, T-cells and basophils can interact with each other. Eosinophils secrete a variety of proinflammatory mediators and cytolytic enzymes that disrupt epithelial integrity, inflict substantial damage to the extracellular matrix, neurons and airway inflammatory cells, and are also known to stimulate degranulation of mast cells and basophils (Pearlman 1999). Once activated and recruited to the airways, the inflammatory cells can easily induce a chronic inflammatory response followed by structural changes of the lung (airway remodeling).

2. AIRWAY HYPERREACTIVITY (AHR)

To date, no satisfying concept linking inflammation with persistent symptoms of asthma is available, but it is known that chronic inflammation is associated with clinical consequences including nonspecific airway hyperresponsiveness (AHR). Nonspecific AHR may be defined as an increase in the ease in degree of airway narrowing in response to a wide range of bronchoconstrictor stimuli (Bousquet *et al.* 2000). The development of AHR in response to allergic inflammation is mediated by multiple independent and additive pathways working in concert (Herz *et al.* 1998; Wilder *et al.* 1999; Wills-Karp 1999). Several mechanisms have been identified to be involved in the occurrence of AHR. The airway changes leading to airway narrowing mainly include: (a) altered neuronal regulation of airway tone, (b) increases in muscle content or function, (c) increased epithelial mucus production and airway edema (Bousquet *et al.* 2000; Wills-Karp 1999). Therefore it is not surprising that to date different modes of measuring AHR have been established both for clinical diagnostics and experimental approaches to characterize the pathophysiological mechanisms of AHR. It has been previously shown that *in vitro* EFS of tracheal segments reflects specifically neuronal airway dysfunction since addition of both atropin (disruption of cholinergic pathways) and capsaicin (depletion of sensory neurons) completely blocks any reaction of the airway to electric field stimulation (Andersson and Grundstrom 1983; Ellis and Undem 1992). By using specific stimuli, measuring of lung function in animal models allows to distinguish between broncho-constriction due to direct stimulation of airways smooth muscle and alteration of sensory nerves (Donnerer *et al.* 1992; Ek *et al.* 1998). Capsaicin is a potent stimulant of sensory nerves and induces a characteristic modification of the normal breathing pattern. In contrast, methacholine acts via direct stimulation of airways smooth muscle cells. Other stimuli including histamine can affect both nerve- and smooth muscle cells. The mechanisms by which inflammatory cells and their mediators interact with the resident lung cells, e.g. neurons, smooth muscle and epithelial cells, awaits further elucidation.

3. INNERVATION OF THE LUNG

The human lung and airways are innervated via a complex system of autonomic nerve fibers with afferent and efferent effector functions. Neuronal control of airway function was

originally thought to be solely dependent upon a balance between cholinergic and adrenergic neurons. Results from experiments performed about 100 years ago suggested the existence of a third innervation pathway. However, it was not until about 40 years ago that the existence of the non-adrenergic non-cholinergic system (NANC) was established (Burnstock *et al.* 1963). The neurotransmitters involved in this system are peptides and, therefore, this nervous system has been referred to as the "peptidergic nervous system" (Figure 1).

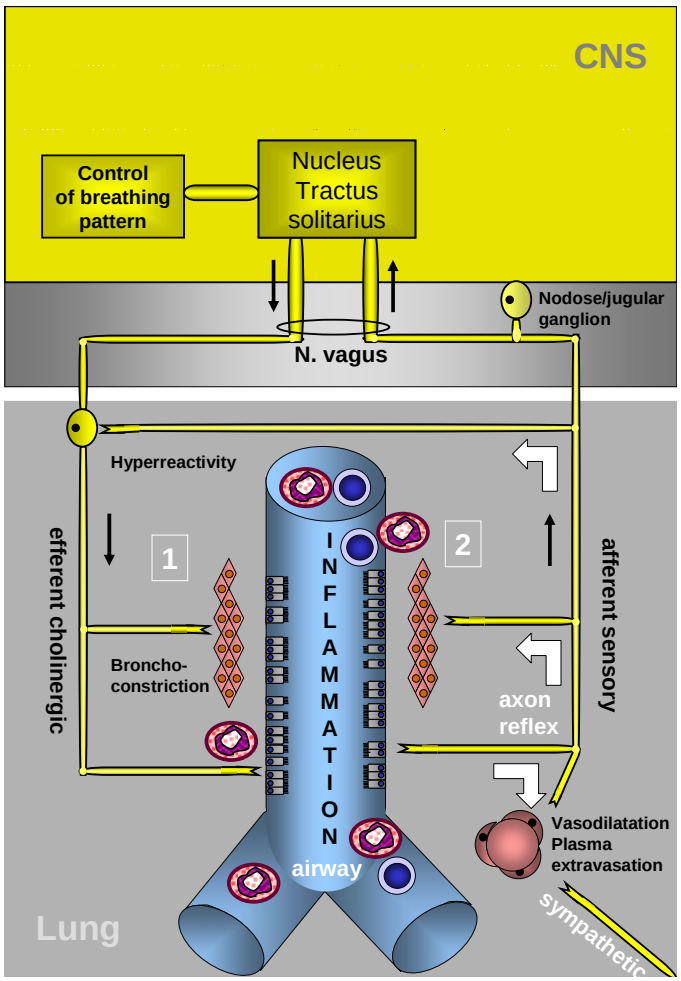

Figure 1. Neuronal plasticity in chronic allergic asthma. The airways are under close control of the lung's autonomic nervous system which predominantly consists of the cholinergic [1] and the non-adrenergic non-cholinergic (NANC) [2] nervous system. For detailed information see Fig. 1.1 and 1.2.

3.1. Parasympathetic Neurons

The parasympathetic nervous system plays an important role in maintaining bronchial smooth muscle tone via mediating broncho-constrictory response. The key neurotransmitter in this system is acetylcholine and the effects of the parasympathetic system can be blocked by atropine and augmented by acetylcholine esterase inhibitors (Colebatch and Halmagyui. 1963; Olsen *et al.* 1963). The parasympathetic neurons travel in the N. vagus. The afferent fibers originate from within and around the airway lumen and transmit the signals to the

central nervous system. The efferent fibers innervate airway smooth muscle cells. The post-ganglionic fibers, carried in the N. vagus, extend from the upper tracheobronchial tree down to the small airways (Anderson 1974; Casale 1987; Richardson 1979) (Figure 1).

Acetylcholine exerts its functional activity via muscarinic receptors. Lung muscarinic receptors are present in high density and the distribution is greatest in the large airways and least in the peripheral airways as revealed by radioligand binding and autoradiography studies (Barnes *et al.* 1983; Casale and Ecklund 1988; Joad and Casale 1988). Five different muscarinic receptor subtypes have been sequenced and cloned (Bonner *et al.* 1987; Bonner *et al.* 1988). However, only three receptor subtypes have been identified based on pharmacological studies. They are termed M1, M2 and M3 respectively (Barnes 1989a; Barnes 1989b; Mak and Barnes 1989). On human airway smooth muscle, the M3 subtype is exclusively expressed. This receptor type is found in large and in some peripheral airways as well as in submucosal glands. It is shown to be responsible for airway smooth muscle constriction and mucosecretion. The M1 receptor is expressed in parasympathetic ganglia, facilitating vagal signal transmission, and on submucosal glands, where it is involved in the regulation of mucosecretion. Although both, M1 and M3 receptors are expressed on submucosal glands, a much higher expression profile has been described for the M3 subtype. The M2 receptor is expressed on cholinergic nerves with negative regulatory effects and functions. Therefore, it inhibits as an autoreceptor the acetylcholine release. Although certainly the main effect of stimulating parasympathetic nerves is of excitatory nature and leads to broncho-constriction, it needs to be emphasized that, in case of M2 receptor stimulation, the “paradox” effect of broncho-constriction can be obtained (Barnes 1989a; Barnes 1989b; Mak and Barnes 1989) (Figure 1.1.).

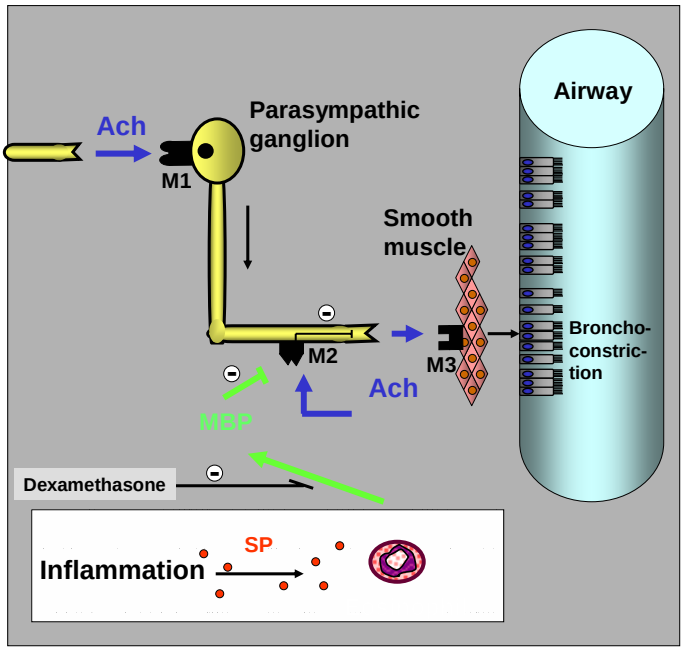

Figure 1.1. The cholinergic system in chronic airway inflammation. In allergic inflammation eosinophils are associated with nerves. The eosinophilic product major basic protein (MBP) acts as antagonist for inhibitory muscarinic M2 receptor. This results in increased release of acetylcholine (Ach) and subsequently to

enhanced bronchoconstriction. This mechanism is augmented by substance P (SP) and can be inhibited by dexamethasone.

3.2. Sympathetic Neurons

Compared to the parasympathetic neurons, the sympathetic nervous system represents only a minor component of the total nerve system in human airways and lung (Anderson 1974; Mann 1971; Partanen *et al.* 1982; Richardson 1979). There is only little evidence for direct sympathetic innervation of human airways. The efferent sympathetic neurons originate from the second to forth thoracic preganglionic fibers ending in the extrapulmonary stelate ganglia. Post-ganglionic sympathetic axons going along with post-ganglionic parasympathetic axons form a dense plexus around airways and blood vessels (Barnes 1986b). But very few of them finally reach airway smooth muscle cells (Richardson 1979). It is important to note that there is considerable variation in sympathetic innervation among various species.

Based on the main effect of the sympathetic nervous system with regard to relaxation of bronchial smooth muscle, this system is termed the “inhibitory” system. Adrenaline, noradrenaline and neuropeptide Y (NPY) represent the major neurotransmitters. They act via stimulation of α- and β-receptors. In general, there is a much higher density of β-compared to α-receptors in the human lung (Barnes *et al.* 1983; Spina *et al.* 1989). These receptors have been identified on a great variety of cell types including airway smooth muscles, alveolar and epithelial cells with generally increasing numbers in the smaller airways of the peripheral lung (Barnes *et al.* 1983; Carstairs *et al.* 1985; Casale and Hart 1987; Casale *et al.* 1987; Spina *et al.* 1989).

The most relevant lung functions to asthma are mediated via β2-receptors. Although human lung contains both, β1- and β2-receptors, a several fold higher expression pattern of the β2-receptors has been observed (Barnes PJ 1984; Carstairs *et al.* 1985; Casale and Hart 1987). Stimulation of the β2-receptors results in airway smooth muscle relaxation (Zaagsma *et al.* 1983), inhibition of antigen-induced mast cell mediator release (Butchers *et al.* 1980; Orange *et al.* 1971a; Orange *et al.* 1971b), release of surfactant and others (Brown and Longmore 1981). Additionally, application of β2 specific agonists inhibits cholinergic nerve activities, indirectly indicating expression of β2-receptors on cholinergic neurons (Rhoden *et al.* 1988; Vermeire and Vanhoutte 1991). The role of α-receptors in this regard is not entirely clear. On the one hand side, stimulation of α1-receptors induces mucosecretion (Culp *et al.* 1990; Lundgren and Shelhamer 1990) and augments mast cell mediator release (Kaliner *et al.* 1972; Moroni *et al.* 1977). On the other side, stimulation of α2-receptor has been demonstrated to inhibit both cholinergic and non-cholinergic excitatory signaling (Grundstrom *et al.* 1981b; Grundstrom *et al.* 1984; Grundstrom and Andersson 1985; Starke 1981; Wikberg *et al.* 1982). The former effects would result in a positive contribution of α1-receptors to the pathogenesis of asthma, whereas the latter effects of α2-receptors suggest a protective role.

3.3. NANC Neurons

The NANC-system has been functionally subdivided into the excitatory (e-NANC) and inhibitory (i-NANC) system. Afferent neurons of the NANC-system are not strictly anatomically separated from vagus nerve. Both systems exhibit their functional activity via a certain panel of neuropeptides. Stimulation of the e-NANC system results in marked broncho-constriction. These effects are mediated by a group of neurotransmitters including substance P (SP), neurokinin A (NKA), neurokinin B (NKB) and calcitonin gene-related peptide (CGRP) (Anderson 1974; Barnes 1986b; Barnes 1987b; Casale TB 1988; Casale TB 1989; Casale 1987; Richardson 1979). This group of mediators is referred to as the tachykinin family. Tachykinins bind to three distinct neurokinin receptors termed NK-1, NK-2 and NK-3, respectively. These three high-affinity tachykinin receptors bind a different kind of tachykinins with preference. NK-1 shows highest affinity for SP, NK-2 for NKA, and NK-3 for NKB, respectively.

Neurons of the e-NANC pathway have been identified in both human and animal lungs (Grundstrom *et al.* 1981a; Lundberg and Saria 1982; Lundberg *et al.* 1983a; Lundberg *et al.* 1983b; Lundberg *et al.* 1984). They consist of non-myelinated vagal afferent fibers. Nerve endings of such non-myelinated sensory C-fibers have been identified in the airway epithelium. NK-2 receptor expression predominates on airway smooth muscle cells, whereas NK-1 receptors are mainly found on blood vessels. NK-3 receptors are mainly expressed on cholinergic nerves.

SP is synthesized in the vagal nodose ganglion and is retrogradually transported down the vagus, where it can be released from the afferent nerve fibers. SP immuno-reactive nerve fibers have been detected near blood vessels, airway epithelium and, to a lesser extend, in airway smooth muscle (Lundberg *et al.* 1984; Wharton *et al.* 1979). NKA is co-localized with SP in sensory airway nerves (Lundberg JM 1985; Lundberg *et al.* 1987). NKA immuno-reactive nerves are predominantly found on airway smooth muscle cells, around blood vessels. They are preferentially detected in the area of the larger airways and, to a lesser extend, in the trachea, bronchioles and alveoli (Lundberg JM 1985; Uchida *et al.* 1987). In contrast NKB has only little effect on human bronchia, at least in-vitro (Barnes 1987a; Naline *et al.* 1989).

CGRP is also co-localized with SP in airway nerves (Martling *et al.* 1988). In this regard, CGRP immuno-reactivity co-exists with that of other tachykinins in cell bodies of the nodose and jugular ganglia, axons and nerve terminals within the airways. In the periphery, CGRP positive nerves have been detected around blood vessels, within the tracheo-bronchial smooth muscle layers and in the respiratory epithelium (Martling *et al.* 1988; Palmer *et al.* 1987; Shimosegawa and Said 1991).

Inhibitory NANC neurons appear to be the predominant neural broncho-dilatatory system in man since there is little evidence for sympathetic innervation of the human airways. Initial evidence for the existence of this system comes from experiments utilizing electrical field stimulation. When both adrenergic and cholinergic receptors were blocked, field stimulation resulted in smooth muscle relaxation (Anderson 1974; Coburn RF 1973; Ichinose *et al.* 1988; Mann 1971; Martling *et al.* 1988; Partanen *et al.* 1982; Richardson and Beland 1976; Richardson 1979). Central neurotransmitters in this system are vasoactive intestinal peptide

(VIP) and nitric oxide (NO) (Boeckxstaens *et al.* 1990; Bult *et al.* 1990; Martling *et al.* 1988).

VIP positive neurons often travel together with cholinergic nerves. Highest density of these nerves is greatest in the upper respiratory tract and least in bronchioli (Dey *et al.* 1981; Martling *et al.* 1988). Furthermore, they have been identified near airway smooth muscle, bronchial glands and vascular smooth muscle. A variety of cell types express VIP receptors. They include pulmonary vascular smooth muscle, smooth muscle of larger but not small airways, airway epithelium, submucosal glands and others (Laitinen *et al.* 1985).

3.4. The Axon Reflex

The axon reflex describes a concept of how the excitatory NANC-system may contribute to the pathophysiology of bronchial asthma (Figure 1). This concept has been originally proposed by Peter Barnes (Barnes 1986a). The axon reflex is initiated by stimulation of vagal afferent nerve endings in the airway epithelium. Any damage to airway epithelium causes such stimulation. A broad range of trigger factors have been identified, including infectious agents, such as viral and bacterial infections, inhaled environmental irritants, such as NO_2, SO_2, ozone and others. Furthermore, various inflammatory mediators released from airway inflammatory cells also stimulate vagal afferent nerve endings. Stimulation of such afferent nerve fibers not only results in a central reflex activity, leading to enhanced efferent cholinergic activities with the result of increased acetylcholine release. In parallel, there exists a “short-circuit” local reflex loop with the non-adrenergic non-cholinergic efferent nerves. This peripheral reflex activity results in an increased release of neuropeptides and tachykinins including SP, NKA, NKB and CGRP. As a consequence, neuropeptide and tachykinin release causes airway smooth muscle restriction, mucus production, edema and release of inflammatory mediators from various inflammatory cells (Figure 1.2).

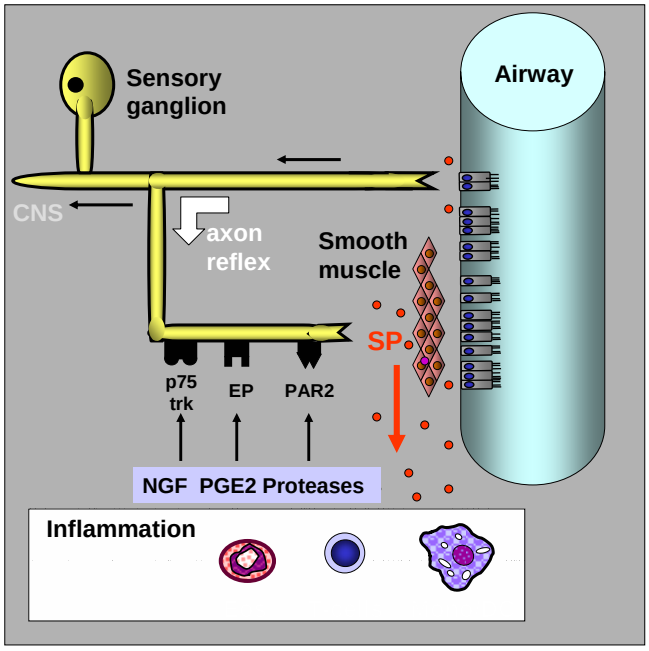

Figure 1.2. The sensory system in chronic airway inflammation. Sensory neurons are affected by various inflammatory stimuli. The best described mediators are neurotrophins including nerve growth factor (NGF) and cytokines like prostaglandine E2 (PGE2). In addition, mast cell products like proteinease could play an important role. These mediators bind to specific receptors on sensory nerve cells, p75 and trk are neurotrophin receptors, EP is the receptor for PGE2 and PAR2 mediates the signal from proteinases, respectively. In the sensory nerve cell they induce increased tachykinin (substance p (SP) or neurokinin (NK) release.

3.5. Neuronal Changes in Allergic Asthma

In the last decade, growing evidence indicates neuronal dysregulation on several levels in allergic BA (Joos *et al.* 2000). Since cholinergic nerves represent the dominant bronchoconstrictory pathway and anticholinergic drugs are very effective bronchodilators in acute severe asthma, cholinergic mechanisms must be considered in the development of AHR. These possible mechanisms include enhanced cholinergic reflex activity, increased acetylcholine (ACh) release, enhanced sensitivity of smooth muscle to ACh and increased density of muscarinic receptors on airway smooth muscle. In addition, sensory nerves are able to modulate cholinergic function. Cholinergic activity was shown to be increased by tachykinins (Delaunois *et al.* 1996; Mackay *et al.* 1998). Contraction of airway smooth muscle is mediated by M3 muscarinic receptors on airway smooth muscle. There is no evidence, however, suggesting that hyperresponsiveness results from any alteration in the function of these M3 muscarinic receptors. In contrast, there is clearly increased release of the neurotransmitter ACh in animal models of hyperreactivity and asthma (Larsen *et al.* 1994). In addition, the loss of function of inhibitory M2 muscarinic receptors on the airway parasympathetic nerves enhances vagally mediated bronchoconstriction and hyperresponsiveness following allergen challenges (Fryer *et al.* 1999). The M2 muscarinic

receptors on the parasympathetic nerves in the lungs normally inhibit release of acetylcholine. When the receptors are blocked, the inhibitory effect is lost and, then, acetylcholine release is increased. Loss of M2 receptor function has been shown as a result of eosinophilic mediations, resulting in airway hyperresponsiveness (Jacoby *et al.* 2001) (Figure 1.1).

The sympathetic nervous system is less prominent than the parasympathetic nervous system within the human airways. It should be highlighted that there is a lack of sympathetic innervation of the human airway smooth muscle compared to other species. Guinea pig models indicate that antigen challenge induces a mast-cell mediated long-lasting increase in synaptic efficacy (Weinreich *et al.* 1995). In asthmatic airways, no difference in the number of NPY-immunoreactive nerves has been found as compared to healthy controls (Howarth *et al.* 1995).

The e-NANC system exhibits a high degree of plasticity in inflammatory conditions. SP and NKA, preferentially released by sensory C-fibers, are closely related members of the neuropeptide family termed tachykinins. They are synthesized preferentially in cell bodies of the sensory ganglia by a complex biosynthetic pathway. These neuropeptides are transferred via axonal transport not only to presynaptic axon endings in the spinal cord and the nucleus of the solitary tract, but also to peripheral sensory nerve endings (Brimijoin *et al.* 1980). Upon exposure to mechanical, thermal, chemical (capsaicin, nicotine) or inflammatory stimuli (bradykinin, histamine, prostaglandins), tachykinins are released from nerve cells through a local (axon) reflex mechanism (Barnes 1986a) (Figure 1.2). Tachykinins act in a dual fashion, as afferent neurotransmitters to the central nervous system as well as efferent neurosecretory mediators diffusing into the peripheral tissue. Increased levels of the neuropeptide substance P (SP) have been detected in the airways of asthmatic patients (Baumgarten *et al.* 1996). Additionally, allergen challenge increased neurokinin A (NKA) levels in bronchoalveolar lavage fluid (BALF) of asthmatic patients (Heaney *et al.* 1998). Nerve fibers containing SP have been described in and around bronchi, bronchioles, the more distal airways and occasionally extend into the alveolar wall. The fibers are located beneath and within the airway epithelium, around blood vessels and sub-mucosal glands, within the bronchial smooth muscle layer and around the local tracheo-bronchial ganglion cells (Lundberg *et al.* 1984). Although there is evidence for an increase in both the number and length of SP immunoreactive nerve fibers in airways from subjects with bronchial asthma as compared to airways from healthy subjects (Ollerenshaw *et al.* 1991), the study from Lilly et al. detected no difference in SP-like immunoreactivity (Lilly *et al.* 1995). One reason for the latter finding may be that the nerve endings release SP due to continued stimulation. In a guinea pig model, it has been shown that sensory innervation of the airways is altered during allergic inflammation (Undem *et al.* 1999). The increase of SP and NKA in the lung in response to allergen challenges has been related to an increased production of these neuropeptides in neurons of the nodose ganglion (Fischer *et al.* 1996). Impaired degradation of tachykinins could further enhance their local activity (van, V and Hulsmann 1999b). Tachykinins are degraded and inactivated by neutral endopeptidase (NEP), a membrane-bound metallopeptidase located mainly on the surface of airway epithelial cells and also present in airway smooth muscle cells, submucosal glands and fibroblasts. As a key role NEP limits the biological actions of mediators like tachykinins via enzymatic degradation.

Allergen exposure, inhalation of cigarette smoke and other respiratory irritants are associated with a reduced NEP activity, thus enhancing the effects of tachykinins within the airways (Di Maria *et al.* 1998; Sont *et al.* 1997; Tudoric *et al.* 2000).

In addition, antigen-induced functional changes in sensory neurons, including the depolarization of the resting membrane potential, changes in membrane resistance, increases in mechanosensitivity (of Aδ vagal afferent airway nerves) and enhanced responses to SP, have been described (Undem *et al.* 1993; Weinreich *et al.* 1997). Since these neuronal alterations in asthma are associated with local inflammation, the concept that inflammatory mediators could be responsible for neuronal dysfunction was developed.

4. INFLAMMATORY MEDIATORS AND THEIR IMPACT ON NERVE CONTROL IN ASTHMA

4.1. Neurotrophins

Neurotrophins are a family of homologous proteins that consist of four members: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin4/5 (NT-4/5). Their role in survival, differentiation and maintainance of neurons is well defined (Lewin and Barde 1996). They exhibit partially overlapping but also distinct patterns of expression and cellular targets. In addition to the central nervous system, neurotrophins also affect peripherial afferent and efferent neurons. The biological effects of neurotrophins are mediated by binding to two different receptor types, the tropomyosin-related kinase (trk) family of receptor tyrosin kinases and the p75 receptor, a member of the tumour necrosis factor superfamily. Neurotrophins bind to the trk receptors with high affinity and specificity, trkA is primarily a receptor to NGF, trkB for BDNF and NT-4/5 and trkC for NT-3. In addition, all neurotrophins bind to the low affinity receptor p75NTR (Kaplan and Miller 2000). Binding of neurotrophins induces receptor dimerization at the cell surface followed by phosphorylation of receptor tyrosine residues. The phosphorylated tyrosine then recruits intracellular signal transduction proteins. These factors initiate functional changes such as survival, proliferation, and differentiation of the corresponding target cell.

4.1.1.Cellular Sources of Neurotrophins in the Immune System

The recruitment of inflammatory cells from the bone marrow may play an important role in the induction of allergic inflammation and asthma (Denburg *et al.* 2000). Before these cells infiltrate the lung and lymphatic tissues, they circulate in the peripheral blood.

In the past years, several cell types have been identified as cellular sources of neurotrophins. There is evidence for local neurotrophin-synthesis in the bone marrow. Laurenzi et al. showed mRNA encoding BDNF and NT-4 in human bone marrow preparations, but the detection of NGF- and NT-3-specific mRNA transcripts failed (Laurenzi *et al.* 1998). In contrast, Labouyrie et al. have demonstrated transcripts for NGF, BDNF, NT-3 and NT-4 in comparable human samples (Labouyrie *et al.* 1999). On a cellular basis, murine stromal cells in long-term bone marrow cultures have been identified for NGF and BDNF synthesis (Dormady *et al.* 2001). NGF is produced by murine bone marrow-derived

cultured mast cells and released upon activation through cross-linkage of the high affinity IgE receptors (Xiang and Nilsson 2000). Human megakaryocytes are a cellular source for BDNF (Fujimura *et al.* 2002).

NGF is elevated in the sera of patients with allergic diseases, and highest levels were found in patients suffering from asthma (Bonini *et al.* 1996), pointing to a neurotrophin synthesis in peripheral blood cells. In fact, neurotrophins can be synthesized by a wide range of white blood cells and the production is often regulated by inflammatory signals.

Human purified monocytes/macrophages derived from peripheral blood (PB) express NGF constitutively. Stimulation with LPS (Caroleo *et al.* 2001) and infection with HIV (Garaci *et al.* 1999) caused a strong upregulation of NGF synthesis in these cells. In contrast, Ehrhard et al. have reported that NGF is not detectable constitutively nor in Staphylococcus aureus Cowain Strain I (SAC)-stimulated cells (Ehrhard *et al.* 1993). Furthermore, human monocytes, which became activated through an isolation process, express BDNF (Kerschensteiner *et al.* 1999).

For T-cells, conflicting data are available. In human purified T-cells, NGF synthesis was not detected (Otten *et al.* 1989). In contrast, Lambiase et al. have demonstrated a constitutive mRNA expression encoding NGF in human CD4/Th1 T-cell clones and Th2 clones, whereas PHA stimulation is required in Th0 cell clones to produce NGF. NGF synthesis in Th2 clones was further increased by PHA stimulation (Lambiase *et al.* 1997). BDNF is expressed by both human Th1 and Th2 polarized T-cells as well as in CD8 T-lymphocytes and can be stimulated by coincubation with allogenic PBMCs and PHA (Kerschensteiner *et al.* 1999).

Human B-cells are also a source of NGF. SAC-stimulation increases NGF production (Torcia *et al.* 1996) and also induces BDNF synthesis (Kerschensteiner *et al.* 1999). Furthermore, Besser and Wank demonstrated NT-3 mRNA expression after IL-4 or PHA stimulation in a human B-cell line (Besser and Wank 1999).

Moreover, human granulocytes contribute to neurotrophin synthesis in peripheral blood. Laurenzi et al. observed mRNA coding for NGF, BDNF, and NT-4, but not NT-3, in granulocyte preparations (Laurenzi *et al.* 1998). NT-4 production was elevated after stimulation with leukotriene B4. Constitutive expression and intracellular storage of NGF and NT-3 was seen in human eosinophils (Kobayashi *et al.* 2002; Solomon *et al.* 1998). New synthesis of NGF is enhanced by Fc-receptor-mediated stimuli, such as immunoglobulin (Ig)A and IgG immune complexes and IL-5 (Kobayashi *et al.* 2002).

Another relevant source of neurotrophins for allergic disease are mast cells. Human umbilical cord mast cells express NGF and BDNF, but no NT-3 (Tam *et al.* 1997).

In the lung, macrophages, alveolar cells type I, T-cells, mast cells, fibroblasts and epithelial cells have been identified to synthesize neurotrophins. Lung macrophages can be divided into two major subclasses, located in various anatomical compartments in the lung: The interstitial macrophages and the alveolar macrophages. Some authors revealed that interstitial macrophages may be the immediate precursors of some alveolar macrophages (Bowden and Adamson 1972). Although differences in morphology, the pattern of surface receptors and function have been shown, recent studies indicate that also interstitial macrophages are highly immunocompetent cells (Johansson *et al.* 1997; Prokhorova *et al.* 1994; Sebring and Lehnert 1992).

Comparing neurotrophin expression in macrophages, it becomes clear that there are differences in the expression pattern. Both alveolar and interstitial macrophages express NGF protein after allergen provocation, as has been demonstrated by Braun et al. In this study, NGF was determined in bronchoalveolar lavage fluid (BALF) cells as well as in bronchial biopsies after allergen challenge in OVA-sensitized and challenged mice in comparison to control animals (Braun *et al.* 1998). The observed increased NGF levels in BALF from OVA-treated mice revealed, besides an allergen-dependent augmentation in NGF production, an allergen-dependent release of NGF. In contrast, BDNF is constitutively expressed in interstitial murine macrophages (Hikawa *et al.* 2002), but synthesis of BDNF in alveolar macrophages requires activation as induced by allergen challenge in a mouse model of asthma (Braun *et al.* 1999a). Investigations for NT-3 production demonstrated a restricted, constitutive synthesis in murine alveolar macrophages, whereas NT-4 was expressed constitutively both from alveolar macrophages as well as from interstitial macrophages (Hikawa *et al.* 2002). In addition to alveolar cells type I (Hikawa *et al.* 2002), also T-cells as well as epithelial cells in the airways serve as a source for BDNF in the mouse (Braun *et al.* 1999a; Lommatzsch *et al.* 1999). Epithelial cells constitutively express BDNF, whereas this neurotrophin was observed in tissue T-cells exclusively after allergen challenge in sensitized mice (Braun *et al.* 1998). In human biopsies, NGF immunoreactivity has been observed in the epithelium also (Kassel *et al.* 2001), but has been detected neither in constitutive nor in inducible form in mice (Braun *et al.* 1998). In human biopsies of patients with asthma, fibroblasts, blood vessels and "a few infiltrating cells" showed a positive NGF immunoreactivity. Low dose allergen challenge increased the mRNA content encoding NGF (Kassel *et al.* 2001).

The data about neurotrophin production in lymph nodes are rare. It could be demonstrated by Torcia et al. that human monocytes and T-cells, in contrast to B-cells, could not express NGF. Even stimulation with SAC has no effect on T-lymphocytes and monocytes, whereas NGF was increased in B-cells (Torcia *et al.* 1996). In rat inguinal lymph nodes, T-cells as well as infiltrating NK-cells were identified for BDNF and NT-3 production (Hammarberg *et al.* 2000).

Overall, it has been shown that neurotrophin synthesis occurs in immune cells and is partly dependent on inflammation. However, the available information about regulation and in particular allergen-dependent regulation of these factors *in vivo* is not satisfactory.

4.1.2. The Role of Neurotrophins in Allergic Inflammation

The role of neurotrophins in asthma-associated inflammation has not yet been well characterized. However, the inflammatory response to allergens is a complex spatiotemporal behavior, which involves several subtypes of immunocompetent cells and a wide variety of mediators. As mentioned by Carr et al., no studies have been published describing the impact of inhibiting neurotrophin actions on airway inflammation in patients with asthma or the effects of exogenously applied neurotrophins in the human lung (Carr *et al.* 2001).

Neurotrophins may influence inflammatory responses either directly, by regulating immune cell functions (Aloe *et al.* 1997), or indirectly through modulation of the synthesis of sensory neuropeptides (Goedert *et al.* 1981; Lindsay and Harmar 1989; Verge *et al.* 1995), including substance P (SP) and calcitonin gene-related peptide (CGRP). The enhanced

release of these neuropeptides is known to modulate immune cell functions (Schaffer *et al.* 1998). This is summarized by the concept of "neurogenic inflammation". A variety of studies have revealed the involvement of neurogenic inflammation in the pathophysiology of inflammatory diseases (Levine *et al.* 1984; Payan 1992).

In the following, we will first focus on the direct effects of neurotrophins on immune cells and then, after discussing the role of neurotrophins in neuronal AHR, describe their involvement in the crosstalk between neurons and immune cells in asthma, referred to as neurogenic inflammation.

Most of the involved immune cells mature in the bone marrow before circulating in the blood and then being recruited to the lung and lymph nodes during immune reactions and inflammation. Therefore, the action of neurotrophins must be described on the levels of sensitization, early phase response and late phase response to completely characterize their functions in asthma.

4.1.3. Neurotrophins and Allergic Sensitization

As demonstrated above, antigen-presenting cells, including dendritic cells and macrophages, as well as T-cells and B-cells are the most relevant cells during sensitization. The cells are bone marrow derived and predominantly localized in the lung and the local draining lymph nodes. Both organs are innervated by different types of nerve fibers including sensory nerves (Hukkanen *et al.* 1992; Lamb and Sparrow 2002; Popper *et al.* 1988) pointing out the potential presence of an intensive crosstalk between inflammatory cells and the peripheral nervous system.

The expression of neurotrophin receptors in antigen-presenting cells is a mandatory prerequisite for a biologic effect of neurotrophins, controversially discussed and probably dependent on localization as well as on the maturation status of the cells.

The myeloid progenitor is the precursor of granulocytes, macrophages, dendritic cells and mast cells. It has been shown that promyelocytes, myelocytes and metamyelocytes in the bone marrow of human individuals express trkC truncated and trkC kinase, but have no immunoreactivity for p75NTR, trkA or trkB (Labouyrie *et al.* 1999).

Macrophages in the stroma of human bone marrow samples show a positive immunoreactivity for trkA, trkB kinase, trkB truncated, trkC kinase and trkC truncated, but no staining for p75NTR (Labouyrie *et al.* 1999). Receptor expression and the first functional observations of neurotrophin effects on hematopoesis (stimulation with NGF enhances granulocyte-macrophage (GM) colony formation from murine bone marrow cells in cooperation with hemopoietic factor(s) (Kannan *et al.* 1993) suggest that in addition to NGF also BDNF, NT-3 and NT-4 may play a central role in hematopoesis.

In human peripheral blood, monocytes/macrophages constitutively express trkA and p75NTR. Stimulation with LPS caused a strong upregulation of trkA but had no effect on p75NTR expression. Treatment with antibodies against NGF to interrupt the autocrine effect of this neurotrophin, markedly reduced trkA expression and caused an increase in p75NTR expression, accompanied by enhanced apoptosis. These results indicate that NGF is an autocrine survival factor which exerts its effects via trkA (Caroleo *et al.* 2001). The results from la Sala et al. are in line with this suggestion. In this study, application of trkA receptor agonists protects monocytes from apoptosis induced by gliotoxin or UVB radiation by up-

regulation of the expression of the anti-apoptotic Bcl-2 family members, Bcl-2, Bcl-XL, and Bfl-1. In addition, it has been shown that trkA stimulation does not change the antigen-presenting function of human monocytes from peripheral blood (la Sala *et al.* 2000).

Hikawa et al. compared the trk receptor expression in interstitial and alveolar macrophages in C57BL/6 mice. In contrast to interstitial macrophages, which express the truncated trkB and the trkC, no expression of neurotrophin receptors was detected in alveolar macrophages (Hikawa *et al.* 2002). In contrast to mice, small populations of neurotrophin receptor expressing alveolar macrophages were found in the human airways. Ricci et al. analyzed cells from human bronchoalveolar lavage fluid and demonstrated a positive immunoreactivity for trkA in 3.5 % of the alveolar macrophages. The full-length isoform for trkB was expressed in 10 %, and 2 % of the cells showed a positive staining for trkC. No low-affinity p75NTR and TrkB[-] truncated isoform receptor immunoreactive alveolar macrophages were found (Ricci *et al.* 2000).

At the moment, the physiologic as well as the pathologic consequences of neurotrophin receptor expression in lung macrophages as well as in lymph nodes remains unclear.

The expression pattern of macrophages in the lymph nodes shows similarities to that of human alveolar macrophages. Shibayama examined the cellular localization of all trks in human non-neuronal tissues from adult individuals. Only a positive immunoreactivity for trkB has been observed in monocytes/makrophages in lymph nodes (Shibayama and Koizumi 1996) which are preferentially located in T-cell zones (Garcia-Suarez *et al.* 1997).

Information about neurotrophin receptor expression on antigen-presenting cells other than macrophages, such as dendritic cells, is rare. As seen above, monocytes from peripheral blood express both functionally active receptors for NGF, the trkA and p75NTR. During differentiation of human monocytes from peripheral blood to dendritic cells *in vitro*, a progressive loss of trkA expression could be observed (la Sala *et al.* 2000), which is accompanied by the loss of survival-promoting characteristics of NGF. In lymph nodes, the expression pattern of neurotrophin receptors has changed. Human follicular dendritic cells isolated from secondary lymph follicles of tonsillar lymph nodes express both p75NTR and trkA but no trkB nor trkC (Garcia-Suarez *et al.* 1997; Labouyrie *et al.* 1997; Pezzati *et al.* 1992). Results about the influence of neurotrophins on dendritic cells are missing completely.

In contrast, the impact of neurotrophins on lymphocyte function is relatively well characterized. Neurotrophin receptor expression in T-lymphocytes of bone marrow or their precursors, respectively, have not been examined. In peripheral blood, the described expression pattern is complex. According to Otten et al., Torcia et al. observed trkA expression in human T-cells (Otten *et al.* 1989; Torcia *et al.* 1996) but no p75NTR (Torcia *et al.* 1996). In contrast, Kittur et al. detected p75NTR human lymphocytes and observed a PHA-dependent regulation (Kittur *et al.* 1992). Furthermore, trkA expression was evaluated in Th1 and Th2 cell clones derived from human circulating mononuclear blood cells and has been detected in both examined subpopulations (Lambiase *et al.* 1997). TrkB is expressed in several subtypes of human T-cells. Constitutive expression of the truncated form has been described in CD4 cells, whereas expression in CD8 cells requires anti-CD3 or PHA stimulation (Besser and Wank 1999). Examination of the truncated trkB isoform in T-cell clones reveals independence from cytokine profile and was observed to be constitutively expressed in Th1, Th2, TH0 as well as γδ+ T-cell clones (Besser and Wank 1999). The full-

length form of trkB, gp145trkB, is also expressed in T-cells from PBMC after adequate stimulation (PHA or anti-CD3). In contrast, Th1 T-cell clones and the $\gamma\delta^+$ T-cell clone expressed gp145trkB constitutively, which could not be located to Th0 nor to Th2 clones. Detection of constitutively expressed trkC in PBMCs failed. Stimulation with anti-CD3 caused an induction of this receptor in the Th0, Th1 and Th2 T-cell clones, whereas no trkC could be observed in the γδ+ T-cell clone. Furthermore, a weak constitutive expression has been shown in two of the examined Th1 T-cell clones (Besser and Wank 1999). T-cells derived from human lymph nodes show trkA, but lack p75NTR (Torcia *et al.* 1996). Anyway, there is strong evidence for physiological importance of neurotrophin receptor expression on T-cells as has been demonstrated in functional trkB deficient mice (Garcia-Suarez *et al.* 2002). The thymus of homozygous functional trkB-deficient animals showed structural and ultrastructural changes consistent with massive death of cortical lymphocytes, pointing out an important role for trkB mediated survival of T-lymphocytes. In addition, signaling of BDNF through trkB, which is expressed in dependence of maturation level, is involved in the T-cell differentiation process as indicated by Maroder et al. (Maroder *et al.* 1996).

Neurotrophin receptor expression in bone marrow B-cells or lymphoid progenitors has not yet been described. In peripheral blood and lymphoid tissue, human B-cells, which can be divided into resting and large B-cells, both express trkA as well as p75NGFR molecules on their surfaces (Otten *et al.* 1989; Torcia *et al.* 1996). Furthermore, expression of the truncated as well as the full-length form of trkB has been demonstrated in human peripheral blood B-cells or a human B-cell line, respectively, and is increased after stimulation with PHA (Besser and Wank 1999). NGF acts as an autocrine survival factor in memory B-cells but not in immature B-lymphocytes, as seen *in vitro* in B-cell preparations from human tonsils and *in vivo* in experiments in the mouse. Incubation of human B-cells with neutralizing antibodies against NGF caused apoptotic cell death in B-cells expressing surface IgA/IgG, but not in "virgin" B-lymphocytes bearing surface IgM/IgD. *In vivo* administration of neutralizing antibodies against NGF reduced the titer of specific IgG in mice immunized with tetanus toxoid, nitrophenol, or arsonate (Torcia *et al.* 1996). The possible mechanism for apoptosis induced by neutralization of endogenously produced NGF may be downregulation of bcl-2 protein and prevention of Bcl-2 phosphorylation, inactivation of p38 MAPK, and cytochrome c release (Torcia *et al.* 1996; Torcia *et al.* 2001). Besides promoting survival, NGF can influence B-cell proliferation and stimulate murine and human B-lymphocytes to produce IgM, IgA and IgG4 (Kimata *et al.* 1991; Otten *et al.* 1989; Torcia *et al.* 1996). Human IgG_4 production is Th2 cytokine-dependent like IgE synthesis. In a mouse model of asthma, Braun et al. have demonstrated that NGF also augments IgE and IgG_1 production in allergen-stimulated mononuclear cells from spleen. Whether this is a direct effect on B-cells or an indirect effect via modulation of Th2 cytokine release from T-cells remains unclear (Braun *et al.* 1998). However, immunoglobulin levels of p75NTR knock-out mice as well as those of CCSP-NGF-tg mice are still normal even after allergic sensitization (Kerzel *et al.* 2003; Path *et al.* 2002).

Studies which elucidate the functional consequences of neurotrophin receptor expression are still absent and, in consideration of maturity-dependent and/or tissue dependent regulation of neurotrophin receptors, further studies are required to define the contribution of neurotrophins during sensitization.

4.1.4. Neurotrophins in Early Phase Response

As demonstrated above, mast cells play a major role in the asthmatic early phase response. Although the NGF function in the immune system was first documented in these cells, little is known about the effects of other neurotrophins than NGF.

In bone marrow, human mastocytes express trkA, both isoforms of trkB and trkC, but no p75NTR (Labouyrie *et al.* 1999). Stimulation of murine bone marrow-derived cultured mast cells with NGF cause proliferation and differentiation after costimulation with IL-3 (Matsuda *et al.* 1991). NGF alone triggers these cells to produce eicosanoids (Marshall *et al.* 1999). In peripheral blood, NGF is responsible for comparable events and has been shown to induce differentiation of human basophil progenitor cells to mature cells (Matsuda *et al.* 1988b). NGF suppresses apoptosis (Kanbe *et al.* 2000), alters degranulation (Mazurek *et al*, 1986), and promotes histamine production of human umbilical cord-derived mast cells in costimulation with IL-3 (Richard *et al.* 1992). Because human cord blood-derived cultured mast cells constitutively express trkA, which is upregulated during NGF-driven culture (Welker *et al.* 2000), but no p75NTR (Tam *et al.* 1997; Welker *et al.* 2000), all effects of NGF may be attributed to binding to this receptor (Kanbe *et al.* 2000). In addition, trkC expression has been shown in these cells (Tam *et al.* 1997), but the influence of NT-3 on mast cell functions is not yet clear. In contrast to mast cells, it has been demonstrated that human basophils in peripheral blood express exclusively trkA and not trkB, trkC and p75NTR (Burgi *et al.* 1996). Stimulation with NGF promotes survival in these cells (Miura *et al.* 2001), causes histamine release, primes basophils to produce lipid mediators in response to various cytokines or complement (Bischoff and Dahinden 1992; Miura *et al.* 2001) and triggers synthesis and release of IL-13 (Sin *et al.* 2001). Interestingly, IL-13 production in response to NGF stimulation is increased in patients with allergic asthma but is not dependent on alterations of trkA surface expression (Sin *et al.* 2001). Intrapulmonary human mature mast cells show a modified neurotrophin receptor expression pattern. TrkA, trkB and trkC expression has been observed in the lung tissue (Tam *et al.* 1997), but the functional consequences of neurotrophin stimulation remain unclear.

A recently published study indicates that abrogation of the NGF signal by intranasal application of neutralizing antibodies against NGF could inhibit allergen-induced early phase reaction in a mouse model of allergic asthma (Path *et al.* 2002). In rats another new study provides *in vivo* evidence that intranasal antagonism of the NGF pathway effectively modifies early phase bronchoconstriction without affecting the LAR or airway inflammation parameters (Glaab *et al.* 2003). Furthermore, the early phase response to allergen was examined in sensitized CCSP-NGF-tg mice and compared to the reaction of wild-type controls. In these animals, bronchoconstriction in response to aerosolized antigen was clearly increased, paralleled by a remarkable increase in serotonin levels in the airways, pointing out that NGF may be involved in mast cell degranulation *in vivo* which are located in the airways of asthmatics (Path *et al.* 2002).

4.1.5. Neurotrophins and Late Phase Response

As described before, allergic late phase response is mainly induced by accumulation of eosinophils in the lung which become activated. Bronchoconstriction and tissue damage are

consequences of mediator release from eosinophils. However, neurotrophins may be potent regulators of allergic late phase response (Figure 1.2).

Eosinophils are presumably recruited from bone marrow into the lung via peripheral blood. In bone marrow, mature eosinophils express neurotrophin receptors, as indicated by Labouyrie et al. (1999) (Labouyrie *et al.* 1999) and therefore fulfill the prerequisite for mediating neurotrophin signals. Expression of trkB kinase, trkC kinase and trkC truncated was observed, but detection of trkA as well as p75NTR failed. In human umbilical cord blood-derived eosinophil progenitors, NGF has no influence on eosinophil differentiation, underlining the absence of trkA and p75NTR (Matsuda *et al.* 1988a). In contrast, peripheral blood eosinophils are responsible for NGF even though no receptor has been demonstrated yet. NGF stimulation causes EPO (eosinophil peroxidase) release both in human and murine cells (Solomon *et al.* 1998; Watanabe *et al.* 2001), and increases chemotactic activity and cytotoxicity as determined by larvicidal activity in human eosinophils (Hamada *et al.* 1996). The effect of NGF on eosinophil survival is controversely discussed (Hamada *et al.* 1996; Solomon *et al.* 1998). New data from our group indicate a causal connection of the endobronchially upregulated neurotrophin levels after segmental allergen provocation (SAP) (Virchow *et al.* 1998) that coincide with an influx of activated eosinophils into the bronchial lumen disposing of an increased viability and CD69 expression which is not present in the peripheral blood. Incubation with NGF, BDNF, NT-3, or NT-4 causes a significant increase of viability and CD69 expression of isolated eosinophils from BALF but not from peripheral blood suggesting a unique sensitivity of endobronchial eosinophils to neurotrophins. To further elucidate the underlying mechanism the expression of the neurotrophin receptors on eosinophils was analysed. After SAP expression of p75NTR, trkA, trkB, and trkC were markedly elevated on eosinophils from BALF (Nassenstein *et al.* 2003). Thus, there is an allergen-dependent up-regulation of the four neurotrophin receptors on eosinophils from BALF. This receptor expression is translated into an increased activation and viability of eosinophils in vitro after incubation with each of the four neurotrophic factors. Recently published studies also indicate that blocking the p75NTR signal *in vivo* could prevent allergic late phase reaction as well as eosinophil accumulation (Kerzel *et al.* 2003; Tokuoka *et al.* 2001). Two groups of mice were examined, demonstrating that local intrapulmonary application of neutralizing p75NTR antibodies could prevent the chronic inflammatory response as well as the general absence of this receptor as seen in p75NTR knock out mice. Because local treatment led to similar changes as observed in p75NTR -/- mice, it could be hypothesized that neurotrophin action on local lung cells plays the most important part in the induction of inflammation.

First evidence of the particular role of NGF in this condition came from a study recently published by Path et al. They investigated the effects of intranasally applied NGF-neutralizing antibodies on inflammation of OVA-sensitized and challenged mice (Path *et al.* 2002). In anti-NGF-treated mice, the number of eosinophils recruited into the airways was significantly reduced, whereas the numbers of macrophages, lymphocytes and neutrophils were not altered. Controversely to the effects of anti-NGF treatment, OVA-sensitized and challenged CCSP-NGF-tg mice, overexpressing NGF in the airways, demonstrated a higher influx of eosinophils, lymphocytes and neutrophils into the airways than corresponding wild-type animals. It is not yet clear whether NGF directly alters eosinophil accumulation, e. g. by

inhibiting apoptosis or increasing chemotactic activity, or whether this observation is due to indirect effects. Firstly, an altered local production of cytokines, which support survival, secondly, the facilitation or induction of mast cell degranulation (as described before) might contribute to eosinophil accumulation, and thirdly, neuronally mediated pathways may represent an additional important mechanism leading to airway inflammation.

4.2. Effects of Lipid Mediators on Sensory Nerve Plasticity

Many inflammatory mediators were shown to influence the peripheral projections of primary spinal afferent neurons. Prostaglandin E2 (PGE2), an inflammatory mediator derived from cyclooxygenase pathway of arachidonic acid metabolism, is released from several the lung invading inflammatory cells. In 1995 Lee et al. reported, that PGE2 augments the pulmonary chemoreflex responses elicited by capsaicin (Lee and Morton 1995). In addition, inhalation of PGE2 has been shown to increase the sensitivity of capsaicin-induced cough reflex in humans. Thus, these observations seem to suggest a potentiating effect of PGE2 on the sensitivity of pulmonary C-fiber endings to capsaicin. More recently the same group (Ho *et al.* 2000) addressed the question, if PGE2 also modulates chemical or mechanical irritants. Indeed, the results of this study clearly demonstrate enhancement of chemical or mechanical sensitivity of pulmonary C-fiber afferents by PGE2, suggesting that endogenous release of this substance may play a role in the modulation of C-fiber response to airway irritation or inflammation (Figure 1.2).

4.3. Effects of Cytokines on Sensory Nerve Plasticity

Also immune cell-derived inflammatory cytokines, predominantly those produced from macrophages, were shown to influence sensory neurons (Horton *et al.* 1998). Interestingly, there are some date showing a close infliction of the cytokines IL-6 an TNF-alpha to the pathology of allergic asthma. Only alveolar macrophages of patients suffering from a allergic Late Asthmatic Response (LAR) have an enhanced secretion of TNF-alpha and IL-6 after stimulation with the allergen or IgE. Patients without LAR have not (Gosset *et al.* 1991; Gosset *et al.* 1992). In a rat model Kips et al. induced a bronchial hyperreagibility on serotonin using TNF-alpha. (Kips *et al.* 1992). In humans inhaled TNF-alpha leads to a hyperreagibility to metacholine (Thomas *et al.* 1995). Therefore therapeutical strategies using TNF-blocking agents are discussed (Thomas 2001). Another cytokine with emerging importance in chronic airway inflammation is the Leukaemia Inhibitory Factor (LIF) (Knight 2001). LIF has been shown to regulate haematopoesis and T cell maturation as well as proliferation, differentiation, mobility and function of nervous tissue. The expression level of Tachykinin receptors is increased in asthmatic patients (Bai *et al.* 1995). LIF induces the expression of neurokinin-1 (NK-1) receptor, up-regulates eNANC and preserves cholinergic nerve activity (Knight *et al.* 2000). This effect appeared to occur on the pre-junctional level since contractile responses to the cholinergic agonist carbachol remained unaffected. Thus,

factors such as LIF that modulate tachykinin receptor expression and enhance tachykinin release from sensory nerves could influence the chronicity of airway inflammation.

4.4. Effects of Mast Cell Products on Sensory Nerve Plasticity

Inflammatory cells also release certain proteinases (e.g. tryptase, trypsin) during the local inflammatory process. Apart from their role in protein degradation, these enzymes function as signaling molecules that regulate cells by cleaving proteinase-activated receptors (PARs), members of a family of G-protein-coupled receptors (Dery *et al.* 1998). Proteases cleave PARs within the extracellular domains to expose tethered ligands that bind to and subsequent activate the cleaved receptor. Recent data show that PARs play an important role in neuronal responses to inflammatory stimuli (Vergnolle *et al.* 2001). Proteinase activated receptor 2 (PAR2) has been shown to be expressed at peripheral as well as central terminals on a subset of afferent sensory neurons and PAR2 agonists elicit neurogenic inflammation by release of SP and CGRP (Vergnolle *et al.* 2001). Within the lung, mast cell containing tryptase is located in close proximity to spinal afferent fibers containing SP and CGRP. During an allergen challenge, mast cells release tryptase, which could directly signal to neurons via PAR2 to stimulate release of neuropetides. Indeed, PAR2 mediates acute inflammation via a neurogenic mechanism and thus, neuropeptides and agonists of PAR2 have similar proinflammatory effects (Steinhoff *et al.* 2000) (Figure 1.2). Taken together, activation of PARs contributes to a newly recognized pathway of neuronal signaling between the inflammatory process and peripheral afferent neurons.

5. NEURONAL SECRETED MEDIATORS AND THEIR INFLUENCE ON IMMUNE CELLS

A substantial amount of studies provide evidence that neuropeptides and neuromediators released from nerve endings of the parasympathetic, sympathetic, and sensory system directly influence immune cells and, thus, participate in immunomodulation .

The major neurotransmitter of parasympathetic nerves is ACh, which exerts its biological activities via binding to the acetylcholine receptors (Figure 1.1). These receptors are classified as M1 to M3 on the basis of functional inhibition by antagonists. The muscarinic receptors M1 and M2 are expressed on T cells and their activation augments anti-CD3-induced mRNA expression of IL-2 and IL-2 receptors as well as T-cell proliferation (Fujino *et al.* 1997). Macrophages express the M3 receptor and subsequent stimulation with Ach triggers the release of chemotactic substances by alveolar macrophages (Sato *et al.* 1998).

Sympathetic nerves predominantly release NA and NPY. NA binds to α- and β-adrenergic receptor subtypes expressed on T cells, monocytes, and mast cells (Carstairs *et al.* 1985; Kammer 1988; Woiciechowsky *et al.* 1998). In contrast to activation of muscarinic receptors, IL-2 production and thus the proliferation of T cells are inhibited by β-adrenergic receptor activation (Kammer 1988). Catecholamine-induced release of IL-10 from unstimulated monocytes appears to be rapid and direct, without involvement of

immunological costimulation (Woiciechowsky *et al.* 1998). Mast cells in the human lung bear the β_2-adrenergic receptor subtype (Carstairs *et al.* 1985). NPY serum levels have been demonstrated to be increased during acute exacerbation of asthma (Cardell *et al.* 1994). NPY is able to modulate immune cell functions such as T cell adhesion to the extracellular fibronectin matrix which is mediated by integrin expression via NPY receptors (Levite *et al.* 1998). NPY was shown to induce Th2 cytokine release from a Th1 cell line and Th1 cytokines from a Th2 cell line breaking the commitment of T-cell effector populations (Levite 1998).

Immunoactive secretory products from the i-NANC system include mediators such as VIP and NO, which appears to be the major neurotransmitter in this system (Belvisi *et al.* 1992). Under normal conditions in mouse lungs, VIP receptors are localized on alveolar macrophages. Immunized and intratracheally challenged mice demonstrated elevated levels of VIP and VIP receptor expression on mononuclear cells and neutrophils in perivascular, peribronchiolar and alveolar inflammatory infiltrates (Kaltreider *et al.* 1997). Direct immunologically effects of VIP (Bellinger *et al.* 1996) include inhibition of T cell proliferation, IL-2, IL-4 and IL-10 cytokine production, inhibition of IgE release by B-cells and inhibition of mediator release from mast cells. By signalling through the $VPAC_1$ receptor, VIP induces the maturation of immature Dendritic cells leading to an up-regulated IL-12 production and an enhanced expression of the DC-maturation marker CD83, especially in the presence of tumour necrosis factor-α (Delneste *et al.* 1999).

Once produced, NO passes membranes by simple diffusion and direct activation of soluble guanylate cyclase. A role of NO has been implied in skewing T lymphocytes towards a Th2 phenotype by inhibition of Th1 cells and their product IFN-γ (Taylor-Robinson *et al.* 1994).

e-NANC system associated neuropeptides with immunomodulatory functions are somatostatin (SOM), calcitonin gene-related peptide (CGRP) and the members of the tachykinin family, SP and NKA, which all act via G protein-coupled receptors and thus share several effector functions. Specific receptors for CGRP and SOM have been demonstrated on monocytes, B cells, and T cells (McGillis *et al.* 1991; van Hagen *et al.* 1994). Similar to NPY, CGRP and SOM have the capacity to induce T cell adhesion to fibronectin and to drive distinct Th1 and Th2 populations to an atypical expression pattern of Th2 or Th1 cytokines respectively and, therefore, break the commitment to a distinct T helper phenotype (Levite *et al.* 1998; Levite 1998).

SOM exerts various inhibitory functions on immune responses via specific receptor activation (reviewed in (van Hagen *et al.* 1994)). SOM affects the suppression of Ig production in B cells, including IgE (Kimata *et al.* 1992), modulation of lymphocyte proliferation (inhibitory effect at low concentrations or stimulatory effect at high concentrations) and reduction of (peritoneal) eosinophil infiltration in experimentally-induced hypereosinophilia. Furthermore, SOM inhibits SP-induced mucus secretion in rats (Wagner *et al.* 1995).

CGRP inhibits SP-induced super oxide production in neutrophils and the proliferation as well as the antigen presentation by peripheral mononuclear cells (Tanabe *et al.* 1996). It also stimulates chemotaxis and adhesion of lymphocytes (Levite *et al.* 1998) and causes eosinophilia in the rat lung (Bellibas 1996). Furthermore, CGRP-containing nerve endings

have been localized in close proximity to skin Langerhans cells (Hosoi *et al.* 1993). CGRP has several suppressive effects on Dendritic Cell activation (Carucci *et al.* 2000). Pretreatment of murine skin DCs with CGRP decreases the alloresponses in the mixed lymphocyte reaction, as well as in ovalbumin-specific T cell responses of syngeneic T cells (Hosoi *et al.* 1993). The intracellular signalling via the type I CGRP receptor which is expressed on human monocyte-derived DCs and long-lived murine DC cell lines leads to an increase in intracellular free Ca^{++} and to a decreased expression of MHC class II, the co-stimulatory molecule CD86. In addition this pathway causes a downregulation of the IL-12 production. This effect could be due to an enhanced production of IL-10 by these DCs (Carucci *et al.* 2000; Torii *et al.* 1997).

The tachykinins including SP, NKA, and NKB exhibit their function via binding to the three neurokinin receptors NK-1 to NK-3. NK-1 receptor expression was identified on immune cells such as B cells, T cells (Braun *et al.* 1999b), monocytes, macrophages (Ho *et al.* 1997), eosinophils and neutrophils (Iwamoto *et al.* 1993). Like VIPR, NK-1R expression by lung-infiltrating leukocytes in systemically and subsequently intratracheally allergen challenged mice is strongly elevated (Kaltreider *et al.* 1997). Since SP binds to NK-1 with the highest affinity, it is the predominant mediator of immunomodulatory effects among tachykinins. The actvities of SP on immune cells include a broad range of functional responses from neutrophils, eosinophils, mast cells, monocytes/macrophages, dendritic cells and lymphocytes (Lambrecht 2001; van der Velden and Hulsmann 1999a). SP stimulates a number of neutrophil functions, including chemotaxis, superoxide production and adherence to epithelium and endothelium. Most of these effects require high concentrations of SP whereas at low doses, SP primes the response to other stimuli that otherwise would be ineffective. SP has a degranulating effect on eosinophils and induces human eosinophil migration *in vitro*. In an *in vivo* study with allergic rhinitis patients, it was shown that SP administered after repeated allergen challenge enhanced the recruitment of eosinophils. It has been demonstrated that SP can cause histamine release from human lung mast cells (Heaney *et al.* 1995). This is underlined by an *in vitro* model using trachea from the SP-hyperresponsive Fisher 344 rat, in which SP stimulation of mast cells represents a major factor leading to bronchoconstriction (Joos *et al.* 1997). Moreover, SP activates monocytes to release inflammatory cytokines, including TNF-α, IL-1, IL-6 and IL-10.

New data show that most immune cells (monocytes, DCs and lymphocytes) secrete SP as well as they express its receptor. This leads to the hypothesis that SP not only acts as a mediator between the nervous and immune system but is also involved in the direct interaction between immune cells in a paracrine or autocrine manner. This happens independently of sensory nerves or neurogenic inflammation (Ho *et al.* 1997; Lai *et al.* 1998; Lambrecht *et al.* 1999). The addition of a specific NK-1R antagonist on co-cultures of DCs and allogeneic or syngeneic ovalbumin-specific T cells leads to a DC induced decrease in T lymphocyte proliferation. This effect was enhanced after blocking the co-stimulatory CD80/86-CD28 pathway. In addition, after the stimulation of purified naive *NK-1R*$^{-/-}$ T cells with stimulatory anti-TCR and anti-CD28 antibodies in the absence of DCs, there was a decrease in T cell proliferation. This reveals an autocrine release of stimulatory SP by T cells themselves (Lambrecht *et al.* 1999). Indeed, T cells have been shown to transcribe the mRNA for Preprotachykinin A and release SP on activation with capsaicin (Lai *et al.* 1998).

Lambrecht et al. doubts a direct autocrine and/or paracrine effect of endogenously released SP on the immunostimulatory capacity of DCs, although it has been shown that SP induces activation of the transcription factor nuclear factor-κB in murine DCs (Kradin *et al.* 1997; Lambrecht *et al.* 1999; Lambrecht 2001; Marriott *et al.* 2000). This transcription factor was shown to be essential in the upregulation of stimulatory activity in DCs by enhanced expression of MHC class II, the co-stimulatory molecules CD86 and CD80, and enhanced secretion of IL-12 (Grohmann *et al.* 1998). SP might enhance T cell responses by recruiting DCs into sites of inflammation. It has been shown to be a chemoattractant for lung-derived DCs *in vitro* and *in vivo.* Therefore it might stimulate the primary immune response by enhancing immune recognition of antigens. Moreover, SP is implied in the recruitment of DCs into sites of inflammation during secondary T cell responses in the lung and skin and its depletion leads to severely reduced delayed hypersensitivity reactions (Kradin *et al.* 1997). Currently, it is unclear how DCs regulate the release and activity of SP during interaction with T cells. Van der Velden et al. demonstrated the presence of aminopeptidase N on the surface of bronchial mucosal $L25^+$ DCs in patients with asthma. This enzyme has the potential to break down SP (van der Velden *et al.* 1998).

In lymphocytes, SP inhibits glucocorticoid-induced thymocyte apoptosis (Dimri *et al.* 2000), stimulates proliferation, cytokine production, chemotaxis (van der Velden and Hulsmann 1999a) and a Th1/Th2 phenotype switch in T cells (Levite *et al.* 1998; Levite 1998) and induces differentiation and immunoglobulin switching in B cells (Braun *et al.* 1999b).

Taken together, the accumulated evidence now points towards direct effects of neurotransmitters and neuropeptides on immune cells. A role of these neurogenic immunodulation in asthma is very likely, but since most of these data were obtained *in vitro* or in animal models, the clinical relevance of these observations requires further evaluation.

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Chapter V

IgE –Mediated Asthma and Rhinitis: A Role of Anthropogenic Substances and Pollutants?

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ABSTRACT

Allergic airway diseases such as asthma and rhinitis are increasing in the westernised countries, presumably explained by several simultaneously acting mechanisms where environmental factors may play a significant role. This review attempts a quantitative evaluation of the adjuvant effects from chemicals and environmental pollutants on the occurrence of sensitisation and allergic airway diseases. Laboratory animal studies showed that tobacco smoke possessed adjuvant effect. In the westernised countries, smoking together with a high exposure to allergens at workplaces had been a dominating factor for development of sensitisation, accounting for 0-71% of the sensitised cases; and accounting for about half of the asthma cases. Smoking among adults and exposures to environmental allergens, which are considered lower than exposures to occupational allergens, also accounted for a substantial number of asthma cases (0- ~23%). Environmental tobacco smoke (ETS) exposures in adult account for less of the asthma cases (0- ~11%). Smoking during pregnancy may explain about 2-8 % of the asthma cases in children, but the mechanism need not be through an adjuvant effect. ETS

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exposures in children may account for about 5%. Laboratory animal studies suggested that indoor pollutants, including house dust, surfactants and quaternary ammonium disinfectants may possess adjuvant effect. However, the epidemiological knowledge is currently too sparse to allow conclusions about a possible risk of these exposures in humans in the westernised countries. Although ozone and nitrogen dioxide had adjuvant effect in animal studies, epidemiological studies showed little effect in humans. Exposure to motor vehicle emission may account for about 0 - <10 % of the asthma cases in the westernised countries. Development of animal models allowing risk assessment of adjuvant effect of chemicals and pollutants should be highly acknowledged, as results from these models may be able to compensate for the lack of relevant epidemiological studies.

INTRODUCTION

In the United States, self-reported asthma has more than doubled between 1980 and 1995 (Redd 2002), a trend that is reflected throughout the industrialized countries (Lundbäck 1998). In a worldwide study involving 56 countries, the ratio of the prevalence in the country with the highest and lowest prevalence of asthma, rhinoconjunctivitis and atopic eczema was 20, 30 and 60, respectively, in children aged 13-14 years (ISAAC 1998), suggesting that environmental factors (in the broadest sense) may be critical to the development of allergic diseases (ISAAC 1998).

Several theories have been forwarded to explain the causes of the increase in asthma. They include obesity (Redd 2002; Kheradmand et al. 2002b), lack of maturation of the immune system due to decreasing exposure to microorganisms and infections, “the hygiene hypothesis” (Redd 2002; Kheradmand et al. 2002b), or lack of protective effects from regular contact with livestock and poultry in farming environments, which could reduce the risk of atopic sensitisation and development of asthma in children (von Mutius 2002). The protective effect of the farming environments may be due to endotoxin exposures that may occur already during pregnancy (von Mutius 2002). Another proposed cause is increased exposures to allergens, for example from house dust mites, cockroach, pets, moulds and pollen (Nielsen et al. 2002). Additionally, it has been suggested that increased exposures to compounds with allergy promoting (adjuvant) effect may play a role, for example from proteases (Kheradmand et al. 2002a; Kheradmand et al. 2002b), outdoor pollutants, for example oxides of nitrogen (NO_x), ozone (O_3), fine and ultra fine particulate matter, diesel exhaust particles (DEP) (Ring et al. 2001), and indoor pollutants (Redd 2002, including environmental tobacco smoke (ETS) (Redd 2002; Kheradmand et al. 2002b; Ring et al. 2001).

Environmental pollutants may aggravate asthma (Koenig 1999) and rhinoconjunctivitis (Riediker et al. 2001). In asthmatics, pollutants may decrease the lung function, increase bronchial hyperresponsiveness, visits to emergency departments, hospital admissions, use of medication and symptom reporting (Koenig 1999). Exacerbation of asthma has, for example, occurred with exposures to ozone (O_3), particulate matters, diesel exhaust particles and endotoxins (Peden 2001; Peden 2002) as well as to sulphur dioxide, sulphuric acid and nitrogen dioxide (NO_2) (Koenig 1999).

It has been claimed that except for car traffic exposure, there is little to support outdoor pollutant exposures have significant adjuvant effects at human exposure levels and thus play an important role in the development of new cases of asthma (Nicolai 2002; Redd 2002). The present review has two purposes: first to suggest in a broad sense where exposures occur to known adjuvants – defined as any material that can increase the humoral or cellular immune response to an antigen (Gupta et al. 1993) - from chemicals and pollutions, and second to estimate how much an exposure may contribute to the development of endpoints related to airway allergy.

METHODS

In the quantitative evaluations, we use the relative risk (RR), which is the event/disease rate in the exposed group divided by the rate in the unexposed or low exposed group, and the attributable risk (AR, also known as attributable fraction or aetiological fraction), which is the fraction (or percentage) of all cases of disease, which are associated with the risk factor (Walter 1978). Health Impact Assessment of epidemiological studies relies on the AR (WHO 2000). The relative risk (RR) and the attributable risk (AR) are calculated according to Walter (1978) and converted to percent. The RRs were estimated from the univariate analyses of the data in the reports. The ARs are obtained from:

$$AR = p(RR-1)/[p(RR-1) + 1]$$

where p is the proportion of all subjects that are exposed in the studied population. The important assumptions for the use of this tool are that there is a causal relationship between the exposure and the health outcome, that the relative risk estimate applies to the all exposed group, and that there is no confounding of the observed effect (WHO 2000), which may not have been fulfilled for several of the calculations. Furthermore, similar populations are needed if results obtained in one population are to be used to predict effects in another (WHO 2000), i.e. effects of exposures in westernised countries may not apply to 3th world countries. Heterogeneity of associations may indicate existence of populations with different sensitivity to the exposure (WHO 2000). Overall, this indicates that caution must be applied in the interpretation of results across the studies.

Development of allergic airway diseases is a stepwise process (Ring et al. 2001) where exposures to allergens may cause sensitisation, a process that is promoted by adjuvants. Exposures to the corresponding allergen may result in new-onset asthma, which is promoted by “asthma risk factors”. Elicitation of an allergic airway response may occur due to exposures to allergens, pollutants, infections and due to exercise (“elicitors”). The different processes are indicated in table 1. Except for this, the term adjuvant has been used in a broader sense as an adjuvant-promoted sensitisation is expected also to increase development of new-onset allergic airway diseases and symptom prevalences.

Table 1. Influence of tobacco smoking on the development of allergen or hapten specific IgE antibodies, airway symptoms or asthma among occupationally exposed workers

Allergen/ hapten (H)	Study design	Subjects [a]	Endpoint and type of effect [b]	RR [c]	AR [c] (%)	Reference
Prawn	Cross-sectional	26	Specific IgE (Ad)	2.36	42	McSharry et al. 1994
Isphagula	Cross-sectional	60	Specific IgE (Ad)	4.97	71	Zetterström et al. 1981
Coffee bean	Cross-sectional	129	Skin prick test (+) (Ad)	4.21	64	Zetterström et al. 1981
Castor bean	Cross-sectional	129	Skin prick test (+) (Ad)	2.87	50	Zetterström et al. 1981
Urinary extract from laboratory animals	Cross-sectional	297	Skin prick test (+) (Ad)	1.40	9	Venables et al. 1988
Laboratory animals	Cross-sectional	156	Airways, eyes and skin symptoms (Es)	2.63	28	Venables et al. 1988
Crab	Cross-sectional	263	Asthma (Arf)	2.20	44	Cartier et al. 1984
Methyltetrahydrophthalic anhydride (H)	Cross-sectional	145	Specific IgE [d] (Ad)	0.42 [e]	-	Welinder et al. 1990
Tetrachlorophthalic anhydride (H)	Cross-sectional	45	Specific IgE [d] (Ad)	0.31 [f]	-	Liss et al. 1993
Phthalic, maleic and/or trimellitic anhydride (H)	Cohort	374	Skin prick test (+) [d] (Ad)	4.30	64	Barker et al. 1998
Platinium (Pt) salt (H)	Cohort	78	Skin prick test (+) (Ad) Respiratory, eye or skin symptoms to Pt in skin prick test (-) subjects (Es)	3.66 1.22	60 11	Calverley et al. 1995

[a] The number of subjects has been extracted from each report and need not be equal to the total number of subjects in the report. The number is included in the calculations.

[b] Adjuvant effect (Ad), asthma risk factor (Arf) and elicitor of symptoms (Es).

[c] The relative risk (RR) and the attributable risk (AR) are calculated according to Walter (1978) and converted to percent.

[d] To human serum albumin conjugate with the corresponding acid anhydride.

[e] The authors mention that smoking did not facilitate sensitisation, but also that healthy worker selection may have occurred in the cross-sectional study.

[f] The authors mention that among nonsmokers and ex-smokers combined, there were a significantly higher proportion of subjects who had specific IgE antibody than among current smokers.

ADJUVANT EFFECT OF TOBACCO SMOKE

Tobacco smoke contains particles and volatile compounds. About 4000 compounds have been identified in the smoke, e.g. aromatic amines, dioxanes, nitrosoamines, polycyclic

aromatic hydrocarbons (PAH), solvents and irritants as formaldehyde and acrolein (Nelson 2001).

Laboratory animal studies support the results from epidemiological studies that tobacco smoke may possess adjuvant effects and that it may exacerbate the IgE mediated asthma. Mice, which repeatedly inhaled environmental tobacco smoke (ETS) and the model allergen, ovalbumin (OVA), developed OVA specific IgE and IgG1 antibodies – both promoted by T helper 2 (Th2) cell cytokine production (Corry and Kheradmand 1999) - whereas animals only inhaling OVA did either produce antibodies or showed a low production. The ETS-OVA group also had airway eosinophilia, which was absent in the OVA group (Rumold et al. 2001). A similar enhancement of the OVA specific IgE antibody production was observed in connection with exposure of rats (Zetterström et al. 1985). Mice sensitised with OVA and afterwards exposed repeatedly to ETS had elevated total serum IgE and serum OVA-specific IgG1, increased blood eosinophilia and infiltration of inflammatory cells in the lungs compared to animals exposed to ambient air. Additionally, OVA provocation showed enhanced antibody production in the ETS exposed animals (Seymour et al. 1997). In a mast cell line, mainstream cigarette smoke stimulated the synthesis of allergy and asthma promoting cytokines, interleukin (IL)-4, 5, 10 and 13 as well as the synthesis of tumor necrosis factor (TNF)-α (Smyth et al. 2000). Histamine release from human mast cells was stimulated directly by tobaccco smoke (Smyth et al. 2000). Overall, the experimental studies suggest both adjuvant effect and exacerbation of asthma from tobacco smoke.

Smoking and Occupational Exposures

Occupational exposures are often related to specific types of compounds at higher levels than in the general population, which may facilitate identification of exposures with adjuvant effect that may also occur in the general population. Thus, occupational exposures to tobacco smoke have indicated adjuvant effect on IgE sensitisation and allergic airway diseases elicited by high molecular weight compounds and low molecular weight haptens, but not on isocyanate and Western Red Cedar induce asthma, which are caused by a non-IgE mediated mechanism (Niven and Pickering 1999). Smoking was associated with chronic bronchitis, but not with asthma in heavy and highway construction workers, who were exposed to diesel exhaust, silica, cement, concrete dust, and to welding and asphalt fumes (Oliver et al. 2001).

Examples of adjuvant effect of smoking include increased risk of sensitisation to house dust mite and storage mite as well as to pollen and grain in Danish farmers (Iversen and Pedersen 1990), asthma at salmon-processing (Douglas et al. 1995), and perhaps also sensitisation to occupational allergens in bakers (odds ratio (OR) 1.75, 95% CI 0.55 to 4.75) (Walusiak et al. 2002). Tobacco smoking increased the frequency of specific IgE formation among workers exposed to the hapten methyltetrahydrophthalic anhydride (Yokota et al. 1997). Additional examples of adjuvant effect of smoking are given in table 1 together with the RRs and the ARs. Thus, tobacco smoking may increase the relative risk of sensitisation to high molecular compounds. This may also apply to exposures to some of the haptens, although the adjuvant effect of smoking on the IgE antibody formation in workers exposed to acid anhydrides may be conflicting (Welinder et al. 1994). Whether this is real or a result of

the study design ("healthy-smoker bias" in cross-sectional studies) may be an open question as one of the cohort studies indicated that a cross-sectional study might miss most of those affected (Barker et al. 1998).

Overall, the median value of the relative risk of adjuvant effects of smoking in the studies in table 1 is 2.63 with a range from 0.31 to 4.97. Refrain from smoking may have prevented 0-71% of the cases of sensitisation, which indicates a considerable preventive potential in relation to workplace exposures with a high allergen load. The proportion of asthma among young adults attributable to occupation has been determined to be about 5-10% in 12 industrialized countries (Kongevinas et al. 1999). Also, the onset of asthma occurs predominantly in early childhood (Oechsli et al. 1987) where no occupational exposure has occurred. Overall, occupational exposures can maximally play a minor role in the increasing asthma prevalence in the westernised countries.

Smoking and Exposures to Environmental Tobacco Smoke among Adults in the General Population

It is assumed that adults in the general population are exposed mainly to outdoor and indoor allergens at lower concentrations than the allergen exposure at workplaces with a high allergen load (table 1). Thus, comparing effects on the development of allergy among smoking individuals, i.e. keeping the adjuvant load constant, may give some indication about the importance of the level of allergen exposure itself. Furthermore, comparing the effects in smokers and individuals exclusively with ETS exposure, who are supposed to be exposed mainly to environmental allergens, may give some indication of the importance of a high versus a low adjuvant load.

A recent review showed that contradicting results appeared about the adjuvant effect of smoking in adults (Ulrik and Lange 2002), also apparent from table 2. Additionally, in a recent case-control study, the incidence of adult asthma was unrelated to ever smoking in males (OR 1.07) and females (OR 1.02), but smoking increased the severity of asthma (Siroux et al. 2000). In another case-control study (Flodin et al. 1995), ever-smokers (current and ex-smokers), who had smoked for three years or more, had a significantly increased risk of developing asthma (OR 1.9).

Table 2. Adjuvant effect of smoking in adults from the general population

Study design [a]	N [a]	Endpoint	RR [b]	AR [b] (%)	Reference
C, ≥ 15 years	1844	Skin prick test (+) (grass, house dust, mould, tree and weed)	0.83	-	Burrows et al. 1981
C, 20-40 years	13002	Specific IgE to: House dust mite, Grass Cat	 1.13 (OR) [c] 0.76 (OR) [c] 0.69 (OR) [c]	 - - -	Jarvis et al. 1999
C, 23 years	8660	Asthma or bronchitis since age 16	1.34	17	Kaplan and Mascie-Taylor 1997
C, 23 years	882	Asthma or bronchitis in the last 12 months	0.83	-	Kaplan and Mascie-Taylor 1997
C, 20-69 years	9742 10832	Wheezing last 12 months: ex-smokers. Smokers	 1.27 2.01	 7 27	Lindström et al. 2001
C, 18-45 years	5687	Ever asthma Current asthma	1.14 (OR) [b](NS) 0.95 (OR) [b](NS)	~4 [b] -	Simpson et al. 2001
C, 19-29 years	624	Ever asthma Current asthma	1.73 1.59	23 19	Backer et al. 2002
Birth Coh followed for 15-17 years	1411 1360	Asthma ever Asthma within last year	1.62 2.19	8 14	Oechsli et al. 1987
Coh, ≥ 18 years	8567	Asthma	1.00	-	Vesterinen et al. 1988
Retrospec-tive Coh, 20-50 years	15813	Incidence rate of adult-onset asthma	1.3 (OR) [c]	-	Torén and Hermansson 1999
Coh, ~20 years	271	Asthma-like symptoms	2.1 (OR) [c]	-	Rasmussen et al. 2000

[a] Abbreviations: C indicates a cross-sectional study; Coh a cohort study, years are age of the studied subjects and N indicates the number of subjects extracted from the report and used in the calculations.

[b] The relative risk (RR) and the attributable risk (AR) are calculated according to Walter (1978) and converted to percent. Calculations are from statistically significant values except where indicated (NS). If the relative risk could not be obtained from the data in the report, the odds ratio (OR > RR for OR >1 and OR < RR for 1 > OR) from the univariate analysis in the report has been given and use for calculation of an estimate of AR by substituting RR with OR in the formula in the Materials section.

[c] The odds ratio is from the logistic regression analysis reported in the study.

Adjuvant effects of smoking may be different for different allergen exposures. Thus, in the European Community Respiratory Health Survey, current smoking showed a statistically significant risk of sensitisation to house dust mite allergen, but sensitisation to grass and cat allergens was statistically decreased when comparing the smokers with lifetime nonsmokers (Jarvis et al. 1999). Also, sensitisation to the allergens was higher in individuals below the age of 30 (Jarvis et al. 1999), suggesting an age-depending effect. Misclassification of asthma and chronic obstructive pulmonary disease may occur (Torén and Hermansson 1999) and hamper the interpretation. Additionally, whether or not causality exists cannot be assessed

from the cross-sectional studies, i.e. which comes first, asthma or smoking? That may not even be solved in a cohort study (Rasmussen et al. 2000). However, in the longitudinal study by Oechsli et al. (1987), it was shown that 90 % of the asthma cases were apparent before the onset of smoking, which is in agreement with the estimated attributable risk of about 10% in adolescents (table 2). In a crude analysis, tobacco smoking may be considered constant over studies in table 2, but the environmental allergen exposures may vary (Nielsen et al. 2002), suggesting that the results from the different studies may be considered supplementary. Thus, comparison of RR and AR from the different studies should be taken cautiously. In adult smokers, an overall comparison between table 1 and 2 suggests that the allergen load itself may be an important risk factor for the occurrence of an adjuvant effect over all endpoints.

Effects of ETS in adults have recently been reviewed comprehensively in two studies (Eisner 2002; Jaakkola and Jaakkola 2002b), both suggesting that ETS increases the incidence of asthma. In Eisner (2002), the range of odds ratios for an association between ETS and asthma was 0.9-2.7 with a median about 1.45 and in the study of Jaakkola and Jaakkola (2002b), the range was 1.60-3.30 for young adults and 1.42-1.62 for adults. Three studies have been selected (table 3) to illustrate the effect of ETS in adults. Thus, the association between ETS exposure and adult onset asthma was studied in non-smoking women in Singapore (Ng et al. 1993). Most of the women (> 97%) did not smoke in Singapore and smoking was normally prohibited in workplaces, cinemas, restaurants, hotels and public transport. Therefore, the principal exposures to ETS occurred at home from a smoking spouse or other members of the household. Light exposure to ETS was not associated with development of asthma, which is in contrast to living together with one or more heavy smokers (table 3). Nevertheless, the lung function decreased both in the light and the heavily exposed women and the decrease was exposure-dependent. Similarly, an adjuvant effect appeared from a study in never-smoking men and women from Switzerland (table 3), where the effect also was exposure-dependent (Leuenberger et al. 1994). A cohort of non-smoking adult Seventh-Day Adventists was followed for 10 years (Greer et al. 1993). New-onset asthma increased in ETS-exposed individuals (table 3). Although not always the case, several studies have indicated that the adjuvant effect of ETS increases with increasing exposure level in the ETS exposure-range. However, when comparing ETS exposure-effects and effects in adult smokers (table 2) a clear picture does not emerge in relation to effects of low and high adjuvant exposure-levels.

Table 3. Adjuvant effect of environmental tobacco smoke in adults

Study design [a]	N [a]	Endpoint	RR [b]	AR [b]	Reference
C, non-smoking women, 20-74 years	1289	Asthma: Light exposed Heavy exposed	 0.84 1.42	 - ≤ 11 [c]	Ng et al. 1993
C, non-smoking men and women, 18-60 years	4197	Physician diagnosed asthma	1.17	5	Leuenberger et al. 1994
Coh, men and women, ≥ 25 years	3577	Asthma	1.45 [d]	-	Greer et al. 1993

[a] Abbreviations: C indicates a cross-sectional study; Coh a cohort study, years are age of the studied subjects and N indicates the number of subjects extracted from the report and used in the calculations.

[b] The relative risk (RR) and the attributable risk (AR) are calculated according to Walter (1978) and converted to percent.

[c] If combining the light and heavy exposed groups and calculating the AR, the result was 6%. As the RR in the light exposed group was below one, it was also assumed that no exposure-effect occurred in the group and it was combined with the non-exposed group and used for the calculation of AR in the heavy exposed group, resulting in an AR of 11%.

[d] The RR is for a 10-year follow-up and taken directly from the logistic regression analysis reported in the study.

In Utero Exposure and Passive Smoking in Children

The literature on ETS has been reviewed (e.g. Halken et al. 1995; Jaakkola and Jaakkola 2002a) and several meta-analyses (Cook and Strachan 1997; Strachan and Cook 1998a; Strachan and Cook 1998b) are available.

Fetal-maternal interaction *in utero* may influence the immune response of infants and cause predisposition to a later development of allergic airway diseases (Warner et al. 2000). However, other effects may also play a role. The effect of tobacco smoke on lung function in children has been investigated in a longitudinal study (Gilliland et al. 2003). Exposure *in utero* of children without asthma decreased the lung function at age 7-18 years. Among children without *in utero* exposure, early asthma onset was associated with a more pronounced decrease in lung function compared with later diagnosed asthma. A disproportionate decrease was observed in children who were exposed both *in utero* and had early onset asthma compared with later onset asthma. The combined effect persisted into adolescence. ETS alone was not associated with lung function effects. This suggests that effects of *in utero* exposure was independent from effects of ETS exposure, and that *in utero* exposure to tobacco smoke may predispose to later debilitating effects of asthma.

The exposure effects of maternal smoking during pregnancy, and childhood exposure to ETS were investigated in a cross-sectional study for asthma promoting effects in 4 th, 7 th, and 10 th grade school children in California (Gilliland et al. 2001). *In utero* exposure to maternal smoking without subsequent postnatal ETS exposure was associated with increased prevalence of physician-diagnosed asthma (OR 1.8). This OR was obtained from a minor

group, 161 out of 5324 children. In contrast, current and previous ETS exposure was not associated with asthma prevalence, but was associated with wheezing. It was proposed that ETS operates as a co-factor with other insults such as intercurrent infections as a trigger of wheezing attacks, rather than as an asthma-inducing factor. This is in contrast to the *in utero* exposure, which increased the asthma prevalence. The incidence of asthma and wheezing illness was followed in a birth cohort until the participants reached the age of 33 (Strachan et al. 1996). Exposure *in utero* together with ETS exposure during the first 16 years of childhood increased the incidence of asthma and wheezing illness at the end of the study (OR 1.40). A German birth cohort was followed until the participants reached to the age of seven. The study showed that maternal smoking during pregnancy was a risk factor of sensitisation to food and aeroallergens during the 2 to 7-year age period (OR 2.27) and for development of asthma (OR 2.46) at the school age (Illi et al. 2001). Estimates on relative risk and attributable risk are given in table 4.

One of the reviews considered effects of parental smoking on IgE levels (neonates and older children), skin prick positivity and allergic rhinitis or eczema in children (Strachan and Cook 1998a). They found no consistent association between parental smoking and allergic rhinitis or eczema. The different studies with skin prick test results were combined in a meta-analysis, which showed no excess risk on prick test positive responses (pooled odds ratio 0.87). The authors conclude that parental smoking, either before or immediate after birth, is unlikely to increase the risk of allergic sensitisation in children. However, the evidence was much less consistent with regard to current exposure. In a meta-analysis comprising 26 studies on paternal smoking effects on development of asthma, the pooled odds ratios for the effects of either parent smoking were 1.21 for asthma (Cook and Strachan 1997). Maternal smoking had a greater effect than paternal smoking, but there was evidence for a small effect from paternal smoking (Cook and Strachan 1997). As the prevalence of asthma increased with the number of parents who smoked, this suggests a causal relationship (Cook and Strachan 1997). Estimates on the relative risk and the attributable risk are given in table 4.

An overall evaluation suggests that *in utero* exposure to tobacco smoke may account for up to 8% of asthma cases among children and ETS exposures for about 5%.

Table 4. *In utero* exposure to tobacco smoke and exposure of children to environmental tobacco smoke (ETS)

Study design [a]	Exposure [a]	N [a]	Endpoint	RR [b]	AR [b] (%)	Reference
C, school children, grades 4,7, and 10	In utero No exposure ETS only *In utero* only ETS and *In utero*	5324 Size of subgroup: 3071 1243 161 849	Physician-diagnosed asthma (all cases: 805)	 1 1 1.60 [c] 1.24 [c]	 0 0 1.7 [c] 3.6 [c]	Gilliland et al. 2001
Birth Coh. followed to 33 years	In utero	18559 new-born, at the end 5801 (31%)	Asthma and wheezing illness	1.27 [d]	5 [d]	Strachan et al. 1996
Birth Coh. followed to 7 years	*In utero*	1314 new-borne	Persistent sensitisation to food and/or aeroallergens Asthma	1.91 [e] 2.21 [e]	14 [e] 8 [e]	Illi et al. 2001
Meta-analysis	ETS	-	Asthma	1.19 [f]	5 [f]	Cook and Strachan 1997

[a] Abbreviations: C indicates a cross-sectional study; Coh. a cohort study, years are age of the studied subjects, ETS indicates environmental tobacco smoke exposure after birth and N the number of subjects studied.

[b] The relative risk (RR) and the attributable risk (AR) are calculated according to Walter (1978) and converted to percent. If the RR was not obtained from the data in the report, odds ratio (OR) has been converted to relative risk RR = OR/[(1-p_{-exp})+ (OR x p_{-exp})] where p_{-exp} is the proportion of events in the reference group (Stoddard and Miller 1995).

[c] The number of preventable asthma cases in each group has been calculated from a•(RR-1)/RR (Stoddard and Miller 1995) where (a) is the number of asthma cases in the group and converted to percent of all asthma cases (805).

[d] The incidence in the reference group was set to 25% as the incidence in atopics and non-atopics was 30.6 and 19.5%, respectively (Strachan et al. 1996). The AR was calculated, using 18.8 % as the proportion of children exposed *in utero* (Gilliland et al. 2001).

[e] The prevalence of sensitization in the non-smoking population has arbitrarily been set to 15%. The prevalence of asthma in the non-smoking population has arbitrarily been set 7.5%, which is the mean asthma prevalence in Danes aged 0-17 years (Mossing and Nielsen 2003). AR was calculated, using 18.8 % as the proportion of the children exposed *in utero* (Gilliland et al. 2001).

[f] The prevalence of asthma in the non-smoking population was set to 7.5% (Mossing and Nielsen 2003). The prevalence of children exposed to ETS has been set to 25%, roughly justified from the fact that Finland has about 27% active male and about 20% active female smokers (Jousilahti and Helakorpi 2000).

ADJUVANT EFFECT FROM INDOOR EXPOSURES

Within Sweden, the prevalence of allergy was highest in the northern part of the country with the cleanest outdoor air. This may suggest that the indoor environment exposures may play an important role in the development of allergic sensitisation and development of allergic diseases (Løvik et al. 1996).

Epidemiological Studies in Cleaners

Due to high occupational exposures, work-related asthma may serve as a model of asthma in the general population (Petsonk 2002). Therefore, inferences about indoor exposure effects may be obtained from cleaners who may be exposed, for example, to indoor allergens from house dust mites, cockroaches, pets, pollens and moulds (Nielsen et al. 2002), and cleaning products (Rosenman et al. 2003).

In a comprehensive study belonging to the European Community Respiratory Health Survey (ECRHS) and comprising 12 industrialized countries, cleaners had an excess risk of asthma. Thus, the risk was elevated in 11 of the 12 participating countries (Kongevinas et al. 1999). In a more recent ECRHS study, the prevalence of asthma was compared between cleaners (14.1%) and office workers (6.9%). Smoking could not account for the elevated prevalence among the cleaners (Zock et al. 2002).

In a Finnish registry study, female cleaners had an increased risk of developing asthma. The tasks were industrial cleaning as well as cleaning of buildings used for administrative purposes, health and social work, and for recreational, cultural and sporting activities. The relative risk of developing asthma among the cleaners was 1.5 when compared with administrative workers. Of the 2414 cleaners with asthma, only 24 (1%) of the cases were recognized as occupational asthma (Karjalainen et al. 2002).

All new cases of occupational asthma diagnosed in five public clinics in the area of the São Paulo Municipality were analysed. The combined group of janitors, house keepers and cleaners had the highest frequency of woman asthmatics (28.8%), whereas the frequency in the same group of males was 0.84%. In women, 38.5% associated cleaning products (i.a. products containing chlorine, ammonia, detergents and waxes) with their symptoms. It was unlikely that smoking was a confounder as only 13.5% of the women were smokers (Mendonca et al 2003).

Spanish cleaners had an increased prevalence ratio (1.7) of asthma, which was highest (3.3) among the private home cleaners. The home cleaners had a prevalence of house dust mite sensitisation of 28% whereas other indoor cleaners had a much lower prevalence of sensitisation (3%), suggesting that exposures to house dust mite may play a role in the onset or aggravation of asthma. Asthma in home cleaners was mainly associated with kitchen cleaning using oven spray or furniture polishing (Zock et al. 2001).

Work-related asthma to cleaning products may be due to work-aggravated asthma or new-onset asthma (Rosenman et al. 2003). Life threatening exacerbation of asthma has been observed after inhalation of strong airway irritants. Thus, a severe asthma attack was elicited in an asthmatic 22-year-old woman, who inhaled chlorine, which was liberated from mixing

sodium hypochlorite and an acid containing detergent (Mapp et al. 2000). Similarly, carpet cleaning with pressure application of a solution containing tripolyphosphate, dipropylene glycol methyl ether and various fragrance compounds in water precipitated a severe asthma attack in a 42-year-old woman with previously diagnosed asthma. The exposure was estimated to be several times the occupational exposure limit (Lynch 2000).

New-onset asthma may be due to direct sensitisation to compounds in the cleaning products as in the case with a hospital worker who had contact with n-alkyl dimethyl benzyl ammonium chloride (benzalkonium chloride) and didecyl dimethyl ammonium chloride. Similarly, asthma was developed in another hospital worker who used a floor cleaner that contained quaternary ammonium compounds (QACs), ethyl alcohol and sodium hydroxide (Rosenman et al. 2003). New-onset asthma may also be due to high exposures to strong irritants such as graffiti-removal products and drain cleaners (Rosenman et al. 2003).

In pig farmers, atopic sensitisation with immunoglobulin E (IgE) production to common aeroallergens was more frequent in farmers who used spray application of disinfectants containing QACs. In atopics, use of the disinfectants was related to respiratory symptoms consistent with asthma. No farmer had specific IgE to chloramines-T and only two out of 40 farmers using QACs had specific IgE to QACs. Thus, this suggests that occupational exposure to QAC disinfectants may induce IgE sensitisation to common aeroallergens and that the combination of atopy and exposure to QACs disinfectants is a risk factor for development of symptoms consistent with asthma (Preller et al. 1996).

On the whole, development of work-related asthma in cleaners has been related to exposures to allergens and chemicals. One of the studies suggested that QACs may possess adjuvant effects. Similar types of reaction may be expected in the general population but with a lower frequency due to the lower exposure levels.

Indoor Dust

House dust (deposits on surfaces) is a complex matrix, which may vary in composition from room to room. It may be divided in fibrous and nonfibrous materials. The fibrous materials may contain, for example, synthetic fibres and natural fibres as hair and cotton. The nonfibrous materials may contain i.a. heavy metals as lead, soot particles, soil, paint, pesticides, phthalates, phenols, aldehydes, polycyclic aromatic hydrocarbons, substances with hormonal or carcinogenic activity, skin cells, and allergenic materials, i.a. from house dust mites, fungi, microorganisms and pollens (Lioy et al. 2002). Samples of airborne indoor particulate matter (mean aerodynamic diameter < 10 μm, PM_{10} fraction) from homes in the city of Oslo contained mainly particles less than 2.5 μm in diameter (fine particles). The fine particles were mainly carbon aggregates (soot) and sulphur containing particles that include gypsum particles. The soot particles may partly be combustion products from car traffic, especially diesel engines, and from indoor smoking (Ormstad et al. 1997). Examples of indoor airborne dust levels are given in table 5, which shows that the airborne dust levels are strongly influenced by daily use of the buildings.

Table 5. Examples of airborne dust levels in buildings

Sampling place	Particles [a)]	Concentration ($\mu g/m^3$)	Reference
29 households in Oslo, Norway	PM_{10}	26 (9-56) [b)]	Ormstad et al. (1997)
Office building in Denmark	Total dust [c)] Respirable dust [e)]	64 (51-76) [d)] 54 (44-65) [d)]	Kildesø et al. 1998 -
Kindergarden in Denmark	Total dust [c)] Respirable dust [e)]	267 (240-294) [d)] 133 (118-148) [d)]	Kildesø et al. 1998 -
School in Denmark	Total dust [c)] Respirable dust [e)]	174 (145-203) [d)] 102 (83-120) [d)]	Kildesø et al. 1998 -
Three offices at a university in the US	$PM_{2.5}$ PM_{10}	5.2 (4.9-5.7) [f)] 8.2 (7.3-9.3) [f)]	Kildesø et al. 1999 -
Lecture room in Prague, Czech Republic	PM_1	18 (11-25) [b)]	Branis et al. 2002
Pizzeria in Prague, Czech Republic	PM_1	213 (146-250) [b)]	Branis et al. 2002
Office (smoking) in Prague,Czech Republic	PM_1	17 (8-35) [b)]	Branis et al. 2002
Office (non-smoking) in Prague,Czech Republic	PM_1	12 (7-18) [b)]	Branis et al. 2002

[a)] Suspended particulate matter. PM_1, $PM_{2.5}$ and PM_{10} are particles with an aerodynamic mean diameter < 1, < 2.5 and < 10 μm, respectively.
[b)] Median and range in parenthesis.
[c)] Collected with a 25 mm filter cassette with an inlet opening of 5.7 mm and the air velocity in the opening of 1.25 m/s.
[d)] Mean value and 95% confidence limits in parenthesis.
[e)] A cyclone was used for the sampling.
[f)] Average concentration and range.

Fine particles may transport allergens to the deep parts of the lungs. The indoor suspended particulate matter (PM_{10}) from the homes in Oslo contained a large number of soot particles (< 1μm), which mainly may be DEP. The soot particles had adhered cat, dog and birch allergens, but not allergens from house dust mites (Ormstad 2000). In an *in vitro* study, it was confirmed that DEP was able to adsorb all four allergens (Ormstad 2000).

Direct evidence for adjuvant effect of suspended particulate matter has been obtained from laboratory animal studies. Thus, injection of the PM_{10} particles together with OVA in the hind foot pad of mice resulted in an enhanced OVA specific IgE content in the serum compared to the level in the control group that only received OVA (Ormstad 2000).

Support from humans for adjuvant effect of indoor exposures may i.a. come from a recent study from India (Mishra 2003). Thus, using biomass fuel (wood, crop residues or dung cakes) for cooking was associated with increased asthma among elderly men and women (≥ 60 years old) when compared to the prevalence in a similar age group that used cleaner fuel (coal, coke, lignit or charcoal). Together with the data from tobacco smoke and DEP studies, this also supports the hypothesis that indoor combustion product exposures may possess adjuvant effect.

However, assessment of adjuvant effect of compounds in the suspended particulate matter is complicated by the fact that house dust also contains endotoxins (Gereda et al. 2000; Gehring et al. 2002), a component of the cell wall of gram-negative bacteria. Endotoxins promote the development of type-1 T-helper cell (Th1) cytokines that inhibit the development of type-2 T-helper cell (Th2) cytokines, which are a prerequisite for the development of allergic sensitisation and the development of allergic asthma and rhinitis (Gereda et al. 2000). Indeed, both studies showed that high endotoxin exposures were associated with protection against allergen-induced sensitisation in children (Gereda et al. 2000; Gehring et al. 2002).

Assessment of the effect of indoor dust is complicated by the fact that indoor dust is a complex and variable matrix, by the highly different exposure levels (table 5), and by the content of substances with adjuvant effect as well as by substances that counteract allergic sensitisation and development of allergic airway diseases. The focus has mainly been on the commonly encountered allergens from house dust mites, cockroach, animal dander, fungal spores and pollens (Clark et al. 1999; Nielsen et al. 2002). However, as several of the allergens, including these from house dust mites, cat, pollen and fungi, may possess protease activity, they may themselves act as immunological adjuvants (Kheradmand et al. 2002a; Kheradmand et al. 2002b). Overall, the role of chemicals and anthropogenic pollutants in the adjuvant effect of indoor dust is not clear from the scientific literature.

Surfactants

In the Western part of the world, surfactants are used in huge amounts, mainly in laundry detergents, cleaning agents and cosmetics. The entire population is exposed daily to residues in floor dust (Clausen et al. 1998; Vejrup and Wolkoff 2002), in clothes (Vejrup and Wolkoff 2002), residues after dishwashing and from contact with cosmetics, including toothpaste, soap and shampoo (Singer and Tjeerdema 1993).

Surfactants only seldom induce asthma in the detergent industry (Stenton et al. 1990; Markham and Wilkie 1976) although it has been described (Stenton et al. 1990). Adjuvant effects of surfactants has been reported. Thus, intraperitoneal administration in mice of non-ionic or anionic surfactants together with a protein from *Neisseria gonorrhoeae* showed adjuvant effect of the surfactants on the IgG antibody production (Teerlink et al. 1987). In guinea pigs, aerosol sensitisation with an inactivated protease and an anionic surfactant also increased antibody production (Markham and Wilkie 1976). However, from the antibody production in a screening model in guinea pigs where diphtheria toxoid was administered subcutaneously (s.c.) together with the test substance, it was concluded that the anionic agents appeared to be inactive, but several non-ionic surface active agents showed a slight effect (Gall 1966). In mice, the protease Alcalase was administered intratracheally in a detergent matrix. The detergent promoted the development of specific IgE and IgG1 antibodies, and the adjuvant effect increased with increasing doses of the detergent (Kawabata et al. 1996). Using intranasal administration of Alcalase together with a detergent, adjuvant effect appeared on the IgG1 production (Robinson et al. 1996). Additionally, the adjuvant effect of the detergent increased with increasing doses and virtually all of the IgG

response was on the IgG1 subclass (Robinson et al. 1996). – The murine $CD4^+$ Th2 cells promote the interleukin (IL) 4 production and isotype switch to IgE and IgG1 production in specific B-cells (Robinson et al. 1996; Poulsen et al. 2000) why the IgG1 production was proposed as a surrogate marker for assessing enzyme allergenicity (Robinson et al. 1996).

Overall, there was little data to support the hypothesis that commonly used surfactants possess allergy promoting effects (Poulsen et al. 2000). However, due to the ubiquitous occurrence of the substances, we investigated their role in a screening study (Clausen et al. 2000). We selected four substances representing the most used types of anionic and non-ionic surfactants in Denmark. Additionally, from each type of surfactant, a single widely used compound was selected as a representative. The anionic substances were sodium dodecyl benzene sulfonate (SDBS), a representative of the linear alkylbenzene sulfonates (LAS-compounds), sodium dodecyl sulfate (SDS), a representative of the alkyl sulfates, and the sodium salt of the coconut oil acids, a representative of soaps. The non-ionic substance Genapol X-80 (nC12, EO8) was used as the representative of alcohol ethoxylates. The test substances were injected s.c. in mice in the concentrations from 1-1000 mg/l together with 1 μg ovalbumin (OVA). Later on the animals were boosted with 0.1 μg OVA s.c.; the surfactant test concentrations overlapped the use concentrations for indoor cleaning. The OVA specific IgE was determined in serum. Only SDS promoted the specific IgE antibody production. The screening study by-passed the external biological barriers and presented the model substances and the model allergen directly to the immune system, which is expected to maximize the responses. Thus, a positive result in the screening study requires testing under natural conditions to determine whether it would be possible to expect an adjuvant effect at human exposure conditions and levels. Overall, the screening study demonstrated that most of the commonly used surfactants did not possess adjuvant effect.

Quaternary Ammonium Compounds

Quaternary ammonium compounds are used as disinfectants, antiseptics, detergents and preservatives. Thus, QACs are used in hospitals to clean medical and surgical instrument, and for cleaning floors and surface. As preservatives and antiseptics, QACs may be found in cosmetics, lotions, skin care products, shampoos, eye drops and contact lens cleaning solutions. Despite the large-scale use in hospitals of QACs, only few cases of asthma have been related to their use (Purohit et al. 2000).

Information about adjuvant effects of QACs are available from the comprehensive screening study of Gall (1966). Diphtheria toxoid mixed with a QAC was injected subcutaneously in guinea-pigs. Animals were boosted once with the toxoid alone and later the antibody titre was determined. Overall, substances with short alkyl chains (e.g. acetylcholine) showed no adjuvant effect. For the trimethyl alkyl ammonium compounds, hexadecyl, octadecyl and longer chain length substances tended to be more active than shorter congeners. Also among the alkyl benzyl ammonium compounds, the adjuvant effect increased with the length of the alkyl chain. In itself, the benzyl group appeared to decrease the adjuvant activity. Dimethyl dialkyl ammonium compounds were more active than their trimethyl alkyl ammonium congeners. One of the most active compounds tested was dimethyl

dioctadecyl ammonium chloride (DDA). In another study, cetyltrimethylammonium bromide (CPC) was administered intraperitoneally in mice together with a protein from *Neisseria gonorrhoea*, which showed an adjuvant effect of the CPC on the IgG production. If the CPC-protein complex was adsorbed to aluminium phosphate, the enhancement was much more pronounced. However, higher concentrations of CPC decreased the antibody production (Teerlink et al. 1987). Our group has recently studied a series of commercially important QACs with the previously mentioned screening method used for the investigation of surfactants. Several QACs, including the widely used compound benzalkonium chloride, showed an adjuvant effect on the OVA specific IgE and IgG1 production (Larsen et al. unpublished results).

DDA is a potent adjuvant where the halide counter ion (Cl^- or Br^-) plays no role in the adjuvant effect. DDA is used as an adjuvant in vaccines. Substances with similar structure are used in cosmetics and laundry softeners where their affinity for negatively charged surfaces are employed in antistatics and hair preparations. DDA stimulates both the humoral and the cell-mediated immune responses (reviewed by Hilgers and Snippe (1992)). The response where DDA was combined with another adjuvant or an immunomodulating agent showed antagonistic, additive or synergistic effect, depending on the admixed substances (Hilgers and Snippe 1992).

In animal testing of QACs, the route of administration of an adjuvant may be less important for the detection of an effect. Thus in mice, DDA showed an adjuvant effect with subcutaneous injection of an experimental tuberculosis subunit vaccine (Lindblad et al. 1997), and DDA showed an adjuvant effect with intranasal (Klinguer et al. 2001) and intramuscular injection (Klinguer-Hamour et al. 2002) of a subunit vaccine against respiratory syncytial virus.

Overall, QACs disinfectants showed a limited direct risk of induction of occupational asthma, which suggests that these substances should possess a low risk in the general population. The adjuvant effect in the general population is also suggested to be minimal due to the lower exposure levels compared to that with occupational purposes. As demonstrated from numerous experimental studies there is no doubt that DDA and substances with a similar structure are potent adjuvants. However, their role, if any, in the development of asthma in the general population is difficult to deduce due to the limited data about exposure levels and use patterns.

ADJUVANT EFFECTS FROM OUTDOOR EXPOSURES

Ozone

Outdoor air ozone is a reaction product from nitrogen oxides, volatile organic compounds in combination with strong sunlight (Peden 2001). Indoor air O_3 levels may be generated, for example, from photocopying machines (Selway et al. 1980) and ozone-generating air-purifying devices (Esswein and Boeniger 1994).

Effects of intermittent O_3 (180-500 $\mu g/m^3$) and OVA aerosol exposures were studied in an IgE high responder strain, BALB/c, and an IgE low responder strain, C57BL/6. In the

BALB/c mice, addition of O_3 to the OVA exposure promoted a positive "skin prick test" to OVA, increased OVA specific IgG1 in serum, increased eosinophils and leokotrienes in the bronchoalveolar lavage fluid, and caused airway hyperresponsiveness. In C57BL/6 mice, addition of O_3 to the OVA exposures promoted a "positive skin prick test" to OVA and OVA specific IgG1 antibodies in serum (Neuhaus-Steinmetz et al. 2000). On the whole, O_3 promoted an asthma-like response in the "atopic" BALB/c strain and sensitisation in the "non-atopic" C57BL/6 strain.

In monkeys sensitised with house dust mite allergen, repeated aerosol exposures to the allergen together with cyclic exposures to air or O_3 were compared. The O_3 exposure increased serum IgE, serum histamine and airway eosinophilia. The combined exposure also resulted in greater alterations in the airway structure, and elevated airway resistance and reactivity (Schelegle et al. 2003), which supports that O_3 may exacerbate asthma reactions.

In a French cross-sectional study, exposures to high and low air pollutant levels were used to study airway effects of SO_2, O_3 and NO_2 children aged 13-14 years. The mean O_3 exposure levels were positively associated with the prevalence of 12-months wheezing and asthma attacks. However, due to the cross-sectional type of study, it was not possible to distinguish between asthma induction and irritant effects that may exacerbate asthma in sensitised individuals (Ramadour et al. 2000). It is well established that O_3 can exacerbate symptoms in asthmatics (Nicolai 2002).

A 10-year prospective study was conducted in adult non-smoking Seventh-Day Adventists (about 4000 participants) to compare incidence of asthma and outdoor O_3 concentrations (Greer et al. 1993). New-onset asthma occurred in 76 cases, 27 men and 51 women. The relative risk of asthma increased with long-term O_3 exposures in men, but not in women. The gender difference was suggested to be due to more time spent outdoors among men and a possible ability of O_3 to potentiate effects of other ambient and occupational respiratory irritants. A 15-year follow-up of the Greer-cohort was performed (McDonnell et al. 1999), showing 115 asthma cases (33 males and 82 females) in the period. The relative risk in men was two-fold for a 27-ppb increment in mean 8-h average O_3 concentration. As in the Greer et al. (1993) study, no association was found between O_3 exposures and the asthma incidence in women (McDonnell et al. 1999).

In a 5-year follow-up in a cohort of 3535 children in California, 265 new asthma cases appeared in the period. A significant increased risk of asthma in children who played three or more team sports (a proxy for heavy exercise increased ventilation) was observed in high O_3 communities, but not in communities with low O_3 levels (McConnelle et al. 2002). Taking into account that the number of subjects playing three or more team sports was low (~8%) and the relative risk of asthma in the group, it suggests an attributable risk of about 5%.

Overall, high O_3 exposures in animals suggest an adjuvant effect of O_3, which also may appear with high exposures in humans. However, the effect can probably only account for a low percentage of asthma cases and thus may at maximum play a marginal role in relation to the increase in the asthma prevalence in the general population.

Nitrogene Oxides

Outdoor air NO_2 is mainly due to emissions from stationary sources, e.g. power plants and steel mills, and mobile sources, e.g. automobiles, aircraft, water-going vessels and lawn mowers (Peden 2001). Indoor air NO_2 derive from emission from unvented cooking and heating appliances and the indoor level may exceed the outdoor level (Shima and Adachi 2000).

Interaction between NO_2 exposure and the model allergen OVA was studied in rats (Siegel et al. 1997). A single one-hour exposure to 87 ppm NO_2 was given one day prior to or on the same day prior to a 30 min OVA aerosol exposure. The OVA exposure was repeated once a week for a total of six exposures. In itself, the one-hour NO_2 exposure caused inflammation in the lungs that resolved within seven days. After three weeks, both groups had a 10 times higher serum OVA specific IgE level than the control group, which only received saline and OVA. Thus, a NO_2 exposure that caused airway inflammation showed adjuvant effect.

Exposure to NO_2 may exacerbate existing asthma, but may not increase the incidence of asthma (Parnia et al. 2002)

Effects of gas cooking, an important indoor NO_2 source, was investigated in a cross-sectional study in 2668 non-smoking women in Singapore (Ng et al. 1993). There was no significant association between the weekly frequency of gas cooking and physician diagnosed asthma, although a non-significant trend was observed with increasing cooking frequency.

In a French cross-sectional study, exposures to high and low air pollutant levels were used to study airway effects of SO_2, O_3 and NO_2 in 2445 children aged 13-14 years. There was no consistent association between the NO_2 levels and prevalence of rhinitis, asthma or asthma-like symptoms (Ramadour et al. 2000). In Japanese communities, 842 children, aged 9-10 years, were followed over three years. The annual mean indoor NO_2 concentration was 18.4 ppb (winter: 24 ppb and summer: 15.2 ppb) in 270 children living in homes with a vented heating system and the concentration was 32.4 ppb (winter: 75.1 ppb) in 572 children living in homes with an unvented heating system. The mean outdoor concentration varied from 7-31.3 ppb between the different communities. The asthma prevalence was increased in girls in relation to indoor NO_2 exposures in the first two years of the study, but not in the third year. There was no NO_2 induced increase in the boys. Logistic regression analysis showed that the incidence of asthma increased with increasing outdoor NO_2 concentration whereas effects of sex, indoor NO_2, parental smoking habit, and use of unvented heating system was not significant (Shima and Adachi 2000). Taking into account that the indoor NO_2 concentrations were as high or higher than the outdoor levels, this suggests that the outdoor NO_2 levels were a surrogate for other outdoor exposures. Thus, there was no clear adjuvant effect of the NO_2 exposure itself at the studied levels. In a later study (Shima et al. 2002), 3049 ten-year-old Japanese school children were followed for 6 years in a prospective cohort study where ambient NO_2 levels were used as surrogate for road traffic pollutants. The prevalence of asthma was not associated with NO_2 concentrations whereas the incidence of new asthma cases was associated with the concentrations. This suggested that traffic-related air pollution contributed to the development of asthma as the use of unvented heaters, which

are a major source of indoor pollution in Japan, was not associated with the prevalence or incidence of asthma.

Overall, there is little evidence that NO_2 could possess an important adjuvant effect at levels humans normally are exposed to.

Road Traffic Pollution

With respect to traffic related pollution, diesel exhaust has attracted much attention in relation to development of allergy and allergic airway diseases. Diesel exhaust consists of gases, including volatile organic compounds, CO, NO, NO_2 and SO_2, and particulate matter, including polycyclic aromatic hydrocarbons adsorbed to carbon particles and acid aerosols (Pandya et al. 2002).

Experimental Studies in Humans and Animals

In atopic humans, intranasal immunization with keyhole limpet hemocyanin (KLH) resulted in development of KLP-specific IgG and IgA antibodies that were detected in nasal lavage fluid. Administration of DEP 24 hours before KLH immunization also caused KLH specific IgE antibody formation in addition to the IgG and IgA antibodies. Nasal lavage fluid contained elevated IL-4 only in the DEP treated group. This indicated that DEP acted as a mucosal adjuvant, which was a prerequisite for formation of specific IgE antibodies against the new allergen (Diaz-Sanchez et al. 1999).

Inhalation of diesel exhaust (i.e. gasses and DEP) in mice and intranasal administration of OVA increased the spleen weight and serum OVA-specific IgE antibodies. Production of IL-4 and IL-10 (Th2 cytokines) increased whereas interferon (INF)-γ (Th1 cell dependent) decreased in the cultured spleen cells (Fujimaki et al. 1997).

Several animal studies have demonstrated adjuvant effects of the DEP fraction. Intranasal administration of DEP and OVA in mice increased the number of animals responding with production of OVA-specific IgE antibodies and the antibody levels. A similar increase was shown with carbon black particles and OVA administration, suggesting that not only the organic matter adsorbed to the DEP but also the carbon core of DEP was involved in the adjuvant effect of DEP (Nilsen et al. 1997). Also, outdoor suspended particulate matter (mainly exhaust particles and soil dust) showed adjuvant effect in mice with intranasal administration with OVA. The adjuvant effect was independent of the size of the particles in the two fractions, ≥ 7 μm and 2-3.3 μm. Both fractions were able to adsorb OVA, but for the same mass of particles, the smaller particles were much more efficient to adsorb OVA (Takafuji et al. 1989), suggesting an increased ability of small particles to transport more allergen into the lungs. In mice, intranasal instillation of DEP and OVA promoted cervical lymph node cell proliferation, IL-4 production and suppressed INF-γ production *in vitro* (Fujimaki et al. 1995), indicating a Th2 promoting effect of DEP in a local airway lymph node. Intratracheal administration of DEP and OVA or Japanese cedar pollen in mice showed an adjuvant effect on serum allergen specific IgE levels. *In vitro,* mediastinal lymph node cell proliferation was enhanced in the DEP co-administered groups as were IL-2 and IL-4

productions (Fujimaki et al. 1994). Additionally, intratracheal administration of DEP and OVA in mice also caused airway hyperresponsiveness (Takano et al. 1998).

Similar results were obtained in animals with non-airway administrations. Thus, intraperitoneal administration of DEP together with OVA, dinitrophenyl-OVA or Japanese cedar pollen allergens enhanced the allergen specific IgE serum levels compared with the levels after allergen administration alone (Muranaka et al. 1986). In mice, intraperitoneal administration of DEP and OVA increased OVA-specific IgE antibodies, but also another Th2 cytokine dependent antibody, OVA-specific IgG1, as well as the Th1 cytokine dependent antibody type, OVA- specific IgG2a. Extraction of the adsorbed hydrocarbons from DEP and co-administration together with OVA enhanced OVA-specific IgE and IgG1 formation. A similar effect was seen with co-administration of the core carbon particles from DEP and OVA (Heo et al. 2001). The adjuvant effect of DEP and carbon black (a model of the core DEP particle) on the OVA specific IgE production was confirmed with co-administration of OVA into the hind footpad in mice. Additionally, the draining lymph node showed increased weight, cell number and cell proliferation *in vitro* (Løvik et al. 1997).

Overall, DEP has shown an adjuvant effect in human and in animal studies. The adjuvant effect was robust over different routes of administration. Furthermore, a considerable amount of knowledge about the immunological mechanisms supports that DEP has adjuvant effects (Pandya et al. 2002).

Reactive Airways Dysfunction Syndrome

This rare form of asthma has been described after overexposure to diesel locomotive emissions (Wade and Newman 1993), where exposures occurred immediately behind the lead engines of a train. It has probably little relevance for non-occupational exposures.

Epidemiology

Air concentrations of motor vehicle emissions, CO, black carbon (BC), ultrafine particles (diameter < 0.1 μm) and particle mass, were studied as a function of the distance from a busy freeway. The major vehicle exhaust particles were in the size range of 20-130 nm for diesel engines and 20-60 nm for gasoline engines. On a flat area downwind the road, ultrafine particulate number concentrations and CO and BC mass concentrations decreased exponentially, reaching background levels about 150-300 m downwind. The total particulate mass (~50 $\mu g/m^3$) was approximately constant up to 300 m downwind, indicating that the ultrafine particles contributed little to the total particle mass along busy roads (Zhu et al. 2002). Health effects also depended on the distance from a busy road. For children living 0-30, 31-60, 61-90, 91-120 and 121-150 m from a main road in Nottingham, the odds ratio for wheeze was approximately 1.8 (statistically significant), 1.2 (not significant), 1.1 (not significant), 0.9 (not significant) and 1.0, respectively. Associations of similar magnitudes were seen for diagnosed asthma. Thus, road traffic emissions have health implications for the population who are living within about 90 m of a main road, corresponding to about 12% of the Nottingham area (Venn et al. 2001). In the Netherlands, girls between 7-12 years old living within 100 m from a freeway had higher prevalence of chronic cough and wheeze compared to girls living within 1000 m from a freeway. The prevalence of asthma attacks

was not associated with proximity to a freeway although asthma attacks were associated with the density of truck traffic. No association between proximity to a freeway or density of truck traffic and chronic cough, wheeze, asthma attacks or rhinitis was apparent in the boys (van Vliet et al. 1997).

Living in an unpolluted forest with exposures to Japanese cedar pollen caused pollinosis (main symptoms: sneezing, rhinorrhea, nasal obstruction, and itching of the eyes) in 5.1% of the individuals. However, exposures to air pollution and road traffic increased the occurrence of pollinosis (Ishizaki et al. 1987), suggesting an adjuvant effect of road traffic pollutants (table 6). Similarly, a Swedish study (Montnémery et al. 2001) found an adjuvant effect of road traffic (table 6). However, no association between road traffic density and allergy, asthma or use of respiratory medication (table 6) was found in another study in adults (Oosterlee et al. 1996). Similarly, in a cross-sectional study in 18-60 years old individuals (N=820) in Switzerland (Wyler at al. 2000), neither car nor truck traffic at the home address was significantly associated with sensitisation to common aeroallergens. A subgroup analysis showed that individuals who had been living at their domicile for 10 years or more, sensitisation to pollen increased significantly with traffic density from about 12% in the group with the lowest traffic density to 28% in the group with the highest car traffic density (RR~2). There was no association between traffic density and hay fever, pollen-related rhinitis or asthma symptoms.

Several epidemiological studies have been performed to elucidate effects of traffic exposures in children, including those mentioned in table 7. In Munich, a cross-sectional study in 4678 school children, aged 9-11 years, showed that an increase in car density traffic was associated with a decrease in lung function and an increase in prevalence of wheezing and dyspnoea. Neither the response to a cold air challenge nor the prevalence of asthma or allergic rhinitis was associated with traffic density (Wjst et al. 1993). In a cross-sectional study in Taiwan, the asthma prevalence in about 14 years old school children (N~300 000) was associated with traffic-related air pollution where CO and NO_x served as indicators (Guo et al. 1999). In a cross-sectional study in 9 years old children (N=317) living near major roads in Germany, outdoor and personal NO_2 exposure levels were measured and correlated with occurrence of rhinitis, wheezing, asthma and atopy, i.e. sensitisation to pollens, house dust mite, cat, milk or egg. No significant correlation was found with personal NO_2 exposure levels. In the children from the urban areas, increasing outdoor NO_2 levels (indicator of traffic pollution) were associated with increasing hay fever, wheezing and atopy (Krämer et al. 2000). Effects of traffic pollution was studied in children between 7-12 years old (N~2000) attending 24 schools located within 400 m from busy motorways in The Netherlands. Traffic pollution indicators were $PM_{2.5}$, NO_2, and reflectance of $PM_{2.5}$ (mainly diesel “soot”). Significant associations were found between current conjunctivitis and truck traffic, $PM_{2.5}$, soot and NO_2 exposures, between hay fever ever and $PM_{2.5}$ exposure, and between elevated total IgE and soot and NO_2. Skin prick test (SPT) reactivity to any indoor or outdoor aeroallergen was associated with NO_2 exposure levels. Reactivity to indoor (cat, dog and house dust mite) allergens were associated with NO_2 exposures and reactivity to outdoor (grasses and trees) allergens with truck traffic and $PM_{2.5}$ exposures. No association was apparent for asthma and traffic or traffic pollutant indicators (Janssen et al. 2003).

Table 6. Effect of road traffic pollution on sensitisation and allergic airway diseases in populations comprising adults

Study design [a]	Response and exposure	N [a]	Endpoint	RR [b]	AR [b]	Reference
C, <10 to > 80 years	Self-reported symptoms from Japanese cedar (JC) pollen (JCP):		Pollinosis:			Ishizaki et al. 1987
	Mountain with no JCP	58		0.33	-	
	JC forest with no pollution	78		1	-	
	City or farming area near JC forest	1199		1.73	15	
	City or farming area with air pollution but few JCP	1193		1.88	18	
	Living within 200 m from a busy road lined with old JC trees.	605		2.59	16	
C, adults	Self-reported symptoms and estimated traffic pollution in 673 living along busy traffic streets and 812 living along quiet streets.	1485	"Allergy – doctor's diagnosis ever"	1.0(OR)	-	Oosterlee et al. 1996
			"Doctors diagnosis of asthma – ever"	1.1(OR)	-	
			Respiratory medication – occasionally"	0.9(OR)	-	
C, 20-59 years	Self-reported symptoms and exposures to heavy traffic	8468	Heavy versus not heavy traffic: Asthma	1.25	8	Montnémery et al. 2001

[a] A cross-sectional study is indicated by C, years are the age of the studied individuals and N is the number of studied individuals or the number obtained from the reference and included in the calculation of the relative risk (RR) and the attributable risk (AR).

[b] The relative risk (RR) and the attributable risk (AR) are calculated according to Walter (1978) and converted to percent. For the data from Ishizaki et al. (1987), the AR (%) was calculated from a•(RR-1)/RR (Stoddard and Miller 1995) where (a) is the number of pollinosis cases in the group and converting to percent of all asthma cases (305). Crude odds ratio (OR) was taken directly from the report of Oosterlee et al. (1996).

Table 7. Effect of road traffic pollution on sensitisation and allergic airway diseases in children and adolescents

Study design [a]	Response and exposure	N [a]	Endpoint	RR [b]	AR [b]	Reference
C, 12-15 years	Self-reported symptoms and truck traffic exposures on residential street	3529 3700	Never versus traffic exposed: Wheezing Rhinitis	1.25 1.64	15 32	Duhme et al. 1996
C, ~8 years	Questionnaire based symptoms and measured outdoor NO_2. Highest exposed (>14 ppb NO_2) versus lowest exposed (≤ 7 ppb)	843	Wheeze last 12 months Asthma last 12 months Ever asthma	1.3 3.8 4.0	- - -	Studnicka et al. 1997
C, 6-14 years	Questionnaire based symptoms and lorry traffic density in the residential street [c]	6997 6658	Never versus lorry traffic: Wheezing with exercise Current asthma	1.31 1.13(NS)	12 -	Ciccone et al. 1998
C, 5-11 years	Questionnaire based symptoms, skin prick test and specific IgE to aeroallergens (atopy), determined air pollution at school and home [d]	5325 5282 3188	Wheezing within the past 12 months Doctor's diagnosis of asthma Atopy	1.24 1.15(NS) 0.98	14 - -	Hirsch et al. 1999
C, 4-11 years and 11-16 years	Questionnaire based symptoms and traffic activity index (TAI) at school [e]	22863 27668	Current wheeze Current wheeze	1.10 0.98	6 -	Venn et al. 2000

[a] A cross-sectional study is indicated by C, years are age of the studied individuals and N is the number of studied individuals or the number obtained from the reference and included in the calculation of the relative risk (RR) and the attributable risk (AR).

[b] The RR and the attributable risk AR was calculated according to Walter (1978) and converted to percent. Not significant is indicated by NS.

[c] Significant results were obtained from a subpopulation of children living in Torino, Milano and Roma, whereas no significant results were obtained from less urbanized areas.

[d] The air concentration of benzene (~90 % is due to road traffic emission (Hirsch et al. 1999)) was used to dichotomise exposed groups into low (≤ 3.5 $\mu g/m^3$) versus higher exposed groups (>3.5 $\mu g/m^3$) and the two groups were used for calculation of RR and AR.

[e] Traffic flow was obtained from roads in the vicinity of the schools and expressed as traffic activity index (TAI, vehicle metres/day/km^2), i.e. the total number of metres travelled by vehicles in one kilometre square area over a period of one day. TAI was dichotomised into low exposure (≤ TAI x 10^6) and higher exposure groups (>TAI x 10^6) for calculation of RR and AR. The results for asthma were approximately similar to the results of wheeze (Venn et al. 2000).

Overall, the traffic pollution might roughly account for about 0 - 20 % of the allergic rhinitis reactions and between 0 - 10 % of the asthma reactions in adults. In children, the corresponding figure for rhinitis was between 0 - 30 %. An asthma promoting effect was absent in about half of the studies dealing with school children. The interpretation of these

figures must clearly be taken cautiously. First, a prerequisite for the conclusions if ascribed to new-onset reactions is that there is a direct causal relationship between traffic exposures and sensitisation or diseases. This might be suggested from the animal studies. However, exacerbation of asthma reactions in already sensitised individuals or individuals with bronchial hyperresponsiveness is another possibility (Janssen et al. 2003). As exacerbation may account for a part of the reported asthma cases, the figures for new-onset asthma may be lower than 10%. Also, individuals living near busy roads may more often close the windows to keep pollutants and noise out of the home, which may change the indoor climate, promoting indoor allergen loads (Duhme et al. 1996) and thereby increase sensitisation in an adjuvant independent manner.

CONCLUSION

Both laboratory animal studies and epidemiological studies provided evidence for adjuvant effects of smoke from tobacco, most evident in relation to occupational exposures, and road traffic pollution. These pollutants promoted sensitisation, development of respiratory symptoms and allergic airway diseases in westernised countries. The epidemiological studies on road traffic effects were less consistent for the allergic effects, but were more consistent for wheeze, which also may be triggered i.a. by viral infections (Taussig 1997). The outdoor pollutants, ozone and nitrogen dioxide, possessed adjuvant effect in animal studies, but little effect was apparent from the epidemiological studies. The routes of exposure were of little importance for the detection of the adjuvant effect in the animal studies. As most of the animal studies were with concentrations considerably higher than the human exposure levels and many of the studies were with routes that were not relevant for human exposures, these studies may be used for hazard identification but not for risk assessment. This is also indicated from the results from the ozone and nitrogen dioxide studies. Adjuvant effects of several indoor pollutants, including house dust and chemicals belonging to surfactants and quaternary ammonium disinfectants, have been demonstrated in animal studies. However, the limited epidemiological studies do not allow a conclusion about their role in promoting sensitisation and development of allergic diseases in humans.

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Chapter VI

Urban Air Pollution, Outdoor Air Allergens and the Increasing Trend of Allergic Respiratory Diseases

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ABSTRACT

Respiratory allergic diseases (rhinitis, rhinosinusitis, bronchial asthma and its equivalents) appear to be increasing in most countries and subjects living in urban and industrialized areas are more likely to experience respiratory allergic symptoms than those living in rural areas. This increase has been linked, among various factors, to air pollution, which is now an important public health hazard. Laboratory studies confirm the epidemiologic evidence that inhalation of some pollutants, either individually or in combination, adversely affect lung function in asthmatics. The most abundant air pollutants in urban areas with high levels of vehicle traffic are respirable particulate matter, nitrogen dioxide and ozone.

In particular ozone, respirable particulate matter and allergens impair lung function and lead to increased airway responsiveness and bronchial obstruction in predisposed subjects. However, besides acting as irritants, airborne pollutants can modulate the allergenicity of antigens carried by airborne particles. By attaching to the surface of pollen grains and of plant-derived paucimicronic particles, pollutants can modify the morphology of these antigen-carrying agents and alter their allergenic potential. In addition, by inducing airway inflammation, which increases airway epithelial permeability, pollutans overcome the mucosal barrier and so facilitate the allergen-induced inflammatory responses. Experimental studies have shown that diesel exhaust particulate (DEP) can modify the immune response in predisposed animals and humans.

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Indeed, DEP increases in vivo IgE and cytokine production at the human respiratory mucosa thereby inducing allergic inflammation of the respiratory airway and the subsequent development of clinical respiratory symptoms . All these results implicate DEP in the enhanced allergic inflammatory response Pollen allergy is a useful model with which to study of the relationship between air pollution and respiratory allergic diseases . It has been suggested that air pollutants promote airway sensitisation by modulating the allergenicity of airborne allergens. Furthermore, airway mucosal damage induced by air pollution may facilitate the access of inhaled allergens to the cells of the immune system. Several factors can influence this interaction: type of air pollutant, plant species, climatic factors, degree of airway sensitisation and hyperresponsiveness of exposed subjects However, the role of climatic factors such as barometric pressure, temperature and humidity in triggering and/or exacerbating respiratory allergic symptoms is still poorly understood.

Key words: Air pollution; Allergy; Allergic asthma; Bronchial asthma; Environmental diseases; Pollen allergy; Respiratory allergy; Urban air pollution.

A dramatic increase in the prevalence of allergic respiratory diseases such as rhinosinusitis and bronchial asthma has been observed in the last two decades in the industrialised countries [1-4]. While the reasons for this increase are still largely unknown, there is some evidence to indicate that these rising trends are associated with increased levels of sensitization to common environmental allergens and early exposure to adjuvant factors such as components of air pollution. In other words, although various factors could play a role in the recent increase in morbidity associated with allergic respiratory diseases, one commonly proposed reason is the costant degradation of air quality with the incresing level of outdoor air pollutants such as those derived from cars and other vehicles.

Several studies have demonstrated that urbanisation with its high levels of vehicle emissions and westernised lifestyle are correlated with the rising trend of pollen-induced respiratory allergy in most industrialised countries [5-9]. However, it is well known that the onset of allergic diseases requires two factors: genetically committed individuals and sensitising agents (allergens such as those derived from pollen grains and adjuvant factors such as components of urban air pollution derived from cars). Of course, the understanding of the interplay between genetic background and environment may lead to preventive and therapeutic interventions for asthma that are able to prevent the progression of the disease, the development of airways inflammation with bronchial hyperresponsiveness to various specific and aspecific stimuli and the development of irreversible changes in airway function.

Several studies suggest that air pollution contributes to facilitate allergic sensitization of the airways in predisposed subjects [10-16] and the increase in allergic respiratory diseases appears to be paralleled by increasing atmospheric concentration of such pollutants as gases and respirable particulate matter.

On the other hand we know that the key feature of bronchial asthma is the development of the airways inflammation and bronchial hyperresponsiveness such as an exaggerated bronchoconstrictor response not only to allergens to which the subjects are sensitized, but also to a range of non-specific stimuly, including agents such as air pollutants, cold air etc..

People who live in urban areas tend to be more affected by pollen-induced respiratory allergy than those of rural areas. In the context of rural communities, subjects exposed to

traffic usually experience a higher frequency of allergic respiratory diseases than those less exposed and urban westernized lifestyle has been found to be associated with a greater risk of allergic sensitization. Urban living means exposure to increased indoor and outdoor pollution, and, because of possible interactions between inhalant allergens such as those carried by pollen grains and other environmental factors, it may enhance the risk of both atopic sensitization and aggravation of symptoms in already sensitized patients.

The most abundant air pollutants in urban areas with high levels of vehicle traffic are respirable particulate matter (PM), nitrogen dioxide and ozone. PM is usually a mixture of organic and inorganic, solid and liquid particles that vary in size, composition and origin. Penetration of PM into the tracheobronchial tract is related to its size and to the efficiency of the airways defence mechanisms. Particles with a diameter less than 10 um (PM10) can penetrate into the lower airways and fine particulate, which includes particles with an aerodynamic diameter equal to or below 2.5 (PM2.5) is thought to constitute a notable health risk since it can be inhaled more deeply into the lungs.

A recent study in London [17] demonstrated that asthma aggravation evaluated by increased emergency department visits is associated with athmospheric concentrations of common outdoor urban pollutants.

It has been also observed that in children with high levels of serum total IgE and with bronchial hyperresponsiveness the prevalence of upper and lower respiratory symptoms enhanced up to 139% for every 100 ug/m3 increase in athmospheric particulate matter [18].

Several studies have reported on the association between asthma or its equivalents and road traffic. Positive associations have been observed between self-reported wheezing in school children and road traffic [19,20]; between urban air pollution and respiratory symptoms [21] and between prevalence of respiratory symptoms in children and adults and proximity to major roads [22-24].

Daily levels of PM10 have been correlated with respiratory symptoms, peak expiratory flow rates, use of antiasthma medications and visits to emergency rooms for respiratory symptoms [25,26]. In the literature some studies also show a significant association between daily mortality from respiratory and cardiovascular diseases and particulate air pollution [27-29].

It is intriguing that whilst the prevalence of allergic rhinitis and allergic asthma is increasing in some European cities, the atmospheric concentration of grass pollen is decreasing [30,31]. The decrease has been attribuited to substantial reductions in grassland over large areas of the European continent. In fact, over the last 20 years, grassland in western Europe has decreased by more than 20%. As a consequence, the increase in cases of allergic rhinitis and asthma induced by grass pollen is probably related, among other factors, to increased air pollution.

However, despite evidence of a correlation between the increasing frequency of respiratory allergy and the increasing trend in air pollution, the link and interaction between the two elements is still within the realm of speculation. One difficulty in interpreting the results of several studies on this topic is the confounding effect of cigarette smoke and exposure to allergens in atopic subjects. Another factor clouding the issue is that laboratory evaluations do not reflect what happens during natural exposition. As a consequence, even if

it is plausible that ambient air pollution plays a role in the onset and in the increasing frequency of respiratory allergy, it is not easy to show that this happens at public health level.

In general, the effects of air pollutants on lung function depend on the environmental concentration of the pollutant, the duration of pollutant exposure and the total ventilation of exposed persons. Aeroallergens, e.g. those derived from pollen grains, lead to bronchial obstruction in predisposed subjects and one of the most frequent models used to study the interrelationship between air pollution and respiratory allergic diseases has been pollen allergy [5-9]. In other words, biological aerosols such as pollen grains or their derived paucimicronic components carrying allergens can act as aerocontaminants in producing these effects [7-9,31].

The most frequent interaction is the synergistic action between airborne biological and chemical (gaseous or particulate) pollutants. The impaired mucociliary clearance induced by chemical pollutants may facilitate the penetration and the access of inhaled allergens to the cells of the immune system [10-11].

INTERACTION BETWEEN AIR POLLUTION AND PLANT-DERIVED ALLERGEN-CARRYING PARTICLES

Vegetation reacts with air pollution over a wide range of pollutant concentrations and environmental conditions. Many factors influence this interaction, including type of air pollutants, plant species, nutrient balance, soil conditions and climatic factors. At low levels of exposure for a given species and pollutant, no significant effect is observed. However, as the exposure level increases, a series of potential injuries may occur (biochemical alterations of the plants etc.).

It has been hypothesized that components of air pollution can influence the plant allergenic content. Pollen grains collected from areas with high levels of air pollution are covered with airborne microparticulate [5] and it has been suggested that the interaction between particulate components of air pollution and pollen allergens alters the antigenicity of pollen allergens.However, pollen allergens should be carried also by paucimicronic particles (diameter between 2 and 5 um) derived from plants [32,33]. By virtue of their small size, paucimicronic particles are able to reach the peripheral airways with inhaled air, so inducing asthma in sensitized patients [34,35]

It has been also observed [36] that exposure to 100 ppb of NO2 adversely affected the pollen germination of various trees (birch, alder and hazel) and so altered their content of proteins, including allergens.

Another possibility is that pollen allergens may be transferred, by physical contact or by elution, to other small particles of air pollution, e.g. those of the diesel exhaust particulate, which can penetrate deep into the airways [37].

CLIMATIC CHANGES AND POLLEN ALLERGY

The role of climate factors such as weather (e.g. pressure, temperature, humidity etc) in the initiation and / or exacerbations of respiratory allergic symptoms in predisposed subjects is still poorly understood. For example, while the relationship between thunderstorm and exacerbations of pollen allergy during the pollen season is now better known, since it seems to be characterized by sudden release of paucimicronic starch granules from grass pollen, which are ruptured by osmotic shock [38], the relationship between respiratory allergy and barometric pressure is not clear, since in some studies there is association with low atmospheric pressure [39] and in some others [40] with high pressure. As a consequence more study is necessary to clarify this aspect. It is also limited our knowledge on the effects of other climate factors which seem to be of major importance to asthma chrisis such as wind speed, passage of cold front, etc. However, as for the cold air it is well known that its inhalation reduces lung function in asthmatics favouring broncho-obstruction.

As for the association between a variety of atmospheric factors and asthma, the question arises as to how increasing levels of greenhouse gases and concomitant climate changes influence the frequency and severity of pollen-induced respiratory allergy.

There is a variety of direct and indirect evidence to suggest that climate with its changes may affect pollen release and consequentely pollen-related asthma. In fact climate variations are likely to influence vegetation with consequent changes in the growth, reproductive cycle etc, and also in the production of allergenic pollen (seasonal period and intensity) with greater proliferation of weed species. However, there is a variation from a region to another, since some areas receive increases in ultraviolet radiotion and/or rainfall frequency and some others receive a reduced quantity. Moreover, ultraviolet radiations in urban polluted atmosphere favour the formation of ozone which is most impacted by elevated daytime temperatures, low wind speeds and clear skies (combinations characteristics of some regions such as Mediterranean area, California, Central and South American regions etc. all with high levels of car traffic). Increased levels in tropospheric ozone are related to increased risk of asthma exacerbation in susceptible asthmatic patients. As for other pollutants most commonly correlated with asthma exacerbation (SO2, NO2, and PM) various weather situations may induce varying effects. In particular inversions of temperature are usually associated with highest levels of PM, SO2, and NOx.

Other than a direct role of weather (winds, rains, changes of temperature etc) as asthma trigger, it can have an indirect influence on allergic respiratory responses acting on plants producing allergenic pollen.

The major air pollutants which are toxic for plants, especially in long term exposure, are ozone, SO2, NO2 and particulate matter (PM).

As for the influence of weather and air pollution on vegetation this reacts over a wide range of pollutant concentrations and environmental conditions. Many factors influence the outcome including plant species, age, soil conditions, nutrient balance, temperature, humidity and sunlight. At low levels of exposure for a given plant species and given pollutants and/or quality of allergenic pollen produced. However, some susceptible plant species should be unable to survive and prosper in a such modified ecosystem.

AIR-POLLUTION-INDUCED INCREASE IN IGE-MEDIATED RESPONSE IN PREDISPOSED SUBJECTS

Since a large part of airborne particulate matter of the urban air originates from diesel-powered engines, this aspect is now an area of research which warrants greater attention in view of the fact that the proportion of new cars with diesel engines is increasing in all industrialized countries.

Moreover, in the context of airborne particulate emissions, interest has focussed on diesel exhaust particulate (DEP) and on the components such as particle-polyaromatic hydrocarbons (PAHs), because experimental studies showed that DEP-PAHs can modify the immune response in predisposed animals and humans modulating also the inflammatory airway process. In other words DEP seems to exert an adjuvant immunological effect on IgE synthesis in atopic subjects, thereby influencing sensitization to airborne allergens [41].

In vivo studies challenging mice and humans by using nasal provocation tests with DEPs have showed an adjuvant immunologic activity on specific and total IgE production [42,43]. It has been also observed that combined diesel exhaust particulate and ragweed allergen challenge markedly enhances human in vivo nasal ragweed specific IgE and skews cytokine production to a T helper cell 2-type pattern [44].

AIR POLLUTION-INDUCED INCREASE IN AIRWAY REACTIVITY IN ALLERGIC SUBJECTS

Several trials have evaluated the ability of air pollution exposure to reduce the threshold dose of aeroallergens able to induce airway responsiveness to the specific bronchial challenge in sensitized subjects**.**

Devalia et al [9] investigated the effect of previous exposure to nitrogen dioxide or ozone on subsequent allergen-induced changes in the nasal mucosa of subjects affected by seasonal allergic rhinitis or perennial allergic asthma. They found that exposure to this pollutants significanly increase the allergen-induced release of eosinophil cationic protein in nasal lavage. These results suggest that exposure to NO2 and ozone may prime the eosinophils to subsequent activation by inhaled antigen in atopic subjects. In other words, atopic state can be upregulated by environmental influence, and some subjects develop atopic disease by response to these environmental factors when they are inhaled in combination with allergens.

Molfino et al [45] showed that the mean provocative dose of ragweed required to induce a 15% decrease in FEV1 was significantly reduced to about half the dose of allergen required when these patients allergic to ragweed were preexposed to ozone versus preexopuse to air. These results were confirmed by Jorres et al [46], who showed that prior exposure to a higher concentration of ozone is able to induce an increase of airway responsiveness to inhaled allergen in exercising atopic asthmatics.In other words these studies have demonstrated that exposure to ozone may enhance the airway responsiveness of already sensitized subjects by lowering the threshold concentration of allergen able to induce the appearance of bronchial obstruction. However, it has been also observed [13] that ozone exposure has both a priming

effect on allergen-induced responses as well as an intrinsic inflammatory action in the nasal airways of perennial allergic asthmatics.

Recently has been observed that the incidence of new diagnoses of asthma is associated with heavy exercise in communities with high concentration of ozone, thus, air pollution and outdoor exercise could contribute to the development of asthma in children [47].

In the context of the interrelationship between air pollution and aeroallergens which are both able (in isolated or combined way) to enhance airway hyperresponsiveness in exposed subjects the city of Naples is a good natural model, since it is characterized by very high levels of urban traffic and year-long sunny days. As a consequence, in Naples there are prolonged high atmospheric levels of ozone and PM. Moreover, throughout the city grows in abundance *Parietaria* (Pellitory-of-the-wall), the well-known plant, which has very strong allergenic properties and which is responsible for IgE-mediated sensitization, with clinical symptoms, frequently also severe,of rhinosinusitis and bronchial asthma or its equivalents, in about 30% of Neapolitans [9,30,48,49]. However, although millions of people suffer from pollinosis due to *Parietaria* allergens, only relatively few reports on this allergy have been published, compared to articles on other seasonal inhalant allergens such as those delivered, for example, by grasses, ragweed, birch. The main *Parietaria* cycle of flowering in the Naples area begins in March and continues until July. Peak pollination occurs in May and June. In September and October, a lower peak is detectable. In other words in Naples *Parietaria's* pollen season is very long, since it can persist from February to December, resulting in almost perennial symptoms. However, in the Naples atmosphere there is a parallel, progressive increase of *Parietaria* pollens, ozone and PM from February to July, after which the production and release of *Parietaria* pollen usually decreases while ozone and PM remain high into the autumn.

We have observed that cases of *Parietaria*-induced respiratory disorders with a peak in the number of emergency room visits for allergic asthma attacks, tend to increase from April to the end of June when there is a contemporaneous increase in the Naples atmosphere of Parietaria pollens, ozone and PM.

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