Tone Sjåheim

Immunopathology of occupational asthma in aluminium potroom workers

Faculty of Medicine
University of Oslo
2008

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Workers in primary aluminium production have an increased risk of developing work-related asthmatic symptoms, referred to as "potroom asthma". The pathogenetic mechanisms are unknown. This thesis aimed to identify the immunopathological characteristics and possible mechanisms of occupational asthma among aluminium potroom workers. Bronchial biopsies were examined with multi-colour immunohistofluorescent staining for identifying different mucosal leukocyte subsets. In addition, exhaled nitric oxide (NO) concentrations, reticular basement membrane (RBM) thickness and blood eosinophils were measured. The studies revealed that potroom asthma is associated with inflammatory changes in peripheral blood and bronchial mucosa, and that the changes in non-smokers are similar to those previously reported in non-occupational and other occupational asthma. Smoking appeared to inhibit the asthma associated mucosal leukocyte accumulation in the asthmatic workers, suggesting an immunomodulating effect of smoking on the asthmatic reaction. The studies revealed further several novel phenotypic characteristics of bronchial T-cells in asthma, in particular regulatory T-cells and T-cell proliferation.

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Immunopathology of occupational asthma in aluminium potroom workers

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1 Preface

1.1 Acknowledgement

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1.2 Summary

Background: Workers in primary aluminium production have an increased risk of developing work-related asthmatic symptoms, referred to as "potroom asthma". The causal agent(s) has not been identified. The pathogenetic mechanisms are unknown and it has been debated whether potroom asthma is associated with chronic inflammatory changes in the airways.

Aim: To identify the immunopathological characteristics and possible mechanisms of occupational asthma among aluminium potroom workers.

Results: A cross-sectional study of potroom workers performed at the Årdal aluminium smelter demonstrated a positive association between potroom asthma and the number of blood eosinophils, being more pronounced in non-smokers than in smokers (paper I).

Paper II, III and IV are based on investigations of bronchial biopsies from workers with and without potroom asthma (non-smokers and smokers), and non-smoking non-exposed controls, using multiple immunohistofluorescent staining for identifying different mucosal leukocyte subsets. In addition, exhaled nitric oxide (NO) concentrations and reticular basement membrane (RBM) thickness were measured (paper II). All parameters investigated, except RBM thickness, were influenced by smoking in the asthmatics.

Non-smokers with potroom asthma had increased RBM thickness, exhaled NO concentrations and increased subepithelial density of eosinophils, macrophages, CD4+ T-cells, effector T-cells (CD25+Foxp3^{neg}), regulatory T-cells (CD25+Foxp3+), proliferating T-cells and HLA-DR+ T-cells compared to controls. The asthma-associated accumulation of subepithelial bronchial leucocytes was more or less inhibited in smokers with potroom asthma. Apart from increased RBM thickness and slightly increased density of eosinophils and percentage of effector T-cells, the smoking asthmatics did not differ from controls. Signs of some bronchial inflammatory reactions were also noted in asymptomatic healthy workers.

Conclusions: This was the first time that pathological changes in the airways of workers with potroom asthma were examined. The studies revealed that potroom asthma is associated with inflammatory changes in peripheral blood and bronchial mucosa, and that the changes in non-smokers are similar to those previously reported in non-occupational and other occupational asthma. Smoking appeared to inhibit the asthma associated mucosal leukocyte accumulation in the asthmatic workers, suggesting an immunomodulating effect of smoking on the asthmatic reaction. The studies revealed further several novel phenotypic characteristics of bronchial T-cells in asthma, in particular regulatory T-cells and T-cell proliferation.

1.3 Selected abbreviations

APC	antigen presenting cell
BAL	broncho alveolar lavage
BHR	bronchial hyperresponsiveness
COPD	chronic obstructive pulmonary disease
CD	Core differentiation
DIC	differential interference contrast
FEV_1	forced expiratory volume in one second
FVC	forced vital capacity
Ig	immunoglobulin
IL	interleukin
IFN	interferon
GM-CSF	granulocyte-macrophage colony stimulating factor
HF	hydrogen fluoride
HLA	human leukocyte antigen
HMW	high molecular weight
LMW	low molecular weight
NO	nitric oxide
OA	occupational asthma
PEF	peak expiratory flow
RADS	reactive airway dysfunction syndrome
SO_2	sulfur dioxide
RBM	reticular basement membrane
TCR	T-cell receptor
TDI	toluene diisocyanate
TGF	tumour growth factor
Th	T helper
TNF	tumour necrosis factor

1.4 List of papers

I. **Sjåheim T,** Kongerud J, Søyseth V.
Blood eosinophils in workers with aluminium potroom asthma are increased to higher levels in non-smokers than in smokers.
Am. J. Occup. Med. 50:443-448, 2007.

II. **Sjåheim T**, Halstensen TS, Lund MB, Bjørtuft Ø, Drabløs PA, Malterud D, Kongerud.
Airway inflammation in aluminium potroom asthma.
Occup Environ Med 2004 61: 779-785.

III. **Sjåheim T,** Kongerud J, Bjørtuft Ø, Drabløs PA, Malterud D, Halstensen TS.
Reduced bronchial $CD4^+$ T-cell density in smokers with occupational asthma.
Eur Respir J 2006; 28: 1138-1144.

IV. **Sjåheim T,** Bjørtuft Ø, Drabløs PA, Kongerud J, Halstensen TS.
Increased bronchial density of CD25+Foxp3+ regulatory T-cells in nonsmokers with occupational asthma.
Submitted.

2 Introduction

2.1 Aluminium production in Norway

Primary aluminium production is an important industry in Norway with a total production capacity in the electrolysis of 1.3-1.4 million tons of aluminium per year. The demand for aluminium products is increasing year by year and aluminium has become the world's second most used metal after steel. Today, there are seven operating Norwegian plants located in Karmøy, Sunndalsøra, Årdal, Høyanger (all Hydro Aluminium), Lista, Mosjøen (Elkem Aluminium) and Husnes (Sør-Norge Aluminium), employing about 1450 potroom workers. Aluminium production is accompanied by emissions of dust and gasses, potentially harmful to the workers and the local environment. Asthma-like symptoms have been the most important health problem for the workforce.

The Norwegian aluminium industry started with a number of small aluminium plants, build in the period 1908-1928. The first plants were typically located close to the hydro-electric power source, often in narrow valleys (Vigeland, Eydehavn, Tyssedal, Stongfjorden, Haugvik, Høyanger). Of these early plants, only Høyanger remains today. Later, power transmission technology made it possible to locate the plants far from the power source. The next period of development came after the Second World War, and six more plants were built between 1948 and 1971. Since then the plants have gradually been expanded and upgraded. Lately an expansion in Sunndal made that plant the biggest aluminium smelter in Europe. At present, Hydro Aluminium expands abroad and the largest aluminium plant ever build in one stage is now under construction in Qatar.

Aluminium (Al) is present in nature in its oxidised form and is most commonly obtained from bauxite which in a first step is refined into alumina (Al_2O_3), using the Bayer process. Secondly, alumina is transformed to aluminium by electrolysis, in the Hall-Hèroult process, used in 1886. It is the latter process which seems to be associated with the development of asthma.
The reduction of alumina takes place in electrolytic cells usually called pots and the large buildings where 100-300 pots are located are known as potrooms (figure 1). Accordingly, the work-related respiratory symptoms in potroom workers are termed potroom asthma (in Norwegian: hallastma). In the pots, alumina is dissolved in an electrolytic bath of molten cryolite, (Na_3AlF_6) at about 950 °C. Molten aluminium is deposited at the bottom of the pots

and is tapped regularly. The pots are of two types, Søderberg and prebake. After its invention in the late 1930s the Søderberg continuous self-baking process was the dominant technology for many years. Today the prebake technology accounts for about 85 % of the aluminium production in the Norwegian smelters.

Figure 1. Potroom with prebake pots. From Sunndal SU4.
Photo by A. Johnsen, Hydro aluminium

The prebake technology permits better collection of the gases and dust from the electrolysis process, with hoods covering the pots. However, some of the hoods have to be removed when aluminium is tapped or the anode has to be changed. In these situations, the collection of pollutants is reduced, and gases and dust are emitted. Peak levels of exposure are consequently more likely to occur in prebake than in Søderberg pots. Independent of pot technology, potroom asthma is nevertheless reported to a similar extent (1).

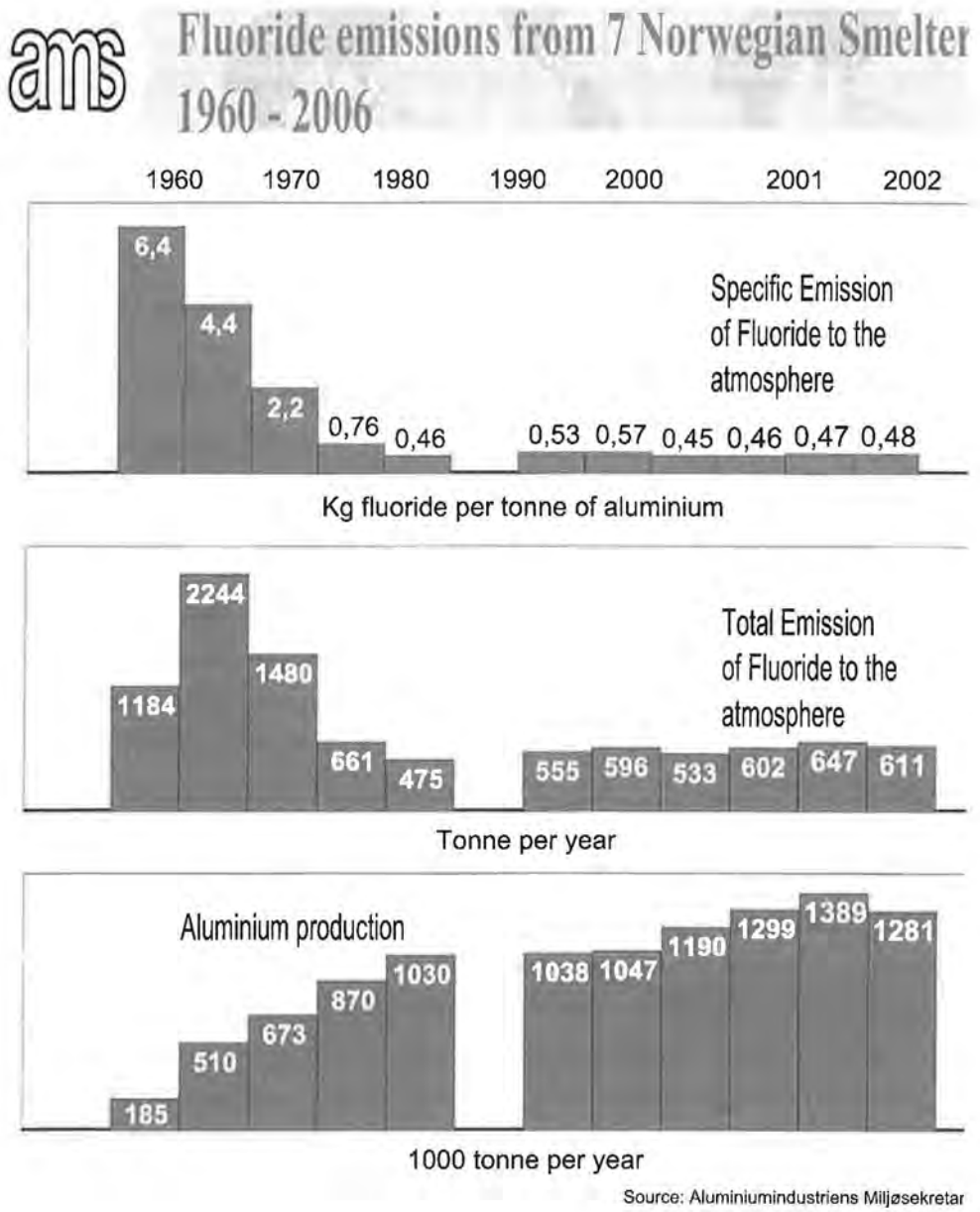
Pot fume emissions

The potroom workers are exposed to a complex pot fume emission of particulates and gases and the causal agent(s) of potroom asthma has not been identified. The respirable particulates are mainly composed of alumina, carbon dust, cryolite and sodium aluminium tetrafluoride ($NaAlF_4$). In addition, minor amounts of metals such as nickel, chromium and vanadium occur. Gases are mainly hydrogen fluoride (HF) and sulfur dioxide (SO_2), which may absorb to the surface of particulates. In a Søderberg plant, also traces of polyaromatic hydrocarbons (PAH) are present. The exposure to particulate fluorides and SO_2 is significantly higher in prebake than in Søderberg pots, while HF is reported to be highest in Søderberg pots (2). The many agents and complex compounds present are often closely related in the working environment. Some studies suggest fluorides as the causal agent (3-5). Whether fluorides, present in both particulate and gaseous form, are the causative agent, co-agent or simply a marker for the causative agent(s) of potroom asthma remains to be determined. A dose-response relationship has been described for fluoride exposure and airway hyperresponsiveness in potroom workers (6).

As a part of the national authority's mandatory surveillance program of the working atmosphere, measurements of fluorides and particulates are regularly carried out in the Norwegian aluminium industry. Potroom workers are randomly selected to wear personal samplers for 8 hour shifts at different intervals (10-20 measurements a year) and eight hour time weighted mean estimates of exposure of total fluorides and particulates are calculated. The mean levels of exposure are mostly far below the Norwegian threshold limit value (total fluorides 0.60 mg/m^3 and total particulates 5 mg/m^3), but peak exposures frequently occur. In the period 1990-1999 about 40 % of the peak measurements for fluorides and 10 % of the measurements for particulates exceeded the Norwegian threshold limit values (7).

Emission of pollutants from some of the smelters to ambient air has been a major local concern for many years. The air quality criteria recommended by the State Pollution Control Authority of Norway have been exceeded at some of the plants causing damage to the local environment. Fluorine emissions into air formerly caused severe environmental effects on conifers (mainly pine) and certain mammals near some of the smelters (8). In Årdal and Sunndal, emissions in the 1960 and 70s killed or seriously damaged many pine trees and other forest vegetation (figure 2) (8). Serious dental and bone damages occurred in wildlife and domestic animals.

The situation has clearly improved following emission reductions, particularly since the mid-1980s (figure 3). Fluoride content in conifers and grass are now routinely monitored in the vicinity of the smelters. In the period 1973-1993 fluoride emissions have been reduced by almost 70 %, while the aluminium production increased by more than 50 % (se below).



Source: Aluminiumindustriens Miljøsekretar

Figure 2. Anode change at Karmøy plant. Note the open pot with emission in the background. *Photo by P.A. Drabløs*

Figure 3. Søderberg-sooting. Note the amount of dust surrounding the pot. *Photo by P.A. Drabløs*

Figure 4. Damaged pine forest in Årdal. *Photo by T. Schelderup.*

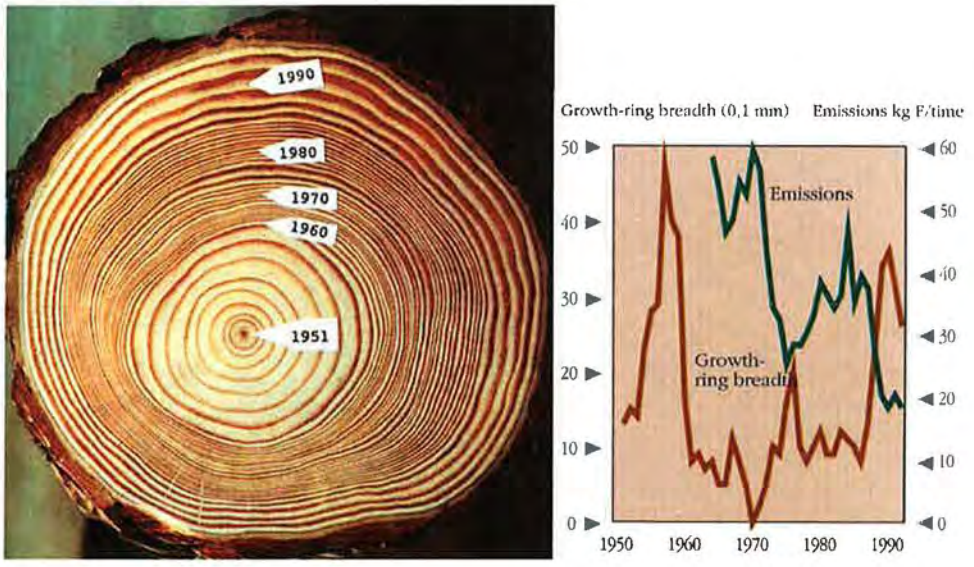


Figure 5. Section through a pine trunk from Hjelle in Årdal. The annual rings are broad and distinct up to 1960. After that they grow very narrow, only to increase again from 1987. The diagram showing annual-ring width and emissions demonstrates a clear relationship between them. Adapted from (8).

2.2 Asthma

Definition of asthma

Asthma is a disorder defined by its clinical, physiological and pathological characteristics. Both genetic and environmental components are involved, but since the pathogenesis of asthma is not clear, much of its definition is descriptive. Based on the functional consequences of airway inflammation, an operational description of asthma has been worked out by the Global Initiative for asthma (GINA).

> *"Asthma is a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. The chronic inflammation is associated with airway hyperresponsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night or in the early morning. These episodes are usually associated with widespread, but variable, airflow obstruction within the lung that is often reversible either spontaneously or with treatment"* (9).

Prevalence and risk factors for asthma

There is good evidence for a real increase in asthma prevalence during the last 30-40 years, at least in Western countries (10). The increase in asthma has in particular been documented in children (11) and has been attributed to aspects of Western lifestyle, including indoor and outdoor pollutants, childhood immunizations and cleaner living conditions. No single cause has, however, been identified. It appears that the global prevalence of asthma ranges from 1 to 18 % (9). In the mid-1990s doctor-diagnosed asthma was reported in 8-9 % of Norwegian school children (12;13). The most recent reported prevalence rates in Norway vary from 5 % in children and adolescents (14) to 20 % in 10-year-old children (15). In adults, the asthma prevalence is about 9 % (10). A review of studies estimating the incidence of adult asthma confirms the increase in asthma and reports the adult incidence of asthma to be approximately 4/1000 per year (16).

Factors influencing the risk of asthma can be divided into those that *cause* the development of asthma (host factors, which are primarily genetic) and those that *trigger* asthma symptoms (indoor/outdoor allergens and pollution, infections, occupational sensitizers, tobacco smoke, and diet). The mechanisms whereby they influence the development and expression of asthma

are, however, complex and interactive. Passive exposure to cigarette smoke both *in utero* and during early life increases the subsequent risk of developing allergy and asthma. Once asthma is established, active cigarette smoking increases symptom severity, hospital admissions, morbidity and mortality (17). Moreover, it is now recognized that patients with asthma who smoke have an impaired response to treatment with corticosteroids (18). Despite this, smoking rates among patients with asthma remain high, and similar to those in the general population (17).

Allergic and non-allergic asthma

The terminology used to characterize allergic and allergy-like reactions appears confusing. To standardize the nomenclature of allergy, the European Academy of Allergology and Clinical Immunology (EAACI) published their official EAACI Position Statement (19) which has gained substantial international recognition. A later update (20) propose the following definitions:

> *"The term hypersensitivity should be used to describe objectively reproducible symptoms or signs initiated by exposure to a defined stimulus at a dose tolerated by normal persons".*
> *"Allergy is a hypersensitivity reaction initiated by specific immunologic mechanisms".*
>
> *"Atopy is a personal and/or familial tendency, usually in childhood or adolescence, to become sensitized and produce IgE antibodies in response to ordinary exposure to allergens, usually proteins. As a consequence, these persons can develop typical symptoms of asthma, rhinoconjunctivitis, or eczema".*

As allergy is defined as a hypersensitivity reaction initiated by immunologic mechanisms, it must be separated from non-allergic hypersensitivity in which an immunologic mechanisms is excluded. When other mechanisms can be proven, as in hypersensitivity to aspirin, the term *non-allergic hypersensitivity* should be used.

Allergy can be antibody-mediated or cell-mediated. In most patients, the antibody typically responsible for an allergic reaction belongs to the immunoglobulin (Ig) E isotype, and these patients may be said to suffer from *IgE-mediated allergy*. In *non-IgE-mediated allergy*, the antibody may belong to the IgG isotype, as in anaphylaxis due to immune complexes

containing dextran and serum sickness. Non-IgE-mediated allergy can also be cell-mediated, as in allergic contact dermatitis and celiac disease, in which allergen-specific lymphocytes play a major role.

The terms atopic and non-atopic asthma have been widely used, and atopic asthma is still often used synonymously with IgE-mediated asthma. According to the revised nomenclature for allergy (19;20), atopy is a clinical definition of an IgE-antibody high-responder and should not be used to describe diseases. Asthma resulting from immunological reactions should be termed *allergic asthma* (figure 6). When there are indications of IgE-mediated mechanisms, the term should be *IgE-mediated asthma.* Other non-immunologic types of asthma should be called *non-allergic* asthma. The old terms, "extrinsic or exogenous" (allergic asthma) and "intrinsic or endogenous" (non-allergic asthma) should no longer be used to distinguish between the allergic and non-allergic subgroups of asthma. About 80 % of childhood asthma and > 50% of adult asthma has been reported to be allergic (19).

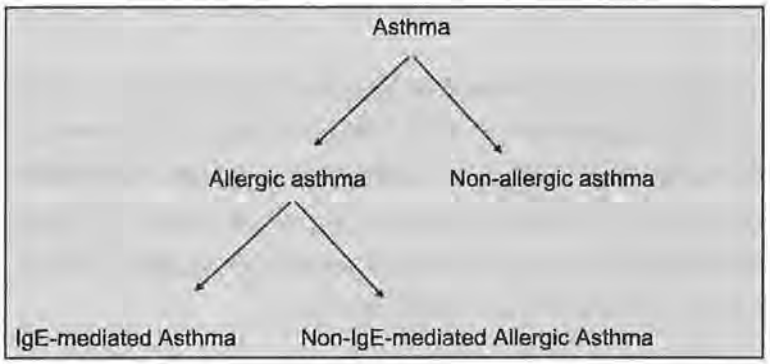


Figure 6. Allergic and non-allergic asthma. Adapted from (19).

2.3 Occupational asthma

Asthma in the workplace can be classified as asthma caused by the working environment (occupational asthma; OA) or asthma exacerbated by the working environment. Substantial epidemiologic and clinical evidence indicate that agents inhaled at work can induce asthma (21) or trigger asthma (22). The symptoms and functional alterations in OA are similar to those found in non-occupational asthma.

Definition of occupational asthma

At present there is no internationally agreed definition of OA. Several definitions have been proposed, and evidence of a direct causal relationship between workplace exposure and the development of asthma remains the common key element in the diagnosis of OA (23-29). Early definitions stipulated that there should also be a sensitizing mechanism (24;26;27). However, evidence of sensitization is only found in a minority of cases and occupational exposure can cause asthma without immune sensitization. A recent editorial consensus definition was reached in the textbook "Asthma in the Workplace";

> *"Occupational asthma is a disease characterized by variable airflow limitation and/or hyperresponsiveness and/or inflammation due to causes and conditions attributable to a particular occupational environment and not to stimuli encountered outside the workplace" (30).*

More than 250 natural and synthetic substances have been associated with OA (31). These agents are categorized into high-molecular-weight (HMW) and low-molecular-weight (LMW) agents, according to whether their molecular weight is above or below 1kD (1000 Dalton). HMW agents are proteins of animal and plant origin acting through an IgE-mediated mechanism. LMW agents include a wide variety of organic and inorganic compounds that, with few exceptions, are not associated with an IgE mechanism.

According to the textbook definition, two types of OA are defined based on their appearance after a latency period. OA occurring after a latency period after an exposure long enough to acquire immunological mediated sensitization to the causal agent, is classified as allergic. Allergic OA includes OA that is induced by an IgE mechanism (most HMW and some LMW agents) and OA in which an IgE mechanism has not consistently been demonstrated (LMW agents such as diisocyanates, western red cedar and acrylates). OA without a latency period is classified as non-allergic and include irritant-induced asthma or reactive airways dysfunction syndrome (RADS), which may occur after single or multiple exposures to nonspecific irritants (dust, vapor, fume, smoke) at high concentrations (32;33). The underlying mechanism of irritant-induced asthma is unknown (34).

Recently published evidence-based guidelines for the identification of OA also propose two types of OA but avoid implying a specific immunological mechanism (35). The authors define OA as being either hypersensitivity induced OA (characterized by a latency period and non-irritant mechanism) or irritant-induced OA (due to an irritant mechanism and not requiring a latent interval).

The possibility of extending the spectrum of irritant-induced asthma further to include the onset of asthma following repeated exposure to low concentrations of irritants ("low dose irritant-induced asthma") has been suggested (36-38). Repeated peak exposure to SO_2 and other irritant gases increased the incidence of asthma during work in sulphite pulp mills (39).

Increases in asthma symptoms, or reactivation of quiescent asthma in individuals with pre-existing asthma due to workplace exposure are normally excluded by definitions of OA. A variety of terms are used to define this concept, such as "work-aggravated asthma" and "work-exacerbated asthma" (30;40). Work-exacerbated asthma is more common compared to OA, but the prevalence is unknown.

Almost 90 % of OA are of the allergic/hypersensitivity type (35) and has a latency period of months to years after the onset of exposure, although OA is most likely to develop in the first few years of exposure. For HMW agents, atopy has consistently, but weakly, been associated with OA development (41). Smoking has been associated with the development of OA in workers exposed to platinum salts and anhydride compounds (42). In general, non-atopic individuals and non-smokers are reported to be at greater risk of developing OA than atopic individuals and smokers (43).

The reported risk of asthma attributed to the workplace is highly variable, and the prevalence is reported between 9 % to 15 % (21;44;45). Asthma is the most prevalent occupational lung disease in developed countries. In Norway, the fraction of new cases of asthma attributable to occupational airborne exposure (dust or fumes) was 14 %, measured in a general population (46). Asthma acquired in the workplace is a diagnosis that is commonly missed and the frequency systematically underestimated (47). Because of its insidious onset, OA is often misdiagnosed as chronic bronchitis or chronic obstructive pulmonary disease (COPD), especially in smokers and the elderly. Occupations associated with a high risk for asthma, include farming and agricultural work, painting, cleaning work and plastic manufacturing (43).

Common identified agents of OA with latency are diisocyanates, flours, allergens from laboratory animals and insects, enzymes, solders, wood dust, natural rubber latex and acrylates.

2.4 Occupational asthma in aluminium smelters (potroom asthma)

The work-related respiratory symptoms occurring in potroom workers, referred to as potroom asthma, has been a major health issue in aluminum plants since it was first described in 1936 (48). Despite a gradual decrease in the pot fume emissions and improved technology of airway protection, potroom asthma is still the most important health problem for the workers.
According to many authors, potroom fume emissions have not been included among causal agents of OA because different mechanisms could be involved, including an immunologically mediated reaction against trace amounts of metals, or an irritant effect resulting from exposure to HF and SO_2 (21).

Definition of potroom asthma
There is no consensus report concerning the definition of potroom asthma. The following definition proposed by Abramson and co-workers is frequently used in clinical studies;

> *"no previous history of asthma; an initial period of symptom free exposure; symptoms such as dyspnea, chest tightness, cough, and wheeze; a temporal relationship to work; improvement off work; documentation of either reversible airways obstruction or bronchial hyperreactivity"* (3).

The occurrence of potroom asthma
The existence of potroom asthma has been demonstrated in cross-sectional (3;49;50) and longitudinal studies (1;3), while other studies have been unable to confirm the existence of occupational asthma in this industry (51-53).

The reported incidence of potroom asthma varies from 0.06 to 4 % of exposed workers per year (3). This observed difference could be related to the study design, different criteria used for definition of asthma, employment selection criteria, healthy worker effects, and over-or underreporting of asthma symptoms in certain industrial populations. Studies from Norway, The Netherlands, Australia and New Zealand have estimated the annual incidence of potroom asthma to be approximately 2 %, with prevalence as high as 10 % in long term workers. In

North America and Canada there has until now been disagreement regarding the existence of potroom asthma. In a recently published study, however, the annual incidence of asthma observed in American potroom workers in the period 1996-2002 was 1.17 % (50). In Norway, the number of cases yearly reported to the authorities increased from 37 cases per year in 1990 to 153 cases per year in 1995, and then gradually decreased under the 1990 level.

Clinical characteristics of potroom asthma

The clinical symptoms of potroom asthma do not differ from those described in traditional, non-occupational asthma or OA acquired in other occupations. The characteristic symptoms of potroom asthma are recurrent episodes of wheezing, breathlessness, chest tightness and coughing. The symptoms may occur immediately after exposure, but generally the onset of symptoms is delayed (54;55). Usually the symptoms occur several hours after exposure or during sleep, and improve during periods off work and worsen on return to work. Only one study has documented a dual asthmatic reaction (55). An important characteristic is a latency period between the first exposure to the offending agent and the onset of asthmatic symptoms. Most cases of potroom asthma develop within the first 1-2 years of employment, but the duration of exposure before the first attack ranges from a few weeks to several years (1;56).

Risk factors for potroom asthma

Bronchial hyperresponsiveness (BHR) is verified by exaggerated airway narrowing in response to a wide variety of stimuli measured by histamine and methacholine inhalation challenge. Although BHR is a common finding in potroom asthma and has been found to be a risk factor (57), a negative BHR test does not exclude the diagnosis, as potroom asthma also occurs in employees with normal bronchial responsiveness. The usefulness of the metacholine test as a tool for detection of work-related asthmatic symptoms appears to be of limited value due to its low sensitivity (58). In the Netherlands, subjects with BHR have since 1982 been screened out by introduction of histamine provocation test (59). Accordingly, the incidence of potroom asthma decreased considerably (from more than 5 % to less than 2 %), but the occurrence of new cases was not completely prevented.

A positive association between hay fever and potroom asthma has been demonstrated (60), and an atopic history as well as increased eosinophil counts in peripheral blood have been reported to be a risk factor for developing potroom asthma (61). However, most studies have failed to

demonstrate an association between atopy and potroom asthma (3;62), and total IgE-levels are reported within the normal range in most cases (63).

The role of smoking in potroom asthma is inconsistently reported. A longitudinal study from Norwegian aluminium smelters revealed that smoking was a strong risk factor for developing potroom asthma (1). This finding has not been confirmed in later studies (60;61). In general, smokers with potroom asthma report more coughing, but less asthma exacerbations and less use of asthma medication (64).

Prognosis of potroom asthma

The prognosis of potroom asthma is variable and depends on the duration of symptoms before withdrawal from exposure. Early diagnosis and removal from exposure result in improvement of symptoms and decrease in bronchial hyperreactivity (65). Workers who continue to be exposed for prolonged periods more often remain symptomatic after removal (66).

2.5 Airway inflammation in asthma

Although the clinical spectrum of asthma is highly variable and different cellular patterns have been observed, the presence of airway inflammation remains a consistent feature of asthma. Even though the symptoms are episodic, the airway inflammation in asthma is persistent. The inflammation affects both large and small airways, but its physiological effects are most pronounced in medium-sized airways during airway narrowing. The pattern of airway inflammation appears to be similar in children and adults and in all clinical forms of asthma, whether allergic, non-allergic or occupational.

Regardless of the heterogeneity of the inflammatory response in asthma, some characteristic pathophysiological changes occur in the asthmatic airway of non-smokers. The airway wall is infiltrated with leucocytes, where the most important cells are supposed to be the CD4+ T-lymphocytes producing proinflammatory T helper (Th) 2 cytokines. Frequently, an increased density of eosinophils is observed, but this finding seems not mandatory and there is an increasing recognition of non-eosinophilic forms of asthma (67;68). Further, macrophages, neutrophils, plasma cells and mast cells are variably increased in the airway of asthmatics. More than 100 different mediators are now recognized to be involved in asthma and mediate the complex inflammatory response in the airways (69). Key mediators of asthma include

cytokines (interleukin (IL)-4, IL-5, IL-13, IL-1β, tumour necrosis factor (TNF)-α and granulocyte-macrophage colony stimulating factor (GM-CSF), NO, histamine, chemokines, cysteinyl leukotrienes and prostaglandin D_2.

Airway remodeling

In response to chronic inflammation and tissue injury, abnormal tissue repair and restructuring may result in thickening of the airway wall which becomes unable to maintain normal function. Such structural changes in the asthmatic airways are referred to as airway wall remodeling and include hypertrophy and hyperplasia of airway smooth muscle, proliferation of airway wall blood vessels, increased numbers of goblet cells in the airway epithelium, increased size of submucosal glands and subepithelial fibrosis by deposition of collagen fibers and proteoglycans under the basement membrane (70).

Airway narrowing is the final common pathway leading to the symptoms and physiological changes in asthma. The predominant mechanism of airway narrowing in asthma is airway smooth muscle contraction in response to multiple bronchoconstrictor mediators and neurotransmitters. In addition, airway edema due to increased microvascular leakage, airway wall thickening due to structural changes and mucus hypersecretion contribute.

T lymphocytes

T lymphocytes (T-cells) are mandatory for acquired cell-mediated immunity and play a central role in the pathogenesis of asthma. After their maturation in thymus, T-cells enter the circulation expressing T-cell receptor (TCR) and either the co-receptors CD4 or CD8. TCR is a two-chain transmembrane molecule and about 95 % of T-cells express the αβ chains. T-cells recognize membrane bound peptide antigens by their TCR. The peptide antigens have to be internalized and processed into a membrane glycoprotein called the major histocompatibility (MHC) molecules on antigen presenting cells (in humans called human leukocyte antigen (HLA) molecules). HLA class I molecules are expressed on all nucleated cells while HLA class II molecules are expressed constitutively only on antigen presenting cells (APC) such as dendritic cells and B cells.

Exogenous protein antigens are taken into APCs where they interact with HLA class II molecules and are transported to the cell surface where they interacts with the TCR expressed by a CD4+ T-cell. *Endogenous* protein antigens, generally derived from infectious pathogens,

are processed to proteins in the cytosol where they interact with HLA class I molecules and are transported to the cell surface where they interact with the TCR expressed by a CD8+ T-cell. Accordingly, CD4+ T-cells are referred to as HLA class II restricted while CD8+ T-cells are referred to as HLA class I restricted.

The differentiation of effector T-cells is initiated by signals from the TCR, co-stimulatory molecules and cytokine receptors. T-cell activation involves a cascade of events that induce gene transcription coding for cytokines and cytokine receptors and results in proliferation and differentiation of the antigen specific T-cell clone. CD25 (IL-2 receptor α-chain) appears on the cell surface 6-8 hours after activation and IL-2 is produced by activated CD4+ T-cells after 8-10 hours. Naïve T-cells differentiate into effector cells that appear within 3-4 days after antigen exposure. T-cells demonstrate their effector function for days to weeks and once the antigen has been destroyed, activated T-cells are eliminated by activation-induced cell death. A minor population of long-lived antigen-specific cells survives, and constitutes the memory population for that antigen.

CD4+ T-cells

CD4+ T-cells play an important role in the initiation of immune responses by providing help to other cells and are referred to as T helper cells (Th). Upon antigen stimulation, naïve CD4+ T-cells activate, expand and differentiate into different effector subsets termed Th1, Th2 and Th3 and are characterized by the production of distinct cytokines and effector functions. During the past five years, Th17 cells have been recognized as an independent T-cell subset that similar to Th1 and Th2 cells, require specific cytokines and transcription factors for their differentiation (71).

Th1 and Th2 differentiation is initiated by activation of T-cells by specific cytokines. Interferon (IFN)-γ and IL-12 signals are important for Th1 cell differentiation while IL-4 induces the development of Th2 cells. Th1 cells promote immune protection against bacterial and viral infections, and viruses and bacteria favour the production of Th1 cells. Th2 cells protect the body from helminthes infections and allergens and parasites favour Th2 cell induction. Th1 cells produce large quantities of IFN-γ. These cells elicit delayed type hypersensitivity responses, activate macrophages and are effective in clearing intracellular pathogens. Th2 cells secrete IL-4, IL-5, IL-13 and IL-25 and are especially important for IgE

production, eosinohilic inflammation and for the elimination of extracellular pathogens. Dysregulated expansion of CD4+ Th effector T-cells causes immunopathology. Excessive Th1 responses are associated with various autoimmune and inflammatory disorders whereas enhanced Th2 cytokine production is involved in allergies and asthma.

The function of Th17 cells is incompletely elucidated, but Th17 cells appear to be highly proinflammatory. Th17 cells produce mainly IL-17 and IL-22 and are critical to enhance host protection against certain extracellular pathogens, which are not efficiently cleared by Th1 and Th2 responses. In addition, Th17 cells have emerged as potent inducers of autoimmune diseases. Tumour growth factor (TGF)-β and IL-6 are crucial factors for Th17 differentiation. Developing Th subsets cross-regulate expansion and function of each other. While IL-4 antagonizes differentiation of Th1 cells and IFN-γ inhibits the development of Th2 cells, both cytokines antagonize the development of Th17 cells (72). IL-2, which is a growth factor for most T-cells and T-cell subsets, appears nevertheless, to have inhibitory effect on the expansion of Th17 cells.

CD8+ T-cells

The principal function of CD8+ T-cells is to kill cells that have been infected by bacteria and viruses and are frequently referred to as T killer cells or cytotoxic T lymphocytes (Tc). CD8+ T-cells are also involved in killing tumour cells and transplanted foreign cells during graft rejection. CD8+ T-cells produce fewer and lower amount of cytokines than CD4+ T-cells. The majority produce Th1-associated cytokines and are named Tc1 cells while a smaller population CD8+ T-cells produce Th2-associated cytokines and are called Tc2. About 2/3 of T-cells in peripheral blood are CD4+ T-cells while about 1/3 are CD8+ T-cells. In the bronchial mucosa in healthy subjects, the CD4 and CD8 subsets are expressed to approximately the same extent (CD4/CD8 ratio ~ 1). In asthma, the mucosal CD4+ T-cell population is increased while in smokers and subject with COPD the CD8+ subpopulation is increased. Viral infections which typically activate CD4 and CD8 IFN-γ producing cells, commonly exacerbate asthma.

γδ T-cells

Some T-cells express the γδ chains of TCR and not the αβ chains. This alternative TCR is known as γδ and the small fraction of T-cells expressing this receptor (~ 5 % in peripheral blood) is referred to as γδ T-cells. Generally, γδ T-cells lack the CD4 co-receptor molecule but

some γδ T-cells co-express the CD8αα homodimer. The function of γδ T-cells is still unknown. They predominantly localise to mucosal surfaces and are probably involved in maintaining mucosal homoeostasis.

Regulatory T-cells

In the 1990s a population of CD4+ T-cells with the ability to downregulate T-cell functions was identified in mice and are referred to as regulatory T-cells (Tregs). These cells express CD25, but as CD25 is expressed on activated effector CD4+ T-cells as well, the characterisation of Tregs has been difficult and probably several subpopulations exist. The Treg cell nomenclature is confusing, but there is general agreement upon the existence of two main subgroups, called naturally occurring Treg and inducible Tregs. Tregs inhibit the proliferation and activation of neighbouring CD4+ and CD8+ T-cells by cell-cell contact, but also through production of TGF-β and IL-10. In addition, Treg may inhibit the activation of dendritic cells. Treg express the nuclear forkhead/winged-helix transcription factor Foxp3. Until recently, Foxp3 was considered to be a specific marker for regulatory T-cells, but are now shown to be expressed upon activation of non-regulatory T- cells as well (73).

TGF-β is a critical differentiation factor for the generation of Treg. It is proposed that there is a functional antagonism between Th17 and Foxp3+ Treg and a reciprocal relationship in the generation of these cells. IL-6 appears to play a critical role in dictating whether the immune response is dominated by pathogenic Th17 cells or protective Treg (74). Foxp3+ Treg comprise about 10-15 % of CD4+ T-cells in secondary lymphoid organs. Treg are considered anergic in vitro, but a rather high proliferative rate has been observed in vivo for murine (75;76) and human peripheral blood Treg (77). There are no available data on quantities or function of pulmonary Tregs in healthy or asthmatic human lungs and this topic is currently under intense investigation.

Cigarette smoking and asthma

Tobacco contains more than 4500 compounds in the particulate and vapor phases, comprising known human carcinogens and many toxic agents. Cigarette smoking has long been known to suppress immune responses in the lungs and render smokers susceptible to respiratory tract infections and cancer (78). Many components of cigarette smoke, including nicotine (79;80),

acrolein (81) and tar components (hydroquinone and catechol) (82) cause profound suppression of T-cell responses.

Smokers have traditionally been excluded from studies of airway inflammation in asthma and the current knowledge of the asthmatic inflammation stems from non-smokers. In general, smoking seems to induce a bronchial inflammatory reaction in healthy subjects even if they remain asymptomatic (83;84), and BHR is reported to be increased in smokers (85). In non-asthmatic monozygotic twins, smoking appears to be associated with increased levels of IL-13, a cytokine that seems to affect allergic inflammation at multiple levels (86).

In mice, smoke exposure inhibited an asthma-induced airway influx of macrophages, CD4+ T-cells and eosinophils (87) as well as dendritic cells (88). Activation and expansion of pulmonary CD4+ T-cells were prevented in a murine adenovirus infection model (88). In humans, asthmatics who smoke have reduced sputum eosinophil counts compared to asthmatic non-smokers (89). Other observations in smoking asthmatics have revealed higher sputum neutrophil counts that drop after smoking cessation (90), increased sputum concentrations of IL-8 (89) and decreased IL-18 (91). It has been reported that smoking increases blood eosinophils in non-asthmatics while the converse has been observed in asthmatics, suggesting that smoking plays a different immunological role in asthmatics and non-asthmatics (92).

It has recently been shown that current smokers with asthma have reduced numbers of mature dendritic cells in the bronchial mucosa (93). Dendritic cells influence the Th1-Th2 balance in the lungs as well as B cell growth and immunoglobulin switching towards IgE and accordingly alter the balance of inflammatory cell phenotypes. Cigarette smoke has been shown to suppress dendritic cell-mediated priming of T-cells and specifically inhibit key Th1-cytokine production, favoring the development of Th2-responses (94).

Airway inflammation in occupational asthma

The pathological alterations in OA with latency are similar to those found in non-occupational asthma where chronic inflammation is the hallmark (21;95), suggesting similar terminal pathogenetic events for asthma independent of its causal agent. The pathophysiology of allergic asthma usually involves an IgE-dependant mechanism. OA induced by IgE-dependent agents is similar to IgE-mediated asthma that is unrelated to work (21). Most HMW agents and some LMW agents induce asthma by producing specific IgE antibodies. The airway

inflammation process is similar in IgE-dependant and in IgE-independent asthma (96-98) and is characterized by the presence of eosinophils, lymphocytes, mast cells, and thickening of the RBM (95;97;99).

Analysis of bronchial biopsies obtained from patients with OA induced by toluene diisocyanate (TDI) has shown an increased number of inflammatory cells and increased RBM thickness (97) and both eosinophils and lymphocytes showed evidence of activation (96). Similar results have been obtained in bronchial biopsies from subjects sensitized to western red cedar, showing increased number of T-cells, especially CD4+ T-cells, which exhibit signs of activation (99). In one study it was suggested that CD8+ cells are key cells in OA with an IgE-independent mechanism (100) but this hypothesis could not be proved in an animal model (101). Histamine and leukotriene E4 are increased in bronchoalveolar lavage (BAL) fluid sampled during early asthmatic reactions by plicatic acid (102). By use of induced sputum, several studies have confirmed increased eosinophil numbers in asthma induced by both HMW and LMW occupational agents (95;103-106). Further, both eosinophilic and non-eosinophilic variants of OA have been identified (107), similar to non-occupational asthma.

OA exhibiting neutrophilic inflammation has been described after exposure to TDI (108) and grain dust (109), but the results are conflicting and the role of neutrophils in OA remains unclear (107;110-113).

There is very little information available concerning histopathological alterations caused by RADS, but bronchial biopsies have shown extensive damage to the airway epithelium, increased basement membrane thickness and submucosal infiltration by predominantly mononuclear cells (114;115). No bronchial biopsy study is available from patients who develop asthma after chronic low-level exposure to irritants.

Airway inflammation in potroom asthma

Few studies have been performed to reveal whether potroom asthma is an inflammatory disorder. The bronchial mucosa from workers with potroom asthma has previously not been investigated. Peripheral blood eosinophil counts increased during asthmatic attacks in potroom workers (116), and case reports of potroom asthma with blood eosinophilia have been described (57). Pre-employment blood eosinophil counts have been shown significantly higher in cases of potroom asthma than in controls, even though the mean number of the eosinophils

in cases where within the normal range (61). Increased concentrations of exhaled NO has been reported in asthmatic potroom workers (117).

3 Objectives

The main purpose of these studies was to identify the immunopathological characteristics and possible mechanisms of occupational asthma in aluminium potroom workers.

More specific the purpose was to;

- develop new and more appropriate immunohistochemical methods to study inflammation in bronchial biopsies from subjects with occupational asthma
- examine whether inflammatory changes as influx of leukocytes to the bronchial mucosa and increase in reticular basement membrane thickness are present in asthmatic workers
- examine whether the levels of blood eosinophils and exhaled nitric oxide are associated with potroom asthma
- examine the influence of cigarette smoking on airway inflammation in asthmatic workers. This last objective was added when the initially results turned out to be different in non-smokers and smokers with asthma.

4 Materials and methods

The thesis is based on two separate study populations. Paper I is based on a cross sectional study performed at the Årdal aluminium smelter in Western Norway, while paper II, III and IV are based on a biopsy study including workers from five different Norwegian aluminium smelters.

4.1 Paper I

A cross-sectional study was carried out at the Årdal aluminium smelter in 1989 by one of the authours (VS). From a total workforce of 380 potroom workers, 338 subjects (89 %) participated. All subjects completed a self-administered, validated questionnaire. Information on respiratory symptoms (dyspnea, wheeze and cough) within the last year, allergy, presence

of familial asthma prior to potroom employment, smoking habits, years of employment in potrooms, and use of respiratory protection were recorded. The characteristics of the participants are shown in table 1 in the paper.

Potroom asthma was defined as a combination of dyspnea and wheezing apart from colds during the last 12 months with improvement on rest days or during vacations, and absence of pre-employment asthma. Any other combination of respiratory symptoms was classified as "Other Symptoms". None of the asthmatics were treated with inhaled corticosteroids and 14 out of 41 subjects (34 %) used inhaled β_2-agonist. Only one of the smoking asthmatics had forced expiratory volume in one second (FEV_1) < 70 % of predicted value (FEV_1 69 %). Accordingly, it was not a major concern that subjects with COPD could have been misclassified as asthmatics. Smoking habits were classified as follows: never smokers were lifetime non-smokers; ex-smokers were those who had stopped smoking at least one year prior to the study, and all other subjects were classified as current smokers. The prevalence of current smoking was 63 %, while 9 % were ex-smokers and 28 % never smokers. The regional Ethical Committee approved the study and informed consent was obtained from all participants.

Blood eosinophil counts, skin prick test and lung function

Capillary blood was collected from all subjects and eosinophils were counted blindly by the last author (VS) using a Fuchs-Rosenthal chamber (118). Eosinophilia was defined as blood eosinophils $\geq 400 \times 10^6$cells/L. Atopy was identified by skin prick test and was performed in 282 subjects (83 %). Registration of the wheal was carried out 15 minutes after application of dog epithelium, common silver birch, timothy grass, mugwort, and house dust mite, with saline and histamine as controls (skin prick test Phazet®, Pharmacia, Uppsala, Sweden). The weal diameter was measured in millimetres as the mean of the long axis and its perpendicular axis, and those who had a positive reaction to at least one of the allergens tested were considered to have atopy. A wheal diameter $\geq$ 1 mm was defined as a positive reaction and $\leq$ 1 mm as a negative reaction. All subjects with a positive skin prick test were regarded as atopic.

Spirometry was performed with a dry bellow spirometer (Jones Pulmonaire, Jones Medical Instruments Co., Oak Brook, Illinois) in accordance with the guidelines recommended by American Thoracic Society (119). Recorded variables were forced vital capacity (FVC) and FEV_1. The prediction equations estimated from a general population in Norway were used to

define levels as percentage of predicted (120). Bronchial obstruction was defined as an FEV_1 < 80 % of the predicted value and FEV_1/FVC < 70 %.

Work exposure

Measurements of total airborne dust and total fluorides (fluorides in gas and particulate forms) were regularly performed using personal samplers. Sampling was carried out over 4-8 hours. The geometric mean of exposure was 1.49 mg/m^3 (SD 0.61) for total dust and 0.39 mg/m^3 (SD 0.12) for total fluorides. The Norwegian threshold limit values are 5.00 mg/m^3 and 0.60 mg/m^3, respectively. Both Søderberg and prebake technologies were used in the plant.

4.2 Paper II, III and IV

A total of 45 subjects were included in the study; 20 potroom workers with occupational asthma (eight non-smokers and 12 smokers), 15 healthy potroom workers (eight non-smokers and seven smokers), and 10 healthy non-exposed controls (all non-smokers). The subjects were included during the period 1998-2002 and investigated at the outpatient clinic at the Department of Respiratory Medicine at Rikshospitalet University Hospital in Oslo. The characteristics of the study population are shown in paper II.

Asthmatic workers

The workers were recruited in cooperation with the Health Departments of the operating Norwegian plants, and subjects from five different aluminium plants were included. Since 1985 all potroom workers in the Norwegian aluminium industry have participated in an annual mandatory medical survey in which respiratory symptoms and spirometric measurements have been recorded. The occupational physicians working at the smelters have enlisted all the workers with a suspected or confirmed diagnosis of occupational asthma. These workers were examined at the plant by the main investigator (TS), and if a diagnosis of occupational potroom asthma was confirmed, the subjects were considered for inclusion.

The diagnosis of potroom asthma was made by confirming the diagnosis of bronchial asthma according to international guidelines (121), and by establishing a causal relation between asthma and the working environment, meeting the criteria for potroom asthma as previously

defined (3). Serial monitoring of peak expiratory flow (PEF) was performed at work and away from work.

The inclusion criteria for potroom asthma in this study were;

- No previous history of asthma.
- An initial exposure period without respiratory symptoms (latency).
- Symptoms such as dyspnea, chest tightness, cough or wheeze, usually occurring several hours after exposure or during sleep, and improvement of the symptoms after absence from work for several days or longer.
- Documentation of a reversible airway obstruction defined as >15 % increase in FEV_1 after inhalation of β_2-agonist or diurnal PEF variability >15 % within the last three months.
- FEV_1 > 70 % of predicted value after β_2-agonist inhalation.

If work aggravation of underlying asthma was suspected, the subject was not included. Workers with the following conditions were not considered for inclusion: oral steroid treatment during the last three months, other lung diseases than asthma, FEV_1< 50 % of predicted value, malign disorder, cardiac disease or pulmonary hypertension.

Most of the workers developing aluminium potroom asthma leave the smelters and for practical reasons former workers were not regarded suitable for participating in the study if they were employed in other occupations or had left the vicinity. The workers spent three days away from work for participating in the study. Thus, the actual asthmatic workers were selected among those still working in the potrooms or those workers relocated to clean working environments within the plant. Accordingly, the number of asthmatic workers potentially available for the study was quite small. From a total asthmatic population of 39 workers, 18 subjects were excluded for the following reasons; significant reversible airway obstruction could no longer be demonstrated, FEV_1 after β_2-agonist inhalation was too low, an anamnesis of atopy, childhood asthma or suspect exercise induced asthma, repetitive respiratory infections and cardiac disease (angina pectoris or hypertension). Of the 21 asthmatic workers who fulfilled the inclusion criteria, only one subject declined the invitation to participate.

Ideally, we wanted to recruit equal numbers of asthmatic smokers and non-smokers. Traditionally, the frequency of smoking is high in this industry, and when the study was carried out about 60 % of the workforce were current smokers. Even among the asthmatics, the majority of the workers were smokers, and accordingly it was not possible to recruit a higher number of non-smoking asthmatic workers. Ex-smokers (who had ceased smoking >1 year previously) and never-smokers were all classified as non-smokers. Two of the asthmatics and three of the healthy workers were ex-smokers. Median smoking load for the asthmatics was 13 pack-years (range 8-31) and 17 pack-years (13-32) for the healthy workers.

About half of the asthmatic workers were still working in the potrooms, despite ongoing asthmatic symptoms and three out of eight of the non-smokers and seven out of 12 of the smokers had been relocated to non-polluted working environments at the smelters. The relocated workers had persistent asthmatic symptoms, although all subjects reported gradual improvement of symptoms after withdrawal from exposure.

Healthy workers (exposed controls)

The healthy workers were all recruited from Karmøy aluminium smelter in cooperation with the physician (PAD) and the main nurse (KW) the plant. The call for healthy volunteers was advertised among the 440 pot operators employed at the smelter. The response was overwhelming and the exposed controls were included successively as they responded and were comparable to the asthmatic workers with respect to age, smoking habits and duration of exposure.

Non-exposed controls

The non-exposed controls were recruited from a healthy never-smoking population, responding to a request for volunteers at the University of Oslo. They were all medical students and had never been exposed to potentially harmful occupational agents. They received NOK 1000 to cover travel expenses and loss of working hours.

The healthy workers and non-exposed controls had no symptoms from the upper or lower airways and their lung function was normal. They were all apparently healthy, defined as being non-asthmatic subject without any known disease. None of the participants had a history of allergy, familiar asthma, or childhood asthma. They claimed to be free from respiratory tract

infections for at least six weeks prior to the investigations. Total IgE levels were within normal range for all participants (both asthmatics and controls).

Methods

All subjects underwent an interview by the main investigator and a medical and occupational history was recorded. A clinical examination, spirometry, chest x-ray, electrocardiogram and measurement of exhaled NO were performed. Blood samples were analysed for total IgE, haemoglobin, CRP, leucocytes, trombocytes, total protein, creatinin, urea, Na, K and INR. Finally, bronchoscopy with bronchial biopsy sampling was carried out. The workers completed a self-administered questionnaire. Duration of exposure was recorded, while other individual exposure data were not available.

Measurement of lung function

Spirometry was performed with a pneumotachograph (Vitalograph, Birmingham, UK) in accordance with the guidelines recommended by European Respiratory Society (122). Recorded variables were FVC, FEV_1, FEV_1/FVC% and PEF. The lung function variables were expressed in absolute values and as percentage of predicted, using the reference values of European Coal and Steel Community (122). If obstruction was demonstrated, a bronchodilator test with β_2 -agonist was performed. The post-bronchodilator values were used in the study.

Measurements of nitric oxide

NO was measured by a chemiluminescence analyser (LR 2000, Logan Research, UK) adapted for on line recording of NO concentrations. The sampling rate of the analyser was set to 250ml/min for all measurements. Exhaled NO measurements were performed in accordance with recommendations outlined in the European Respiratory Task Force Report (123). The measurements were made by slow exhalation (20-30s) from total lung capacity through a Teflon mouthpiece, against a mild resistance (target mouth pressure 4-5 cm H_2O) to avoid nasal contamination. End expiratory NO values were measured at the plateau level of the last part of the exhalation curve. Three technically acceptable measurements were obtained and the mean of the two closest measurements was reported. The analyser was calibrated daily.

Bronchoscopy

Bronchoscopy with biopsy sampling was performed following the guidelines from the European Society of Pneumonology (124). A Pentax FB-19H or Olympus 20D IT bronchoscope with working channel 2.8 mm was used. All subjects were premedicated with atropine 0.6 mg subcutaneously and 5 mg diazepam orally. Alfentanil was given intravenously as needed for mild sedation and analgesic (0-2 mg). Lidocaine was used for local anaesthesia. Endobronchial biopsies were taken from the proximal airways under direct vision by video scope. A maximum of six bronchial biopsies was obtained from the second and third generation carinae of the right lung by a single use forceps (Microvasive 1267, Radial Jaw, Boston Scientific). During the procedure, oxygen saturation and heart rate were monitored and 2 litres per minutes of oxygen was continuously given by nasal administration. After the investigation, the subjects were observed for 4 hours at the hospital and they spent the following night at the hospital hotel. The procedure was well tolerated in all subjects. Several pilot biopsies were performed in selected patients at the hospital prior to the study in order to test different biopsy forceps, and the biopsy quality was overall good. Although large forceps were used, no bleeding events occurred. As other researchers have reported (125), we experienced that biopsies were most easily taken from the asthmatics and it was more challenging to obtain good quality biopsies from the healthy subjects.

Bronchial biopsy processing

The biopsy specimens were immediately orientated on a thin slice of carrot and embedded in OCT (Tissue-Tek Optimal Cutting Temperature Compound, Miles Laboratories, IN) in a tube of aluminium foil, immediately frozen (within 1 minute) in isophentane precooled in liquid nitrogen, and stored at – 70 °C until sectioning. Cryo sections were cut serially at 4 μm, dried overnight in room temperature, wrapped in aluminium foil and stored without fixation at -20 °C until use. This procedure provided well preserved tissue morphology, intact immunoepitopes and the possibility of performing immunohistofluorescent staining for up to three overlapping cell markers.

Immunohistofluorescent staining

To identify the different leucocyte subsets in the bronchial mucosa (with the exception of eosinophils) multicolour immunohistofluorescent staining was performed. Compared with the traditional immunoenzyme staining methods, immunofluorescence is more reliable and with a higher detection sensitivity when analysing two antigens in the same cell simultaneously (126).

Antibodies or other proteins can be conjugated to fluorescent compounds called fluorochromes. The most commonly used fluorochromes for immunohistochemical conjugates emit either green (fluorescein isothiocyanate, FITC, ALEXA-488), red (tetramethylrhodamine isothiocyanate, TRITC, Texas red, derivate of rhodamine B sulphonyl chloride or indocarbocyanine, Cy3, ALEXA-594) or blue (aminomethylcoumarin acetic acid, AMCA, ALEXA-350) colour. These three colours can be observed individually in a fluorescence microscope equipped with the proper filters and two or three different antibodies can be visualised simultaneously by the different fluorescent colours. The fluorochrome can be conjugated directly to the primary antibody (direct technique) or the secondary antibody (indirect technique). Direct techniques often reveal weak reactivity as it gives no amplification of the signal. In the present studies, indirect immunofluorescence technique was applied by the use of unlabelled primary antibodies followed by fluorochrome labelled polyclonal reagents to obtain immunological amplification of the fluorescent signal.

Before staining, all sections were prefixed with acetone for 10 minutes followed by periodate lysine paraformaldehyde (PLP) fixation for 10 minutes. PLP induces variable antigenic masking and the lowest working concentration was preferred. Different concentrations of paraformaldehyd (1 %-4 %) were applied depending on the epitope investigated.

Primary mAb against the following epitopes were used in the present studies; CD45, CD3, CD4, CD8, TCRδ1, CD25, Foxp3, Ki-67, CD68, HLA-DR, mast-cell tryptase and neutrophil elastase. The sections were stained in various combinations for two- and three colour immunohistofluorescent examination as described in paper II-IV. DNA staining was applied to visualize the nucleus when appropriate. In general, primary unlabelled antibodies from different species (commonly mouse and rabbit) were followed by fluorochrome-conjugated goat IgG subclass specific (IgG_1, IgG_{2a}, and IgG_3) reagents from the same species. The basic principles for multiple immunohistofluorescent staining have previously been described (127). Methodological controls included sections stained without primary antibody and/or incubated with non-immune mouse Ig in similar concentrations as the monoclonal antibodies.

Antibody reagents were appropriately diluted in phosphate buffered saline (PBS), pH 7.5, containing 125g/l bovine serum albumin (BSA). To reduce any unwanted unspecific bindings, the sections were incubated with goat serum (5 %) for 15 minutes. The primary antibodies were incubated for 20 hours in 4 °C. and the secondary layer was applied for 1 hour in room

temperature. Between steps, the slides were rinsed for 3 minutes in PBS. Finally, the sections were mounted in buffered polyvinyl alcohol (PVA).

Eosinophils

Eosinophils were identified on hematoxilin and eosin stained slides examined by light microscopy. Combined with differential interference contrast (DIC) microscopy, the eosinophils could easily be identified with their typically granular appearance (figure 6). This method for identification of eosinophils in cryo sections has been shown to be more reliable than immunohistochemical labelling of eosinophil granule proteins (128). In cases of doubt, we additionally used eosin fluorescence to localise the cell, as eosinophils are known for their tendency to absorb immunofluorescent reagents and/or to be autofluorescent.

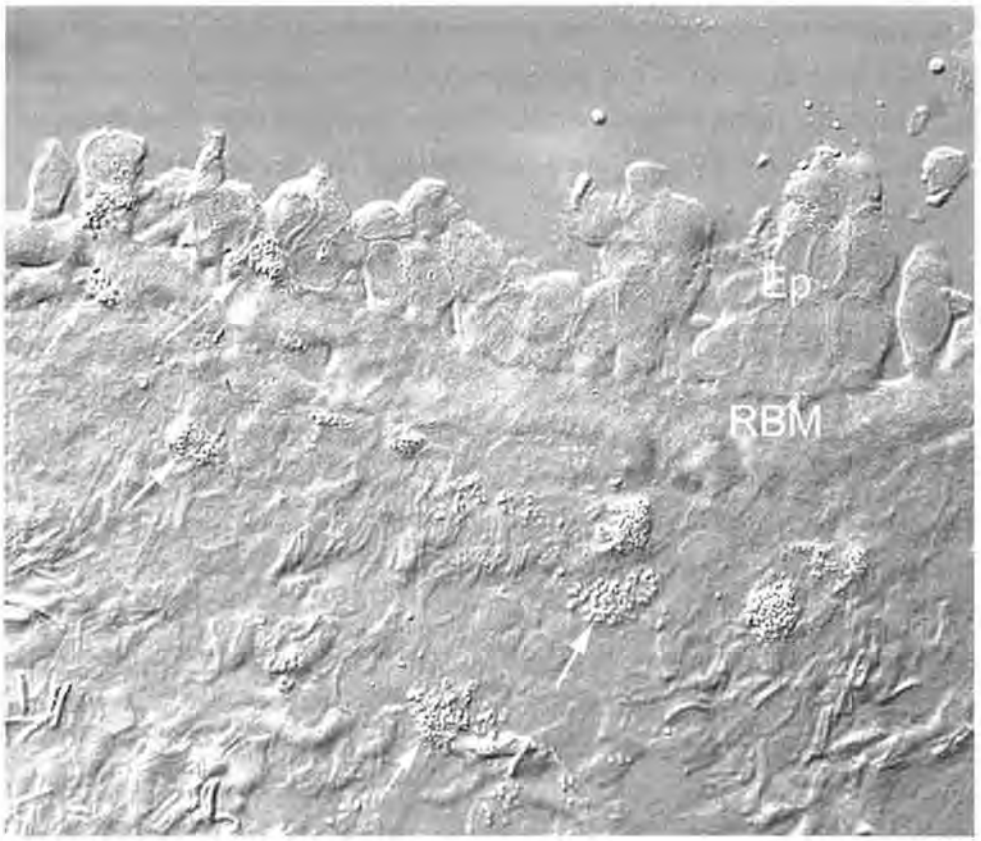


Figure 7. Eosinophil cells identified by DIC microscopy by their typically granular appearance (arrows) in the damaged epithelial layer (Ep) and beneath the reticular basement membrane (RBM).

Microscopy and digital image analysis

The immunostained slides were examined in a Zeiss Axioplane2 microscope with appropriate fluorochrome filters including single-, double-, and triple-colour filter blocks that allow single and simultaneous examinations of green, red, and blue emissions. The microscopes were also equipped with optics for DIC microscopy that enables simultaneous observation of fluorescent emission and tissue morphology. Single-color images were captured with a MicroMax CCD digital camera system and the imagine software package MetaMorph 3.0 or AnalySIS Soft Imagine System.

Quantification of leukocytes

Cryo-sections from two to four different located lower lobe biopsies were examined in each subject until sufficient tissue to obtain representative cell counts were obtained (129). Larger than recommended submucosal areas were always analyzed and varied from median 0.9 mm^2 (paper II), 1.2 mm^2 (paper III) to 1.80 mm^2 (paper IV). All slides were coded and analyzed at 630x magnification. Double and triple labeled cells were identified in single colour filters, any additional markers were then examined and scored in single filter, and its location verified in double-, and/or triple-filters.

Positively stained cells were counted in a subepithelial zone 114 μm beneath the reticular basement membrane and in intact epithelium (defined as the presence of both basal and columnar cells). Excluding submucosal glands and vessels, all available area of a biopsy was analyzed. Subepithelial cell density (cells per square millimeter) of the various cell types within each subject was estimated by adding up the actual number of evaluated cells in each section divided by the total area examined. The number of intraepithelial cells in each section were added up and expressed as number of cells per millimeter of intact epithelium.

Measurements of reticular basement membrane

RBM thickness was measured by use of DIC microscopy combined with light microscope, as illustrated in paper II. A digital captured high power image was used to measure the distance from the basis of the bronchial epithelium to the outer limit of the RBM by AnalySIS Soft Imagine System. Only perpendicular cut sections were examined and median 68 measurements (range 37-161) were performed on each section at approximately 20μm intervals as recommended by Sullivan et al. (129).

4.3 Ethics

The study was approved by the Regional Ethics Committee and informed written consent was obtained from all subjects. The subjects were informed about inconvenience and potential complications connected with the investigations. The Norwegian Data Inspectorate approved the study.

4.4 Statistical analysis

Paper I

The association between each outcome variable and its covariate was analysed using univariate analysis. A multivariate model was then constructed using ordinary least square regression regarding eosinophil count and logistic regression regarding potroom asthma as outcome variable, respectively. Covariates that had $p<0.2$ in the univariate analysis were included in the initial multivariate model. The model was then reduced by backward elimination. Covariates were removed from the model if the association with the outcome variable had $p>0.05$ and the removal did not result in a meaningful change in the association between the outcome and the determinant of interest (confounding).

Paper II, II and IV

Differences between two groups were compared by the Mann-Whitney U test, as the cell counts in the bronchial mucosa did not show normal distribution (129). Correlation coefficients were calculated using Spearman's rank method.

The potroom workers were older than the non-exposed controls and accordingly, all data were controlled for age as a potential confounder by analysis of variance. Multiple linear regression analysis was performed in asthmatics and healthy subjects separately by using exposure, lung function, age and smoking as explanatory variables.

Chi-squared tests were used to calculate differences in the proportion of cells that were so rarely expressed that the overall amount of cells was considered too low to justify inter-individual group evaluation. Two levels of differences were applied. Although p-value ≤ 0.05 was considered to be significant in paper II and IV, the number of variables in paper III made it

advisable to use the $p \leq 0.01$ levels to ensure significance, although $p \leq 0.05$ was additionally used to define differences.

5 Summary of papers

5.1 Paper I.

Sjåheim T, Kongerud J, Søyseth V.

Blood eosinophils in workers with aluminium potroom asthma are increased to higher levels in non-smokers than in smokers.

Am. J. Occup. Med. 50:443-448, 2007.

Aim: To investigate the association between blood eosinophils and potroom asthma and the effect of cigarette smoking.

Results: Potroom asthma was reported by 11.5 % of the workforce, and equally in non-atopic and atopic subjects. Blood eosinophilia was present in 21 % of the workers with asthma and the highest mean number of blood eosinophils was observed in non-smoking asthmatics. Multiple regression analysis showed that the mean eopsinophil count in non-smokers was 177 $x10^6$ cells/L higher among workers with asthma than asymptomatic workers ($p<0.001$), whereas the corresponding difference among smokers was only $63x10^6$ cells/L ($p=0.03$). Multiple logistic regression analysis showed that for workers with blood eosinophils $\geq 400x10^6$ cells/L (eosinophilia) the odds ratio for potroom asthma was 4.2 compared to workers with blood eosinophils $< 200x10^6$ cells/L.

Conclusion: The existence of potroom asthma was positively associated with the number of blood eosinophils. The probability of having potroom asthma was thus increased with increasing blood eosinophils. The association between potroom astma and blood eosinophils was weaker in smokers than in non-smokers, suggesting that smoking reduced the asthma associated increase in peripheral blood eosinophils. The existence of potroom asthma without blood eosinophilia demonstrates that potroom asthma is a heterogeneous disorder with both eosinophilic and non-eosinophilic phenotypes, and supports the view that eosinophil-driven mechanisms are not the only underlying mechanisms in potroom asthma.

5.2 Paper II

Sjåheim T, Halstensen TS, Lund MB, Bjørtuft Ø, Drabløs PA, Malterud D, Kongerud J
Airway inflammation in aluminium potroom asthma.
Occup Environ Med 2004 61: 779-785.

Aim: To investigate whether potroom asthma is associated with inflammatory changes in the airways, and the impact of smoking.
Results: The RBM thickness was the only outcome not influenced by smoking; RBM was significantly thickened in asthmatic workers as well as in healthy workers compared to the non-exposed controls. Asthmatic non-smokers had significantly higher concentrations of exhaled NO and increased density of subepithelial total leucocytes (CD45+ cells) and eosinophils compared to the non-smoking control groups. In contrast, a rather low density of subepithelial leukocytes was observed in asthmatic smokers and eosinophils were the only leukocyte subset increased in asthmatic smokers (when compared to non-exposed control). Approximately half of the asthmatic workers had increased subepithelial eosinophil density and the remaining half had similar density as non-exposed controls. In healthy workers, both RBM thickness and subepithelial eosinophil density were significantly increased compared to non-exposed controls. Asthmatic workers still working in the potrooms did not differ in any of the outcome variables compared to the relocated workers.
Conclusion: Inflammatory changes were present in the airways of workers with potroom asthma. Pot fume emissions may induce a chronic inflammatory reaction in the bronchial mucosa that persists even after relocating from exposure. Non-smokers with potroom asthma had pathological changes in the airway mucosa similar to those reported in occupational and non-occupational asthma. In smokers, the expected asthma associated accumulation of subepithelial leukocytes was not present, suggesting that smoking affects the underlying mechanisms involved in asthma. Healthy workers showed signs of inflammation in the bronchial mucosa although they were asymptomatic, indicating that a subclinical airway inflammation is induced in these workers.

5.3 Paper III

Sjåheim T, Kongerud J, Bjørtuft Ø, Drabløs PA, Malterud D, Halstensen TS.
Reduced bronchial $CD4^+$ T-cell density in smokers with occupational asthma
Eur Respir J 2006; 28: 1138-1144

Aim: To investigate the bronchial mucosal T-cell subsets (CD3+, CD4+, CD8+, γδ-T-cells) and macrophages in non-smokers and smokers with potroom asthma.

Results: Non-smoking asthmatics had significantly increased subepithelial density of CD4+ T-cells compared to all groups ($p \leq 0.01$) while there was no difference in the densities of CD8+ T-cells. A parallel increase in CD4+ T-cells was not observed in smoking asthmatics, which actually had the lowest CD4+ T-cell density of all groups. The CD4+ T-cell subset was reduced to a greater extent than the CD8+ T-cell subset in smoking asthmatics, and accordingly was the CD4+/CD8+ T-cell ratio lowered in smoking asthmatics. The overall amount of γδ- T-cells was low, and this subset was predominantly observed in the epithelium. Both subepithelial and intraepithelial γδ-T-cells were significantly reduced in the asthmatic groups compared with non-exposed controls. The density of subepithelial macrophages was increased in non-smoking asthmatics compared to smoking asthmatics as well as the non-smoking controls ($p<0.05$) while density of macrophages in smoking asthmatics did not differ from controls.

In contrast to the subepithelium, the number of intraepithelial CD8+ T-cells and macrophages was significantly increased in smoking asthmatics compared to non-smoking asthmatics. A parallel increase was observed in smoking healthy workers who in addition demonstrated increase in intraepithelial CD4+ T-cells. Such increase in intraepithelial CD4+ T-cells was not observed in smoking asthmatics.

Conclusion: The increased subepithelial density of CD4+ T-cells and macrophages observed in non-smokers with potroom asthma illustrates the immunopathological similarity between occupational and non-occupational asthma. Smoking affected predominantly CD4+ T-cell accumulation in the asthmatic airways, suggesting different pathogenetic mechanisms in smokers and non-smokers with potroom asthma. Smokers with potroom asthma may represent a separate asthmatic phenotype without the characteristic asthma-associated increase in bronchial CD4+ T-cells.

5.4 Paper IV

Sjåheim T, Bjørtuft Ø, Drabløs PA, Kongerud J, Halstensen TS.

Increased bronchial density of CD25+Foxp3+ regulatory T-cells in nonsmokers with occupational asthma.

Submitted

Aim: To investigate the activation of T-cells through expression of CD25, Foxp3, HLA-DR and the proliferation factor Ki-67 in bronchial biopsies from non-smokers and smokers with potroom asthma.

Results: Non-smoking asthmatics had significantly increased subepithelial density of Foxp3+ T-cells and CD25+ T-cells compared to all the other groups, while smoking asthmatics did not differ from the control groups. Activated CD4+CD25+ T-cells include both regulatory T-cells (Foxp3+) and effector T-cells ($Foxp3^{neg}$) and triple-colour immunohistofluoresence staining revealed that the majority (medians 54-83 %) were Foxp3+, presumably regulatory T-cells (Treg). Non-smoking asthmatics had significantly increased density of putative Treg(CD4+CD25+Foxp3+CD3+) and putative effector T-cells(CD4+CD25+$Foxp3^{neg}$CD3+) in addition to increased density of proliferating (Ki-67+) T-cells (both CD4+ and CD8+), and HLA-DR+ T-cells (predominantly CD8+). A parallel increase was not observed in smoking asthmatics, and this group did not differ from controls except from an increased percentage of the CD25+ T-cells being effector T-cells.

Conclusion: Several markers of T-cell activation were observed in non-smoking asthmatics (subepithelial accumulation of Treg, effector T-cells, proliferating T-cells and HLA-DR+ T-cells) while only increased percentage of subepithelial effector T-cells was observed in smoking asthmatics. Although Treg seems to accumulate in the bronchial mucosa in non-smokers with potroom asthma, their regulatory function appeared insufficient to prevent the asthmatic bronchial inflammatory response.

6 Discussion

6.1 Methodological considerations

Paper I

From the total potroom workforce of 380 subjects at Årdal aluminium plant, 338 (89 %) participated in the study. According to the plant physician (VS) there were no major differences between the dropouts and the participants.

Skin prick test

As most studies define a skin prick test with a weal diameter ≥ 3 mm as positive, we initially considered the skin prick test as positive with a weal diameter > 3 mm and equivocal or negative with diameter > 1 mm but < 3mm. As the mean eosinophil count turned out to be the same in the positive and equivocal group, these two groups were analysed together. Accordingly, we defined a skin prick test with weal diameter ≥ 1 mm as positive and thus subjects with a positive skin prick test were regarded as atopic.

There was 17 % missing data for the skin prick test as shown in table I (16 % in the asymptomatic workers and group and 19 % in symptomatic workers). However, when the statistical analyses were performed without atopy in the models, the association between eosinophils and the symptom groups remained unchanged and thus not influenced by missing data for atopy.

Paper II, III and IV

Initially we aimed to include only asthmatic non-smokers without steroid treatment. However, it became evident that the number of potential participants would be rather small, as most of the workers with potroom asthma were smokers and/or treated with inhaled corticosteroids. To gain a sufficient number of subjects, we decided to include asthmatic smokers and subjects on inhaled corticosteroids. Further on, it turned out that most of the parameters investigated were affected by smoking habits. Consequently, the data had to be presented separately for non-smokers and smokers, making each asthmatic group rather small. Preferably, a higher number of asthmatics should have been included. To increase the number of asthmatic subjects would

require waiting for new cases of potroom asthma to occur, which was beyond the resources available for the study.

The diagnosis of potroom asthma

Diagnosing occupational asthma may be difficult, and by inclusion of the asthmatic subjects in the biopsy study, great attention was paid to attain a correct diagnosis of potroom asthma. In all asthmatic subjects, the diagnosis of potroom asthma was confirmed by the main investigator (TS). The main investigator examined nearly all potroom workers at every plant who reported respiratory symptoms in the annual questionnaire. Although they had respiratory symptoms suggestive of potroom asthma, no significant airway obstruction could be demonstrated in the majority of these subjects. Several subjects had a previously confirmed diagnosis of potroom asthma by a pulmonary specialist and were treated with inhaled corticosteroids. Some were still working in the potrooms and reported ongoing respiratory symptoms, but the majority did no not demonstrate significant airflow obstruction. Accordingly, they did not fulfil the inclusion criteria. For ethical reasons the steroid medication was not withdrawn.

Especially in smokers, great attention was paid to distinguish between asthma and COPD, and a misclassification of COPD as asthma seems unlikely to have occurred in the present study. The asthmatic smokers had history, symptoms and reversible airflow obstruction consistent with asthma and not COPD. The lung function in smoking asthmatic was comparable to that in non-smoking asthmatics (FEV_1 90 % vs. 91 % of predicted value).

The importance of inhaled steroid treatment and previous smoking

Some asthmatic workers on inhaled corticosteroid treatment still claimed respiratory symptoms and airflow obstruction and four asthmatics on inhaled corticosteroid treatment were included (one non-smoker and three smokers). Consequently, the impaired immune response observed in smoking asthmatics could in theory be explained partly by the use of anti-inflammatory treatment. However, the three steroid treated smoking asthmatics had the most inflamed mucosa within the group, with throughout leukocyte phenotype densities in the upper range. This is in line with the observed reduced response to inhaled corticosteroids in smoking asthmatics (18). When the four asthmatic subjects on inhaled corticosteroid treatment were excluded from the analysis the overall results remained unchanged.

Two of the asthmatic workers and three of the healthy workers were ex-smokers and regarded as non-smokers (and similar to never-smokers). The asthmatics stopped smoking three and 13 years before inclusion in the study, and the healthy workers six, 14 and 15 years before. In the initial period of the study, very limited information available concerning airway inflammation in previous smokers. Later studies have demonstrated that in subjects with established COPD, the mucosal bronchial cell infiltrate is similar in ex-smokers and in those who continue to smoke (130;131). Such information is still not available in smoking asthmatics, but our data indicate that it is inappropriate to merge asthmatic non-smokers and ex-smokers into the same group in future studies, due to a potential hangover effect of smoking. Excluding the five ex-smokers (two asthmatics and three healthy workers) in the present study from the analysis did not change the main results. Interestingly, one of the asthmatic ex-smokers still "behaved" as an asthmatic smoker with throughout low density of the different leukocyte subtypes, suggesting a persistent smoking-induced effect.

The non-exposed control group

The non-exposed controls had a mean age of 26 years while the mean age of the potroom workers was 39 years. Due to limited resources and for practical reasons, the non-exposed controls were recruited among non-smoking medical students, who were younger than the workers. Ideally, a non-exposed smoking control group should have been included as well. To control for age as a potential confounder, analysis of variance was performed for all the outcomes. The only parameter that varied with age was neutrophils, and a reduction in subepithelial neutrophil density was observed with increasing age for all groups.

Assessment of airway inflammation in asthma by bronchial biopsies.

Endobronchial biopsy samples are limited in overall size to between 1 and 2 mm in diameter. They may not be representative of the entire conducting airways as they mainly derive from relatively proximal airways and from subcarina rather than the lateral wall. Moreover, bronchial biopsies provide only a snapshot in time of an ongoing disease process as asthma. However, this is the only technique that directly samples the resident cells in the bronchial mucosa and maintains the spatial relationship with structural components in vivo (70).

The exact localisation of airway inflammation in asthma is still a matter of controversy. It is, however, accepted that inflammation is exhibited in both large and small airways. Because inflammation in the asthmatic airway is likely to be unevenly distributed in the airway mucosa

throughout the bronchial tree, randomness in biopsy sampling and the selection of biopsy sections are essential (70). For interpretation of the data, it was assumed that endobronchial biopsy of large airway subcarinae provides unbiased and representative samples of the entire bronchial tree, which currently is unproven and needs to be determined in larger lung samples obtained at surgery or post mortem.

Immunohistofluorescent staining

The immunoenzyme staining methods traditionally used to identify cells in the bronchial mucosa have many limitations. Although double immunoenzyme staining can be performed, it is mainly appropriate to identify two non-overlapping cell subsets, as double labelled cells often appear difficult to separate from strongly single colour stained cells. An alternative method has been single staining on neighbouring sections and co-localization in a two lens, single ocular system called Lucida that permits co-localisation. However, the staining is performed on two separate sections and different cells may be stained in the two sections. Moreover, adjacent thin sections may be difficult to produce.

Most cell activation markers are not restricted to one single cell type and single staining for these activation markers may produce erroneously results when identifying which cell type that is expressing the given marker in situ. Single staining for the cell identification marker CD4 has often been announced as CD4+ T-cells, without taking into consideration that CD4 is expressed on many other cell types, including macrophages. This may be even more pronounced when using the expression of CD25 as a marker for activated CD25+ T-cells, and this our study (paper IV) revealed that the majority of CD25+ cells actually were non-T-cells ($CD3^{neg}$). Thus multi-labelling is required to identify several cell subtypes, and the main advantage of immunohistofluorescent staining is the ability to filter out each label, and to visualize both single labelling and double,-and triple labelling simultaneously.

A challenge when applying fluorescent staining on bronchial mucosa is the inborn tendency of elastic fibres to be autofluorescent, masking single stained cells. However, when examined through double fluorescence filters, autofluorescence becomes yellow-white whereas single labelled cells appear green, red or blue. Thus, viewing trough such double /triple fluorescence filter blocks discriminated between immunofluorescent labelled cells and autofluorescence. The new use of double and triple immunohistofluorescent labelling on the bronchial mucosa have thus revealed several new phenotypic characteristics of the leukocyte subsets in the

asthmatic inflammatory response. Evaluation of multiple stained slides is time-consuming and requires experience. The slides were primarily analyzed by one observer (TS), after frequent counting controls by the supervisor (TSH) and regular discussions of positive cutoff scores.

6.2 Discussion of main results

Although the immunosuppressive effect of smoking is well documented in human studies, it has previously not been investigated in relation to asthma. Since the results turned out to be strikingly different in the asthmatics according to smoking habits, the data are presented for non-smokers and smokers separately. In the healthy, non-asthmatic workers, however, the outcomes were more or less similar in non-smokers and smokers.

Non-smoking asthmatics

The study confirms that potroom asthma is an inflammatory disease of the airways in non-smokers as revealed by increased exhaled NO concentrations, thickened RBM, increased number of intraepithelial mast cells and increased subepithelial density of eosinophils, macrophages, CD4+ T-cells, effector T-cells, regulatory T-cells, proliferating T-cells and HLA-DR+ T-cells. The inflammatory response was also revealed in blood by increased number of eosinophils. These observations illustrate that contaminants in the potrooms may induce chronic inflammatory changes in the airway wall of susceptible, but previously healthy subjects, and that reflex bronchoconstriction alone not can account for the mechanism behind potroom asthma. The current results confirm the immunopathological similarity between occupational and non-occupational asthma as shown in other studies as well (21). The data also demonstrate that the airway inflammation in potroom asthma is not different from what has been shown in occupational asthma caused by other agents, as for instance isocyanates. Accordingly, potroom asthma should be classified as OA and no longer as an asthma-like disorder or asthma-variant syndrome (54). Potroom fume emissions have traditionally not been included among causal agents of OA because different mechanisms could be involved including an immunologically mediated reaction against trace amounts of metals, or an irritant effect resulting from exposure to hydrogen fluoride and sulphur dioxide (21).

The present study does not bring us any closer to identify the offending agent of potroom asthma, but strongly suggests that an allergic cell-mediated immunity is involved in the immunopathology. However, it can not be excluded that chronic exposure to low concentrations of irritants also play a role, perhaps by aggravating the asthmatic symptoms. No bronchial biopsy study is, however, available from patients who develop asthma after chronic low-level exposure to irritants.

The presence of both eosinophilic and non-eosinophilic phenotypes of potroom asthma supports the view that multiple causal pathways may be involved, even in the same individual. Our data do not support the hypothesis that non-eosinophilic asthma is a neutrophil mediated inflammation (67;132;133), as there was no difference in bronchial neutrophil density between eosinophilic and non-eosinophilic asthmatic groups.

Neutrophil involvement has been documented in asthma induced by isocyanates (67;68;108) and grain dust (67;68;109), but increased subepithelial neutrophil density was not observed in the present study. Similarly, the increased subepithelial mast cell density reported in isocyanate induced asthma was not observed in potroom asthma (paper II). However, subepithelial mast cell density in asthma is highly variable (134-136). The present study is rather in line with a study in isocyanate asthma showing increased mast cells only in the epithelium and not in the subepithelial layer (97).

To date, there are no published reports on regulatory T-cells in the bronchial mucosa and no other researchers have used the expression of Foxp3 in CD4+CD25+ T cells (CD3+) to identify putative Treg and effector T-cells in tissue sections. The increased density of Treg (CD4+CD25+ Foxp3+ T-cells) in non-smoking asthmatics contrasts the hypothesis that impaired expansion of Treg leads to the development of asthma (137;138). This hypothesis is nevertheless based on human studies in BAL (139). Although several recent studies indicate that Treg may inhibit Th2 responses and protect against allergy and asthma (137) there are evidenced that Treg may predominantly inhibit Th1 responses (140). By reducing the Th1-induced Th2-inhibition, the predominance of Th1-inhibition may facilitate Th2 type inflammation, as demonstrated in mice (141). The present study indicates that despite the accumulation of Tregs in the inflamed tissue, these cells were unable to fully inhibit the mucosal inflammation and T-cell proliferation.

The identification of proliferating bronchial mucosal T-cells is a new observation. T-cells are assumed to proliferate in the regional lymph nodes. Ki-67+ T-cells were mainly observed in non-smoking asthmatics and relatively few proliferating T-cells were observed in smoking asthmatics and controls. Whether T-cells are induced to proliferate locally in the bronchial mucosa can not be confirmed by the present study, as it can not be excluded that the Ki-67+ T-cells represented newly arrived cells which had been induced to proliferate in organized lymphoid tissue 6-8 hours prior to biopsy sampling. Both CD4+ and CD8+ T-cells proliferated to approximately the same extent in non-smoking asthmatics. This observation supports the participation of the CD8+ T-cell subset in asthma. The involvement of CD8+ T-cells was further supported by the increased density of HLA-DR activated CD8+ T-cells in this group.

Smoking asthmatics

It has previously been hypothesized that smoking amplifies asthmatic airway inflammation (142). Even though smoking may induce profound immunosuppressive effects, smoking in general induces an inflammatory reaction in the airways of asymptomatic smokers (83;84). Increased bronchial CD8+ T-cell and macrophage density are consistent findings in asymptomatic smokers (143;144), and this was likewise observed in the smoking healthy workers in the present study (paper III).

Accordingly, the strikingly lower bronchial density of all the leukocyte subsets in smoking compared to non-smoking asthmatics was somewhat surprising. To date there are still no comparable human study in smoking asthmatics. It appears that smoking affects all the subepithelial leukocyte subsets identified in the present study; i.e. eosinophils, neutrophils, macrophages, mast cells, and T-cells. The CD4+ T-cell subset seemed to be more reduced than the CD8+ subset, a phenomenon that also has been observed in mice (88). The immunmodulating effect of smoking was also observed in blood, as shown in paper I by reduced blood eosinophils in smoking asthmatics when compared to non-smoking asthmatics.
T-cell activation was in general also suppressed in the asthmatic smokers, with reduced subepithelial density of regulatory T-cells, effector T-cells and reduced local T-cell proliferation when compared to non-smoking asthmatics. However, group based data showed that both non-smoking and smoking asthmatics had increased percentage of effector T-cells (of CD25+ T-cells) when compared to the respective control workers. Although the percentage of effector T-cells was higher in smoking than in non-smoking asthmatics, a corresponding

difference was observed in the healthy workers, suggesting a smoke influenced increase in the percentage of effector T-cells and accordingly reduction in the percentage of Treg. Moreover, smoking asthmatics had a slightly increased subepithelial density of HLA-DR+ CD8+ T-cells (group based data). Thus, despite the significantly lower density of CD25+ T-cells in smoking compared to non-smoking asthmatics, the increase in effector T-cells vs. Treg T-cell to Treg suggested that the CD4+ T-cell subset was involved in the immunopathology of smoking asthmatics as well.

In contrast to the subepithelial layer, the number of intraepithelial macrophages and CD8+ T-cells was increased in smoking compared to non-smoking asthmatics. A parallel increase was observed in healthy smokers compared to healthy non-smokers. Consequently it appears that smoking attracts these cells to the epithelium independent of disease status. In line with the previous observation that smoking predominantly suppressed the CD4+ T-cell subset, an asthma-associated increase in intraepithelial CD4+ T-cells was lacking in the smoking asthmatics.

Is smoking asthmatics a separate phenotype?

The RBM thickening was similar in smoking and non-smoking asthmatics. Although debated whether this is specific for asthma, the observation of thickened RBM supported that a chronic inflammatory process is present in the asthmatic smokers. Although asthmatics smokers did not have the asthma-associated bronchial leukocyte accumulation, there were some evidences for mucosal inflammatory reactions as a slightly increased subepithelial eosinophil density and increased percentage of effector T-cells (group based data). Thus, despite the significantly lower density of CD25+ T-cells, they were more often of the effector T-cell phenotype in smoking asthmatics, suggesting that the CD4+ T-cell subset was, after all, involved in the immunopathology of smoking asthmatics. There was no evidence for increased activation of the CD8+ T-cell subset in the smoking asthmatic, apparently discriminating them from smoking induced COPD. The main question is, however, whether this apparently small alteration in effector/regulatory T-cell ratio could be responsible for driving the asthmatic process or whether different immunopathological mechanisms are present in smokers and non-smokers. Nevertheless, our data challenge the present view of asthma as primarily a CD4+ T-cell driven disease.

Exhaled nitric oxide and reticular basement membrane

The low levels of exhaled NO concentrations in smokers was similar to what has been previously shown in smoking subjects (145) including smoking asthmatics (146). Exhaled NO has previously been explored in potroom workers (117). Apart from our findings that exhaled NO concentrations correlated to the density of lamins propria CD45+ leucocytes (total leukocytes) and eosinophils (147), the study could not clarify the role of NO in potroom asthma.

The thickened RBM in non-smoking asthmatics with potroom asthma was consistent with previous studies in conventional asthma (148), and the RBM thickening was independent of smoking habits. Subject with eosinophilic asthma (with eosinophil density above the upper range seen in non-exposed controls) had thickest RBM. Subjecs with eosinophilic asthma had significantly thicker RBM than those with non-eosinophil asthma (8.5 μm vs. 7.6 μm, p= 0.04). When analyzing all subjects together (n=45), a significant correlation between RBM and subepithelial eosinophil density was observed (r=0, 44, p=0,003) (unpublished data).

Persistent inflammation in relocated asthmatic workers.

The study interprets that exposure to contaminants in the potrooms may induce chronic, irreversible inflammatory changes in the airway wall, although we found no correlation between duration of exposure and inflammatory changes. Ten out of the twenty asthmatic workers were relocated to "clean" unpolluted working environments at the smelters. The outcome parameters did not diverge between the relocated workers and those still working in the potrooms, which are in line with the permanent disability and persistent airway inflammation reported in workers with other form of OA who were removed from exposure (41).

The latency

The latency period, i.e. the time from the start of exposure in the potroom until first appearance of respiratory symptoms, was median 7 years and equal in non-smokers (range 1-13) and smokers (range 0.5-13). This appear rather long, taking into account that most cases of potroom asthma in Norwegian smelters occur within the first 2 years of employment (1;49). Workers who develop asthma within the first 1-2 years are probably more prone to leave the smelter and more easily get new jobs than more settled workers. On the other hand, a

cumulative effect of exposure has been suggested by others, as respiratory symptoms increased with years of employment. Those working in potrooms for more than 10 years had more often asthma (49). However, data on mean latency for developing asthma in Norwegian smelters are not available. There are no recognized association between a short latency and more severe disease.

Subclinical inflammation in the healthy workers

In contrast to asthmatic workers, a smoking associated decrease in leukocyte densities was not observed in the healthy workers. While smoking seemed to dampen the inflammatory response in asthmatics, it appeared to induce inflammatory changes in healthy subject as blood and mucosal eosinophils and mucosal macrophages tended to be increased in smoking compared to non-smoking healthy workers. Compared to non-exposed control, the healthy workers (both smokers and non-smokers) had thickened RBM, increased subepithelial density of eosinophils and effector T-cells and their exhaled NO concentrations tended to be elevated. This finding suggested that exposure to pollutants in aluminium smelters affects the bronchial mucosa and induce a subclinical inflammatory reaction, even in healthy asymptomatic workers. Thickening of RBM is regarded as an early sign in the development of asthma, suggesting latent asthma However, none of these healthy workers have until now, (observation time several years), developed asthma and they are all still working in the potrooms.

The number of study participants

The number of participants included was rather limited, a weakness shared with many bronchial biopsy studies. For reasons previously outlined, it was not possible to include more asthmatic subjects. The main problem with such small studies is the risk to exclude real differences that do not reach the level of significance due to low sample size (type II error). By using non-parametric statistic analysis, which is rather sensitive to small sample size, our data clearly suggest that the main conclusions of the study are valid. The surprisingly lack of asthma induced increase in subepithelial leukocyte subsets in smoking asthmatics was observed in a group of 12 subjects, which is not that small compared to other published bronchial biopsy studies in asthma. We compared the result with a smaller group of eight non-smoking asthmatics, where the results turned out as expected and in agreement with several comparable studies showing increased subepithelial leukocyte subset densities in non-smoking asthmatics. The differences between the two asthmatic groups were in general highly

significant, and it then appears unlikely that increasing the group of non-smoking asthmatics would have changed the main results. As previously described, the difference could not be ascribed to the inclusion of steroid-treated subjects or ex-smokers. The control groups were included just to illustrate the different T-cell density levels in exposed and non-exposed non-asthmatic subjects. There were no unexpected observations in these three control groups and their leukocyte densities were more or less the same.

Are the results in asthmatic smokers restricted to potroom asthma?

One obvious question is whether the immunomodulating effect in the asthmatic smokers is induced by smoking per se or by the combination of exposure to cigarette smoke and potroom fume emission. To answer this question a comparable study in smokers with non-occupational asthma has to be performed. A potential interaction effect was, however, not observed in the smoking healthy workers. Further more, seven out of the 12 asthmatic smokers had been relocated to unpolluted working environments for median 2 years prior to examination. The results in these relocated smoking asthmatic workers did not differ from the smoking asthmatics who were still exposed. The immunomodulating effect of smoking has been well documented in a large number of studies. The impact of smoking on bronchial mucosal inflammation in asthmatics has, however, not been investigated. The present observations suggest that the inhibited asthma associated bronchial leukocyte accumulation may be a general phenomenon and not restricted to smokers with potroom astma. Smoking seems to affect underlying mechanisms involved in asthma, and smoking asthmatics may represent an asthmatic phenotype with an overall suppressed bronchial inflammation and especially without the characteristic increase in mucosal CD4+ T-cells.

Is smoking a risk factor for potroom asthma?

Cigarette smoking has been reported to be associated with the development of OA in workers exposed to platinum salts and anhydride compounds, which are chemicals that cause asthma through an IgE mechanism (149;150). In addition, smoking increases the risk of sensitization to HMW agents that cause asthma through an IgE mechanism. In contrast, smoking does not increase the risk of asthma caused by LMW agents, such as diisocyanates and red cedar (151). Diverging results has been reported in the aluminium industry. No significant association between smoking and potroom asthma was observed in an Australian smelter (60). Further, a cross-sectional study reported lower prevalence of respiratory symptoms in smoking potroom

workers than in non-smokers and lack of combined effect of exposure and smoking on respiratory health (152). In the Norwegian aluminium industry, smoking has been reported to be a strong risk factor for work-related asthmatic symptoms and an interaction was found between smoking and fluoride exposure (1;49). However, the present thesis was not designed to further elucidate this question. In paper I, 9 % of the non-smokers and 13 % of the smokers reported potroom asthma. Several potroom workers reported worsening of their asthmatic symptoms when they stopped smoking, and a corresponding increase in airway obstruction has been documented by the plant physician (personal communication). Interestingly, one third of smoking asthmatics reported worsening of their asthmatic symptoms after smoking cessation in a previous study (153). A protective effect of smoking should however not be announced. Smoking may probably mask or delay asthmatic symptoms while immunpathological reactions induce changes in the airways that are not identified with parameters traditionally investigated in asthma.

7 Main conclusions

Potroom asthma was positively associated with the number of peripheral blood eosinophils and inflammatory changes in the bronchial mucosa. Non-smokers with potroom asthma demonstrated similar changes as previously shown in occupational and non-occupational asthma (increased exhaled NO concentrations and RBM thickness, increased subepithelial density of eosinophils, macrophages, CD4+ T-cells, activated T-cells (CD25+ and HLA-DR+ T-cells). In addition, increased density of regulatory T-cells and proliferating T-cells was revealed in non-smokers with asthma, adding new information to the understanding of asthma.

Smoking appeared all over to inhibit the asthma associated accumulation of leucocytes, in particular mucosal CD4+ T-cell subset and T-cell proliferation. Apart from thickened RBM and a slight increase in subepithelial eosinophil density and percentage of effector T-cells, the bronchial leukocyte composition in smokers with potroom asthma did not differ from controls. Although immunomodulation of smoking on the bronchial asthmatic inflammation was demonstrated in occupational asthma, the phenomenon may not be restricted to potroom asthma. Smoking asthmatics may represent a separate asthmatic phenotype without the characteristic CD4+ T-cell dominance, a statement that still has to be proven in traditional asthma.

The mucosal inflammatory changes in the asthmatics who were still working in the potrooms did not differ from those demonstrated in the relocated workers. Accordingly, potroom fume emission induces a chronic bronchial inflammation that persists after removal from exposure. A subclinical bronchial inflammatory reaction was induced in the healthy asymptomatic workers, revealed by increased RBM thickness and increased subepithelial eosinophil density.

The results strongly suggest that an allergic cell-mediated immunity is involved in the immunopathology of potroom asthma, although it can not be excluded that chronic exposure to low concentrations of irritants participates in parallel. Potroom asthma should from now on be classified as occupational asthma and not as an asthma-like disorder or as an asthma-variant syndrome.

8 References

(1) Kongerud J, Samuelsen SO. A longitudinal study of respiratory symptoms in aluminum potroom workers. Am Rev Respir Dis 1991 ;144(1):10-6.

(2) Skaugset NP. Potroom asthma in the production of primary aluminium. (The HAPPA report). STAMI/AMS: 2007

(3) Abramson MJ, Wlodarczyk JH, Saunders NA, Hensley MJ. Does aluminum smelting cause lung disease? AmRev Respir Dis 139(4):1042-57, 1989.

(4) Soyseth V, Kongerud J. Prevalence of respiratory disorders among aluminium potroom workers in relation to exposure to fluoride. Br J Ind Med 1992 ;49(2):125-30.

(5) Steinegger AF, Schlatter C. Evaluation of fluoride exposure in aluminium smelters: state of the art. Medicina del Lavoro 83(5):489-98, 1992

(6) Soyseth V, Kongerud J, Ekstrand J, Boe J. Relation between exposure to fluoride and bronchial responsiveness in aluminium potroom workers with work-related asthma-like symptoms. Thorax 1994; 49(10):984-9.

(7) Bolstad KL. Statistisk gjennomgang av arbeidsmiljømålinger i HAMP i perioden 1990-1999 (In norwegian). Hydro Aluminium Teknologisenter Årdal; 2000. Report No.: TEK 2000/125.

(8) Ongstad L,Stoll C,Aasland T. The Norwegian aluminium industry and the local environment. 1994 ISBN: 82-90861-27-3.

(9) From the *Global Strategy for Asthma Management and Prevention*, Global Initiative for Asthma (GINA) 2006.

(10) Brogger J, Bakke P, Eide GE, Johansen B, Andersen A, Gulsvik A. Long-term changes in adult asthma prevalence. Eur Respir J 2003;21(3):468-72.

(11) Downs SH, Marks GB, Sporik R, Belosouva EG, Car NG, Peat JK. Continued increase in the prevalence of asthma and atopy. Arch Dis Child 2001;84(1):20-3.

(12) Nystad W, Magnus P, Gulsvik A, Skarpaas IJ, Carlsen KH. Changing prevalence of asthma in school children: evidence for diagnostic changes in asthma in two surveys 13 yrs apart. Eur Respir J 1997;10(5):1046-51.

(13) Skjønsberg OH, Clench-Aas J, Leegaard J, Skarpaas I, Giaever P, Bartonova A, et al. Prevalence of bronchial asthma in schoolchildren in Oslo, Norway. Comparison of data obtained in 1993 and 1981. Allergy 1995;50(10):806-10.

(14) Furu.K, Skurtveit S, Langhammer A, Nafstad P. Use of anti-asthmatic medication as a proxy for prevalence of asthma in children and adolescents in Norway: a nationwide prescription database analysis. Eur J Clin Pharmacol 2007;63:693-8.

(15) Carlsen KCL, Haland G, Devulapalli CS, Munthe-Kaas M, Pettersen M, Granum B, et al. Asthma in every fifth child in Oslo, Norway: a 10-year follow up of a birth cohort study*. Allergy 2006;61(4):454-60.

(16) Eagan TML, Brogger JC, Eide GE, Bakke P. The incidence of adult asthma: a review. Int J Tuberc Lung Dis 2005;9(6):603-12.

(17) Thomson NC, Chaudhuri R, Livingston E. Asthma and cigarette smoking. Eur Respir J 2004 (5):822-33.

(18) Thomson NC, Spears M. The influence of smoking on the treatment response in patients with asthma. Curr Opin Allergy Clin Immunol 2005 (1):57-63.

(19) Johansson SGO, Hourihane JO, Bousquet J, Bruijnzeel-Koomen C, Dreborg S, Haahtela T, et al. A revised nomenclature for allergy: An EAACI position statement from the EAACI nomenclature task force. Allergy 2001;56(9):813-24.

(20) Johansson SGO, Bieber T, Dahl R, Friedmann PS, Lanier BQ, Lockey RF, et al. Revised nomenclature for allergy for global use: Report of the Nomenclature Review Committee of the World Allergy Organization, 2003. Journal of Allergy and Clinical Immunology 2004;113(5):832-6.
(21) Mapp CE, Boschetto P, Maestrelli P, Fabbri LM. Occupational Asthma. Am J Respir Crit Care Med 2005;172(3):280-305.
(22) Vandenplas O, Malo JL. Definitions and types of work-related asthma: a nosological approach. Eur Respir J 21(4):706-12, 2003
(23) Brooks SM. Occupational asthma. In: Weiss EB, Segal MS, Stein M, editors. Bronchial Asthma. Boston: Little, Brown; 1985. p. 461-9.
(24) Burge PS. Occupational asthma. In: Barnes P, Rodger IW, Thomson NC, editors. Asthma: Basic Mechanisms and Clinical Management.London: Academic Press; 1988. p. 465-82.
(25) Chan-Yeung M, Malo JL. Occupational asthma. Chest 1987;81:130S-6S.
(26) Cotes JE, Steal J. Work-related lung disorders. Oxford: Blackwell Scientific Publ; 1987.
(27) Newman-Taylor AJ. Occupational asthma. Thorax 1980;35:241-5.
(28) Parkes WR. Occupational Lung Disorders. London: Butterworths; 1982.
(29) Sheppard D. Occupational asthma and byssinosis. In: Murray JF, Nadel JA, editors. Textbook of Respiratory Medicine.Philadelphia: WB Saunders; 1988. p. 1593-605.
(30) Bernstein IL, Bernstein DI, Chan-Yeung M, Malo JL. Definition and classification of asthma in the workplace. In: Bernstein IL, Chan-Yeung M, Malo JL, Bernstein DI, editors. Asthma in the workplace. 3rd edn. New York: Taylor & Francis; 2006. p1-8.
(31) Mapp CE. Agents, old and new, causing occupational asthma. Occup Environ Med 2001 ;58(5):354.
(32) Brooks SM, Weiss MA, Bernstein IL. Reactive airways dysfunction syndrome (RADS). Persistent asthma syndrome after high level irritant exposures. Chest 1985;1;88(3):376-84.
(33) Tarlo SM. Workplace irritant exposure: do they produce true occupational asthma? Ann Allergy Asthma Immunol 2003;90(5 Suppl 2):19-23.
(34) Malo JL. Irritant-induced asthma and reactive airways dysfunction syndrome. Can Respir J 1998;5:66-7.
(35) Nicholson PJ, Cullinan P, Newman Taylor AJ, Burge PS, Boyle C. Evidence based guidelines for the prevention, identification, and management of occupational asthma. Occup Environ Med 2005;62(5):290-9.
(36) Balmes J. Occupational airways diseases from chronic low-level exposures to irritants. Clin Chest Med 2002;23(4):727-35.
(37) Brooks SM, Hammad Y, Richards I, Giovinco-Barbas J, Jenkins K. The spectrum of irritant-induced asthma: sudden and not-so-sudden onset and the role of allergy. Chest 1998;113(1):42-9.
(38) Kipen HM, Blume R, Hutt D. Asthma experience in an occupational and environmental medicine clinic. Low-dose reactive airways dysfunction syndrome. J Occup Med 1995;36(10):1133-7.
(39) Andersson E, Knutsson A, Hagberg S, Nilsson T, Karlsson B, Alfredsson L, et al. Incidence of asthma among workers exposed to sulphur dioxide and other irritant gases. Eur Respir J 2006;27(4):720-5.
(40) Chan-Yeung M. Assessment of asthma in the workplace. ACCP consensus statement. American College of Chest Physicians. Chest 1995;108(4):1084-117.

(41) Becklake MR, Malo JL, Chan-Yeung M. Epidemiological Approches in Occupational asthma. In: Bernstein IL, Chan-Yeung M, Malo JL, Bernstein DI, editors. Asthma in the workplace.New York: Taylor & Francis; 2006. p. 69-72.
(42) Venables KM. Prevention of occupational asthma. Eur Respir J 1994;7(4):768-78.
(43) Venables KM, Chan-Yeung M. Occupational asthma. Lancet 349(9063):1465-9, 1997
(44) Balmes J, Becklake M, Blanc P, Henneberger P, Kreiss K, Mapp C, et al. American Thoracic Society Statement: Occupational Contribution to the Burden of Airway Disease. Am J Respir Crit Care Med 2003;167(5):787-97.
(45) Blanc PD, Toren K. How much adult asthma can be attributed to occupational factors? The American Journal of Medicine 1999;107(6):580-7.
(46) Eagan TML, Gulsvik A, Eide GE, Bakke PS. Occupational Airborne Exposure and the Incidence of Respiratory Symptoms and Asthma. Am J Respir Crit Care Med 2002; 166(7):933-8.
(47) Kogevinas M, Zock JP, Jarvis D, Kromhout H, Lillienberg L, Plana E, et al. Exposure to substances in the workplace and new-onset asthma: an international prospective population-based study (ECRHS-II). The Lancet 370(9584):336-41.
(48) Frostad E. Fluorine intoxication in Norwegian aluminium plants. Tidsskr Nor Laegefor 1936;56(179).
(49) Kongerud J, Gronnesby JK, Magnus P. Respiratory symptoms and lung function of aluminum potroom workers. Scand J Work Environ Health 1990;16(4):270-7.
(50) Taiwo O, Sircar K, Slade M, Cantley L, Vegso S, Rabinowitz P, et al. Incidence of Asthma Among Aluminum Workers. J Occup Environ Med 2006;48:275-82.
(51) Chan-Yeung M, Wong R, MacLean L, Tan F, Schulzer M, Enarson D, et al. Epidemiologic health study of workers in an aluminium smelter in British Columbia. Effects on the respiratory system. Am Rev Respir Dis 1983;127(4):465-9.
(52) Discher D, Breitenstein B. Prevalence of chronic pulmonary disease in aluminium potroom workers. J Occup Med 1976;18:379-86.
(53) Kaltreider N, Elder M, Cralley L, Corwell M. Health survey of aluminium workers with special reference to fluoride exposure. J Occup Med 1976;14:531-41.
(54) Bernstein IL, Merget R. Metals. In: Bernstein IL, Chan-Yeung M, Malo JL, Bernstein DI, editors. Asthma in the workplace. 3rd ed. New York: Taylor and Francis; 2006. p. 544-6.
(55) Desjardins A, Bergeron JP, Ghezzo H, Cartier A, Malo JL. Aluminium potroom asthma confirmed by monitoring of forced expiratory volume in one second. Am J Respir Crit Care Med 1994;150:1714-7.
(56) O'Donnell TV, Welford B, Coleman ED. Potroom asthma:New Zealand experience and follow-up. Am J Ind Med 1989;15:43-9.
(57) Simonsson BG, Sjoberg A, Rolf C, Haeger-Aronsen B. Acute and long-term airway hyperreactivity in aluminium-salt exposed workers with nocturnal asthma. Eur J Respir Dis 66(2):105-18, 1985
(58) Kongerud J, Soyseth V. Methacholine responsiveness, respiratory symptoms and pulmonary function in aluminum potroom workers. Eur Respir J 1991;4(2):159-66.
(59) Sorgdrager B, de Looff AJ, de Monchy JG, Pal TM, Dubois AE, Rijcken B. Occurrence of occupational asthma in aluminum potroom workers in relation to preventive measures. Int Arch Occup Environ Health 71(1):53-9, 1998
(60) Barnard CG, McBride DI, Firth HM, Herbison GP. Assessing individual employee risk factors for occupational asthma in primary aluminium smelting. Occup Environ Med 2004;61(7):604-8.

(61) Sorgdrager B, Pal TM, de Looff AJ, Dubois AE, de Monchy JG. Occupational asthma in aluminium potroom workers related to pre-employment eosinophil count. Eur Respir J 8(9):1520-4, 1995
(62) Saric M, Godnic-Cvar J, Gomzi M, Stilinovic L. The role of atopy in potroom workers' asthma. Am J Ind Med 9(3):239-42, 1986.
(63) Mackay IR, Oliphant RC, Laby B, Smith MM, Fisher JN, Mitchell RJ, et al. An Immunologic and Genetic Study of Asthma in Workers in an Aluminum Smelter. J Occup Med 1990;32(10):1022-6.
(64) Slåstad S, Leira H, Bratt U. Forekomst av arbeidsrelatert astma i Norge. (In Norwegian). Helse Midt-Norge; 2003. Report No.: 4 2003.
(65) Soyseth V, Kongerud J, Boe J, Fonneland T. Bronchial responsiveness and work-related asthma in aluminium potroom workers: effect of removal from exposure. Eur Respir J 1992;5(7):829-33.
(66) Wergeland E, Lund E, Waage J. Respiratory dysfunction after potroom asthma. Am J Ind Med 1987;11:627-36.
(67) Gibson PG, Simpson JL, Saltos N. Heterogeneity of Airway Inflammation in Persistent Asthma. Evidence of Neutrophilic Inflammation and Increased Sputum Interleukin-8. Chest 2001;119:1329-36.
(68) Wenzel SE, Schwartz LB, Langmack EL, Halliday JL, Trudeau JB, Gibbs RL, et al. Evidence that severe asthma can be divided pathologically into two inflammatory subtypes with distinct physiologic and clinical characteristics. Am J Respir Crit Care Med160(3):1001-8, 1999
(69) Barnes PJ, Chung KF, Page CP. Inflammatory Mediators of Asthma: An Update. Pharmacol Rev 1998; 1;50(4):515-96.
(70) Jeffery P, Holgate S, Wenzel S. Methods for the Assessment of Endobronchial Biopsies in Clinical Research: Application to Studies of Pathogenesis and the Effects of Treatment. Am J Respir Crit Care Med 2003;168(6):1S-7.
(71) Schmidt-Weber CB, Akdis M, Akdis CA. TH17 cells in the big picture of immunology. J Allergy Clin Immunol 2007;120(2):247-54.
(72) Bettelli E, Korn T, Kuchroo VK. Th17: the third member of the effector T cell trilogy. Curr Opin Immunol 2007;19(6):652-7.
(73) Wang J, Ioan-Facsinay A, van der Voort E, Huizinga T, Toes R. Transient expression of FOXP3 in human activated nonregulatory CD4+ T cells. Eur J Immunol 2007;37:129-38.
(74) Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, et al. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature 2006:1;441(7090):235-8.
(75) Fisson S, Darrasse-Jeze G, Litvinova E, Septier F, Klatzmann D, Liblau R, et al. Continuous Activation of Autoreactive CD4+ CD25+ Regulatory T Cells in the Steady State. J Exp Med 2003;198(5):737-46.
(76) Walker LSK, Chodos A, Eggena M, Dooms H, Abbas AK. Antigen-dependent Proliferation of CD4+ CD25+ Regulatory T Cells In Vivo. J Exp Med 2003;198(2):249-58.
(77) Vukmanovic-Stejic M, Zhang Y, Cook JE, Fletcher JM, McQuaid A, Masters JE, et al. Human CD4+ CD25hi Foxp3+ regulatory T cells are derived by rapid turnover of memory populations in vivo. J Clin Invest 2006;116(9):2423-33.
(78) Sopori M. Effects of cigarette smoke on the immune system. Nat Rev Immunol 2002 ;2(5):372-7.

(79) Geng Y, Savage SM, Razanai-Boroujerdi S, Sopori ML. Effects of nicotine on the immune response. II. Chronic nicotine treatment induces T cell anergy. J Immunol 1996 ;156(7):2384-90.
(80) Singh SP, Kalra R, Puttfarcken P, Kozak A, Tesfaigzi J, Sopori ML. Acute and chronic nicotine exposures modulate the immune system through different pathways. Toxicology & Applied Pharmacology 164(1):65-72, 2000.
(81) Lambert C, McCue J, Portas M, Ouyang Y, Li J, Rosano TG, et al. Acrolein in cigarette smoke inhibits T-cell responses. J Allergy Clin Immunol 2005;116(4):916-22.
(82) Frazer-Abel AA, McCue JM, Lazis S, Portas M, Lambert C, Freed BM. Cigarette tar phenols impede T cell cycle progression by inhibiting cyclin-dependent kinases. Molecular Immunology 2007;44(4):488-93.
(83) Amin K, Ekberg-Jansson A, Lofdahl CG, Venge P. Relationship between inflammatory cells and structural changes in the lungs of asymptomatic and never smokers: a biopsy study. Thorax 58(2):135-42, 2003.
(84) Cosio MG, Majo J, Cosio MG. Inflammation of the airways and lung parenchyma in COPD: role of T cells. Chest 121(5 Suppl):160S-165S, 2002
(85) Chinn S, Jarvis D, Luczynska CM, ckermann-Liebrich U, Anto JM, Cerveri I, et al. An Increase in Bronchial Responsiveness Is Associated with Continuing or Restarting Smoking. Am J Respir Crit Care Med 2005;172(8):956-61.
(86) Cozen W, Diaz-Sanchez D, Gauderman JJ, Zadnick J, Cockburn MG, Gill PS, et al. Th1 and Th2 Cytokines and IgE levels in Identical Twins with Varying Levels in Identical Twins with Varying Levels of Cigarette Consumption. J Clin Immunol 2004;24:617-22.
(87) Melgert BN, Postma DS, Geerlings M, Luinge MA, Klok PA, van der Strate BW, et al. Short-term smoke exposure attenuates ovalbumin-induced airway inflammation in allergic mice. American Journal of Respiratory Cell & Molecular Biology 30(6):880-5, 2004 J
(88) Robbins CS, Dawe DE, Goncharova SI, Pouladi MA, Drannik AG, Swirski FK, et al. Cigarette smoke decreases pulmonary dendritic cells and impacts antiviral immune responsiveness. Am J Respir Cell Mol Biol 30(2):202-11, 2004
(89) Chalmers GW, MacLeod KJ, Thomson L, Little SA, McSharry C, Thomson NC. Smoking and airway inflammation in patients with mild asthma. Chest 120(6):1917-22, 2001
(90) Chaudhuri R, Livingston E, McMahon AD, Lafferty J, Fraser I, Spears M, et al. Effects of Smoking Cessation on Lung Function and Airway Inflammation in Smokers with Asthma. Am J Respir Crit Care Med 2006;174(2):127-33.
(91) McKay A, Komai-Koma M, MacLeod KJ, Campbell CC, Kitson SM, Chaudhuri R, et al. Interleukin-18 levels in induced sputum are reduced in asthmatic and normal smokers. Clin Exp Allergy 34(6):904-10, 2004
(92) Sunyer J, Springer G, Jamieson B, Conover C, Detels R, Rinaldo C, et al. Effects of asthma on cell components in peripheral blood among smokers and non-smokers. Clin Exp Allergy 33(11):1500-5, 2003
(93) Tsoumakidou M, Elston W, Zhu J, Wang Z, Gamble E, Siafakas NM, et al. Cigarette Smoking Alters Bronchial Mucosal Immunity in Asthma. Am J Respir Crit Care Med 2007 ;175(9):919-25.
(94) Vassallo R, Tamada K, Lau JS, Kroening PR, Chen L. Cigarette Smoke Extract Suppresses Human Dendritic Cell Function Leading to Preferential Induction of Th-2 Priming. J Immunol 2005;175(4):2684-91.

(95) Maestrelli P, Fabbri LM, Mapp C. Pathophysiology. In: Bernstein IL, Chan-Yeung M, Malo JL, Bernstein DI, editors. Asthma in the workplace.New York: Taylor & Francis; 2006. p. 121-9.
(96) Bentley AM, Maestrelli P, Saetta M, Fabbri LM, Robinson DS, Bradley BL, et al. Activated T-lymphocytes and eosinophils in the bronchial mucosa in isocyanate-induced asthma. Journal of Allergy & Clinical Immunology 89(4):821-9, 1992.
(97) Saetta M, Di SA, Maestrelli P, De MN, Milani GF, Pivirotto F, et al. Airway mucosal inflammation in occupational asthma induced by toluene diisocyanate. Am Rev Respir Dis145(1):160-8, 1992
(98) Turato G, Saetta M. Why does airway obstruction persist in asthma due to low-molecular-weight agents? A Pathologistis view. Occup Med 15(2):445-54, 2000
(99) Frew AJ, Chan H, Lam S, Chan-Yeung M. Bronchial inflammation in occupational asthma due to western red cedar. Am J Respir Crit Care Med 151(2 Pt 1):340-4, 1995
(100) Maestrelli P, Del Prete GF, De CM, D'Elios MM, Saetta M, Di SA, et al. CD8 T-cell clones producing interleukin-5 and interferon-gamma in bronchial mucosa of patients with asthma induced by toluene diisocyanate. Scand J Work Environ Health 1920;(5):376-81.
(101) Herrick CA, Das J, Xu L, Wisnewski AV, Redlich CA, Bottomly K. Differential roles for CD4 and CD8 T cells after diisocyanate sensitization: genetic control of TH2-induced lung inflammation. J Allergy Clin Immunol 111(5):1087-94, 2003
(102) Chan-Yeung M, Chan H, Tse KS, Salari H, Lam S. Histamine and leukotrienes release in bronchoalveolar fluid during plicatic acid-induced bronchoconstriction. J Allergy Clin Immunol 1989;84:762-8.
(103) Lemiere C, Pizzichini MM, Balkissoon R, Clelland L, Efthimiadis A, O'Shaughnessy D, et al. Diagnosing occupational asthma: use of induced sputum. Eur Respir J 13(3):482-8, 1999
(104) Lemiere C, Chaboillez S, Malo JL, Cartier A. Changes in sputum cell counts after exposure to occupational agents: what do they mean? J Allergy Clin Immunol 107(6):1063-8, 2001
(105) Lemiere C, Romeo P, Chaboillez S, Tremblay C, Malo JL. Airway inflammation and functional changes after exposure to different concentrations of isocyanates. J Allergy Clin Immunol 110(4):641-6, 2002
(106) Obata H, Dittrick M, Chan H, Chan-Yeung M. Sputum eosinophils and exhaled nitric oxide during late asthmatic reaction in patients with western red cedar. Eur Respir J 1999;13:489-95.
(107) Anees W, Huggins V, Pavord ID, Robertson AS, Burge PS. Occupational asthma due to low molecular weight agents: eosinophilic and non-eosinophilic variants. Thorax 57(3):231-6, 2002
(108) Park HS, Hwang SC, Nahm DH, Yim H. Immunohistochemical characterization of the Cellular infiltrate in airway Mucosa od Toluene Diisocyanate (TDI)-Induced Asthma: Comparison with Allergic Asthma. J Korean Med Sci 1998;13:21-6.
(109) Park HS, Jung KS, Hwang SC, Nahm DH, Yim HE. Neutrophil infiltration and release of IL-8 in airway mucosa from subjects with grain dust-induced occupational asthma. Clin Exp Allergy 1998 15;28(6):724-30.
(110) Di Franco A, Vagaggini B, Bacci E, Bartoli ML, Cianchetti S, Carnevali S, et al. Leukocyte counts in hypertonic saline-induced sputum in subjects with occupational asthma. Respir Med 1998;92(3):550-7.
(111) Jung KS, Park HS. Evidence for neutrophil activation in occupational asthma. Respirology 1999; 24;4(3):303-6.

(112) Lemiere C, Chaboilliez S, Trudeau C, Taha R, Maghni K, Martin JG, et al. Characterization of airway inflammation after repeated exposures to occupational agents. J Allergy Clin Immunol 106(6):1163-70, 2000
(113) Park, Jung, Kim, Nahm, Kang. Neutrophil activation following TDI bronchial challenges to the airway secretion from subjects with TDI-induced asthma. Clin Exp Allergy 1999; 27;29(10):1395-401.
(114) Gautrin D, Boulet LP, Boutet M, Dugas M, Bhèrer L, L'Archeveque J, et al. Is reactive airways dysfunction syndrome a variant of occupational asthma? J Allergy Clin Immunol 1994;93:12-22.
(115) Lemiere C, Malo JL, Boutet M. Reactive airways dysfunction syndrome due to chlorine: sequential bronchial biopsies and functional assessment. Eur Respir J 10(1):241-4, 1997
(116) Midttun O. Bronchial asthma in the aluminium industry. Acta Allergol 1960;15:208-21.
(117) Lund MB, Oksne PI, Hamre R, Kongerud J. Increased nitric oxide in exhaled air: an early marker of asthma in non-smoking aluminium potroom workers? Occup Environ Med 2000;57(4):274-8.
(118) Dacie J. Eosinophil counts by counting chamber method. In: Dacie J, Lewis SM, editors. Practical haematology.Edinburgh: Livingston; 1984. p. 42-3.
(119) American Thoracic Society - ATS Statement. Snowbird workshop on standardization of spirometry. Am Rev Respir Dis 1979;119:831-8.
(120) Gulsvik A. Obstructive lung disease in an urban population 1979. Thesis
(121) International consensus report on diagnosis and treatment of asthma. National Heart, Lung and Blood Institute, National Institutes of Health. Eur Respir J 1992;(5):601-41.
(122) Quanjer P, Tammeling G, Cotes J, Pedersen O, Peslin R, Yernault J. Lung volumes and forced ventilatory flows. report Working Party Standardization of Lung Function Tests, European Respiratory Society. Eur Respir J Suppl 1993;16:5-40.
(123) Kharitonov S, Alving K, Barnes P. Exhaled and nasal nitric oxide measurements; recommendations. Eur Respir J 1997;10:1683-93.
(124) Investigative use of bronchoschopy, lavage and bronchial biopsies in asthma and other airways diseases. Workshop summary and guildelines. Eur Respir J 1992;5:115-21.
(125) Ordonez C, Ferrando R, Hyde DM, Wong HH, Fahy JV. Epithelial Desquamation in Asthma . Artifact or Pathology? Am J Respir Crit Care Med 2000;162(6):2324-9.
(126) Valnes K, Brandtzaeg P. Comparison of paired immunofluorescence and paired immunoenzyme staining methods based on primary antisera from the same species. J Histochem Cytochem 1982;30(6):518-24.
(127) Halstensen TS, Scott H, Farstad IN, Michaelsen TE, Brandtzaeg P. In situ two- and three-color immunofluorescence staining of mucosal T-cells in celiac disease. Increase of TCR gamma/delta+CD8- and TCR alpha/beta+CD45R0+ intraepithelial lymphocytes and IL-2R+ TCR alpha/beta+CD4+CD45R0+ lamina propria lymphocytes. Prog Histochem Cytochem 1992;26:201-10.
(128) Jahnsen FL, Haraldsen G, Rugtveit J, Halstensen TS, Brandtzaeg P. Differential interference contrast microscopy combined with immunofluorescense: a new method to phenotype eosinophils in situ. J Immunol Methods 1994;173:77-91.
(129) Sullivan P, Stephens D, Ansari T, Costello J, Jeffery P. Variation in the measurements of basement membrane thickness and inflammatory cell number in bronchial biopsies. Eur Respir J12(4):811-5, 1998
(130) Gamble E, Grootendorst DC, Hattotuwa K, O'Shaughnessy T, Ram FSF, Qiu Y, et al. Airway mucosal inflammation in COPD is similar in smokers and ex-smokers: a pooled analysis. Eur Respir J 2007 ;30(3):467-71.

(131) Rutgers SR, Postma DS, ten Hacken NH, Kauffman HF, van der Mark TW, Koeter GH, et al. Ongoing airway inflammation in patients with COPD who do not currently smoke. Thorax 55(1):12-8, 2000
(132) Douwes J, Gibson P, Pekkanen J, Pearce N. Non-eosinophilic asthma: importance and possible mechanisms. Thorax 57(7):643-8, 2002 .
(133) Simpson JL, SCOTT R, Boyle MJ, Gibson PG. Inflammatory subtypes in asthma: Assessment and identification using induced sputum. Respirology 2006 ;11(1):54-61.
(134) Di Stefano A, Saetta M, Maestrelli P, Milani G, Pivirotto F, Mapp CE, et al. Mast cells in the airway mucosa and rapid development of occupational asthma induced by toluene diisocyanate. Am Rev Respir Dis 147(4):1005-9, 1993
(135) Djukanovic R, Wilson JW, Britten KM, Wilson SJ, Walls AF, Roche WR, et al. Quantitation of mast cells and eosinophils in the bronchial mucosa of symptomatic atopic asthmatics and healthy control subjects using immunohistochemistry. Am Rev Respir Dis142(4):863-71, 1990
(136) Saetta M, Maestrelli P, Di SA, De MN, Milani GF, Pivirotto F, et al. Effect of cessation of exposure to toluene diisocyanate (TDI) on bronchial mucosa of subjects with TDI-induced asthma. Am Rev Respir Dis 145(1):169-74, 1992
(137) Larche M. Regulatory T Cells in Allergy and Asthma. Chest 2007;132(3):1007-14.
(138) Umetsu DT, DeKruyff RH. The regulation of allergy and asthma. Immunological Reviews 2006;212(1):238-55.
(139) Hartl D, Koller B, Mehlhorn AT, Reinhardt D, Nicolai T, Schendel DJ, et al. Quantitative and functional impairment of pulmonary CD4+CD25hi regulatory T cells in pediatric asthma. J Allergy Clin Immunol 2007;119(5):1258-66.
(140) Wan YY, Flavell RA. Regulatory T-cell functions are subverted and converted owing to attenuated Foxp3 expression. Nature 2007;445(7129):766-70.
(141) Suto A, Nakajima H, Kagami SI, Suzuki K, Saito Y, Iwamoto I. Role of CD4+ CD25+ Regulatory T Cells in T Helper 2 Cell-mediated Allergic Inflammation in the Airways. Am J Respir Crit Care Med 2001;164(4):680-7.
(142) Floreani AA, Rennard SI. The role of cigarette smoke in the pathogenesis of asthma and as a trigger for acute symptoms. Curr Opin PulmMed 5(1):38-46, 1999
(143) Di SA, Caramori G, Ricciardolo FL, Capelli A, Adcock IM, Donner CF. Cellular and molecular mechanisms in chronic obstructive pulmonary disease: an overview. Clin Exp Allergy 34(8):1156-67, 2004
(144) Saetta M, Turato G, Maestrelli P, Mapp CE, Fabbri LM. Cellular and structural bases of chronic obstructive pulmonary disease. Am J Respir Crit Care Med163(6):1304-9, 2001
(145) Kharitonov SA, Robbins RA, Yates D, Keatings V, Barnes PJ. Acute and chronic effect of cigarette smoking on exhaled nitric oxide. Am J Respir Crit Care Med 1995;152(2):609-12.
(146) Verleden GM, Dupont LJ, Verpeut AC, Demedts MG. The Effect of Cigarette Smoking on Exhaled Nitric Oxide in Mild Steroid-Naive Asthmatics. Chest 1999 1;116(1):59-64.
(147) Sjaheim T, Halstensen TS, Lund MB, Bjortuft O, Drablos PA, Malterud D, et al. Airway inflammation in aluminium potroom asthma. Occup Environ Med 61(9):779-85, 2004
(148) Jeffery PK, Laitinen A, Venge P. Biopsy markers of airway inflammation and remodelling. Respiratory Med 94 Suppl F:S9-15, 2000
(149) Venables KM, Upton JL, Hawkins ER, Tee RD, Longbottom JL, Newman Taylor AJ. Smoking, atopy, and laboratory animal allergy. Br J Ind Med 45(10):667-71, 1988

(150) Venables KM, Dally MB, Nunn AJ, Stevens JF, Stephens R, Farrer N, et al. Smoking and occupational allergy in workers in a platinum refinery. BMJ 299(6705):939-42, 1989
(151) Mapp CE, Newman-Taylor AJ. Occupational asthma with latency (sensitizer-induced occupational asthma): factors predisposing to sensitization, development and persistence of symptoms. Proceedings of the first Jack Pepys Occupational Asthma Symposium. Am J Resir Crit Care Med 2003;167:454-6.
(152) Radon K, Nowak D, Szadkowski D. Lack of combined effects of exposure and smoking on respiratory health in aluminium potroom workers. Occup Environ Med 1999 ;56(7):468-72.
(153) Hillerdahl G, Rylander R. Asthma and cessation of smoking. Clin Allergy 14(1):45-7, 1984

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Blood Eosinophils in Workers With Aluminum Potroom Asthma Are Increased to Higher Levels in Non-Smokers Than in Smokers

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Background *Aluminum potroom asthma (PA) has been described in several reports. This study aimed to investigate the association between blood eosinophils and PA.*
Methods *In a cross-sectional study, 338 workers were examined as follows: spirometry, blood eosinophils, skin prick test, and work exposure measurements. They also completed a questionnaire on respiratory symptoms, smoking, allergy, and duration of work exposure.*
Results *The odds ratio for PA was 4.2 (95% confidence interval 1.5–9.7) for workers with eosinophils $\geq 400 \times 10^6$ cells/L compared with workers with eosinophils $< 200 \times 10^6$ cells/L. In non-smokers, the number of eosinophils was 177×10^6 cells/L ($P < 0.001$) higher among workers with PA than asymptomatic workers, whereas the corresponding difference among current smokers was only 63×10^6 cells/L ($P = 0.03$).*
Conclusions *The prevalence of PA was positively associated with blood eosinophils. An attenuation of the blood eosinophil increase was observed in smoking asthmatics, suggesting an immune-modulating effect of smoking.* Am. J. Ind. Med. 50:443–448, 2007.

KEY WORDS: occupational asthma; eosinophil inflammation; smoking; aluminum smelter

INTRODUCTION

Occupational asthma in aluminum potroom workers has been documented in both cross-sectional [Abramson et al., 1989; Kongerud et al., 1990] and longitudinal studies [Kongerud et al., 1990; Kongerud and Samuelsen, 1991], and is often referred to as potroom asthma (PA). Aluminum is produced in pots by electrolysis of alumina (Al_2O_3). Pot fume emissions consist of a complex mixture of dust and gases potentially harmful to the airways. The dust is mainly composed of alumina, aluminum trifluoride, and agglomerates of cryolite, providing a huge surface area on which gases such as hydrogen fluorides and sulfur dioxide are absorbed. The causal agent(s) of PA has or have not been identified, although fluoride compounds have been suggested by several authors [Abramson et al., 1989; Steinegger and Schlatter, 1992; Soyseth et al., 1994]. The pathogenetic mechanisms in PA remain unknown, and it is debated whether the condition is caused by an irritant response or a hypersensitivity response to agents in the working environment. The predictive value of bronchial hyperresponsiveness in PA is low [Kongerud and Soyseth, 1991], and the role of atopy is unclear [Saric et al., 1986; Kongerud et al., 1994].

Eosinophils are principally tissue-dwelling cells, but it is well recognized that asthma and asthma-like symptoms may be reflected by increased levels of blood eosinophils [Lewis

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et al., 2001]. The role of eosinophils in PA has not been studied in any detail. The blood eosinophil count has been shown to increase during asthmatic attacks in potroom workers [Midttun, 1960], and case reports of PA with blood eosinophilia have been described [Simonsson et al., 1985]. In addition, the occurrence of PA has been associated with high pre-employment blood eosinophil counts in a Dutch study [Sorgdrager et al., 1995].

Increased density of eosinophils has been reported in the bronchial mucosa of workers with diagnosed PA and, unexpectedly, the increase in eosinophils was far more pronounced in non-smokers than smokers [Sjaheim et al., 2004]. Accordingly, the present study aimed to investigate the association between PA and peripheral blood eosinophil counts, as well as the effect of cigarette smoking on this association.

MATERIALS AND METHODS

A cross-sectional study was carried out at Årdal aluminum smelter in western Norway in 1989. From a total workforce of 380 potroom workers, 338 subjects (89%) participated in the study. The characteristics of the participants are shown in Table I. All subjects completed a self-administered, validated questionnaire [Kongerud et al., 1989]. Information on respiratory symptoms (dyspnea, wheeze, and cough) within the last year, allergy, presence of familial asthma, asthma prior to potroom employment, smoking habits, years of employment in potrooms, and use of respiratory protection were recorded.

PA was defined as a combination of dyspnea and wheezing apart from colds during the past 12 months with improvement on rest days or during vacations, and absence of pre-employment asthma. Any other combination of respiratory symptoms was classified as Other Symptoms (OS). None of the asthmatics was treated with corticosteroids and 14/41 (34%) used an inhaled β_2-agonist. Smoking habits were classified as follows: never smokers were lifetime non-smokers; ex-smokers were those who had stopped smoking at least 1 year prior to the study, and all other subjects were classified as current smokers. The prevalence of current smoking was 63%, while 9% were ex-smokers, and 28% never smokers. The Regional Ethical Committee approved the study and informed consent was obtained from all participants.

Blood Eosinophil Count

Capillary blood was collected from all subjects and eosinophils were counted blindly by one observer (VS) using a Fuchs-Rosenthal chamber [Dacie, 1984]. The result was given as the mean of two parallel counts measured as number of cells per liter (L). Eosinophilia was defined as blood eosinophils $\geq 400 \times 10^6$ cells/L.

TABLE I. Characteristics of 338 Potroom Workers at Årdal Aluminum Smelter in Western Norway

			Respiratory symptoms present	
		No respiratory symptoms	Potroom asthma	Other symptoms
Workers	n (%)	248 (100)	39 (100)	51 (100)
Male	n (%)	215 (87)	36 (92)	44 (86)
Female	n (%)	33 (13)	3 (8)	7 (14)
Age, years	mean (SD)	36 (12)	35 (11)	36 (13)
Smoking status				
Never smokers	n (%)	74 (30)	7 (18)	14 (27)
Ex-smoker	n (%)	23 (9)	4 (10)	4 (8)
Current smoker	n (%)	151 (61)	28 (72)	33 (65)
Atopy				
Negative	n (%)	159 (64)	26 (67)	27 (53)
Positive	n (%)	50 (20)	6 (15)	14 (27)
Missing	n (%)	39 (16)	7 (18)	10 (20)
FEV_1, L	mean (SD)	4.0 (0.75)	4.1 (0.77)	3.8 (0.84)
FEV_1 % predicted	mean (SD)	92 (12)	89 (13)	87 (11)
Bronchial obstruction	n (%)	13 (5)	7 (18)	7 (14)
Total dust, mg/m^3	mean (SD)	1.51 (0.61)	1.49 (0.72)	1.41 (0.50)
Total fluorides, mg/m^3	mean (SD)	0.40 (0.13)	0.38 (0.14)	0.37 (0.10)
Length of employment, years	mean (SD)	13 (12)	12 (11)	10 (11)

SD, standard deviation; FEV_1, forced expiratory volume in 1 s. Bronchial obstruction: $FEV_1 < 80\%$ of predicted value and $FEV_1/FVC < 70\%$.

Skin Prick Test

Identification of atopy was by skin prick test and was performed in 282 subjects (83%). Registration of the wheal was carried out 15 min after application of dog epithelium, common silver birch, timothy grass, mugwort, and house dust mite, with saline and histamine as controls (skin prick test Phazet®, Pharmacia, Uppsala, Sweden). The wheal diameter was measured in millimeters as the mean of the long axis and its perpendicular axis, and those who had a positive reaction to at least one of the allergens tested were considered to have atopy. A wheal diameter ≥ 1 mm was defined as a positive reaction and <1 mm as a negative reaction. All subjects with a positive skin prick test were regarded as atopic.

Lung Function

Forced expiratory volume in 1 s (FEV_1) and forced vital capacity (FVC) were measured on a dry bellow spirometer (Jones Pulmonaire, Jones Medical Instruments Co., Oak Brook, Illinois) as previously described [Kongerud and Soyseth, 1991]. The prediction equations estimated from a general population in Norway were used to define levels as percentage of predicted [Gulsvik, 1979]. Bronchial obstruction was defined as an $FEV_1 < 80\%$ of the predicted value and an $FEV_1/FVC < 70\%$. Lung function was similar in non-smokers (median FEV_1 4.0 L (SD 0.8), 88% predicted) and smokers (4.1 L (SD 0.77), 92% predicted) with PA. However, a higher percentage of smoking asthmatics (6/28–21%) than non-smoking asthmatics (1/11–9%) were obstructive. Of the non-smoking asthmatics, none had an $FEV_1 < 70\%$ and three had an $FEV_1 < 80\%$, while among smoking asthmatics one subject had an $FEV_1 < 70\%$ and six had an $FEV_1 < 80\%$.

Work Exposure

Measurements of total airborne dust and total fluorides (fluorides in gas and particulate forms) were regularly performed using personal samplers. Sampling was carried out over 4–8 hr. The geometric mean of exposure was 1.49 mg/m^3 (SD 0.61) for total dust and 0.39 mg/m^3 (SD 0.12) for total fluorides. Norwegian hygienic standards are 5.00 mg/m^3 and 0.60 mg/m^3, respectively. Both Søderberg and prebake technology were used in the plant.

Statistical Analyses

The association between each outcome variable and its covariates was analyzed using univariate analyses. Thereafter, a multivariate model was constructed. The selection of covariates in the initial multivariate model was made as follows: all the covariates from the univariate analyses that were associated with the outcome having $P < 0.2$ were entered into the initial multivariate model. The full model was then reduced by backward elimination of each covariate with $P \geq 0.05$, unless the association between the outcome and any of the remaining covariates changed by a meaningful magnitude. Thus, the final model consisted of the covariates of interest, the covariates whose association with the outcome had a $P < 0.05$, and those covariates that were needed in order to control for confounding.

For the analyses of eosinophil count as the outcome variable, ordinary least square regression was used, whereas in the analyses of PA and OS as the dependent variable logistic regression was chosen. In order to investigate any effect modification of the relationship between symptoms and eosinophils by smoking, a product term between smoking and symptoms in addition to smoking and atopic status was included in the ordinary least square regression model. Similarly, a product term between smoking and eosinophils was included in the logistic regression model. Test for trend was assessed using eosinophil count as a continuous variable.

The univariate relationship between eosinophil counts as the dependent variable and each of the covariates was investigated separately. The following covariates were considered: age, gender, PA, OS, smoking, atopy, duration of employment, use of airway protection, total dust, total fluorides, FEV_1, and bronchial obstruction. In the univariate analyses with eosinophil cell count as the dependent variable, $P < 0.2$ was achieved for PA, OS, smoking, atopy, bronchial obstruction, and gender. These variables were therefore included in the initial multivariate model.

Univariate logistic regression analyses were performed separately for PA and OS using the following covariates: age, gender, smoking, duration of employment, use of airway protection, atopy, total dust, total fluorides, FEV_1, bronchial obstruction, and eosinophil counts. Separate multivariate models were developed for PA and OS. According to the criteria for model selection, current smoking, bronchial obstruction, and eosinophils were entered into the initial multivariate logistic model.

RESULTS

The association between respiratory symptoms and blood eosinophils stratified by smoking status is presented in Table II. Asymptomatic smokers had significantly higher eosinophil cell counts ($P = 0.005$, t-test) than asymptomatic non-smokers (mean 225×10^6/L vs. 180×10^6/L). In contrast, in subjects reporting PA or OS, this association was reversed, and the eosinophil cell counts were lower in smokers (mean 285×10^6/L) than in non-smokers (mean 364×10^6/L) with PA, though the difference was not significant ($P = 0.4$). Seventy-nine percent of the workers with PA had no blood eosinophilia, and the presence of

TABLE II. The Association Between Different Categories of Respiratory Symptoms and Eosinophil Cell Count in Peripheral Blood Among 338 Potroom Workers at Årdal Aluminum Smelter in Western Norway

	Eosinophils ($\times 10^6$/L)			Eosinophils
	<200	200–399	≥400	Mean (SD)
	N (%)	N (%)	N (%)	$\times 10^6$/L
No symptoms	140 (56)	87 (35)	21 (9)	207 (130)
Non-smokers	62 (64)	32 (33)	3 (3)	180 (114)
Smokers	78 (52)	55 (36)	18 (12)	225 (136)
Potroom Asthma	11 (28)	20 (51)	8 (21)	307 (176)
Non-smokers	3 (27)	4 (36)	4 (36)	364 (231)
Smokers	8 (29)	16 (57)	4 (14)	285 (147)
Other Symptoms	23 (45)	16 (31)	12 (24)	266 (187)
Non-smokers	8 (44)	5 (28)	5 (28)	300 (248)
Smokers	15 (45)	11 (33)	7 (21)	247 (145)
Total	174 (51)	123 (36)	41 (12)	228 (149)

eosinophilia was more frequent in non-smokers (36%) than in smokers (14%). PA was reported by 12% of non-atopic subjects and 9% of atopic subjects. In subjects with PA, there was no difference in eosinophil count ($P = 0.6$) between subjects who used an inhaled β_2-agonist and those who did not (mean 281×10^6/L vs. 320×10^6/L).

In the analyses with eosinophils as the dependent variable, the product terms OS × smoking and PA × smoking smoking were significant ($P = 0.03$ and 0.005, respectively). Similarly, the product term smoking × atopy status was significant ($P = 0.03$). Thus, we chose to perform the rest of the analyses using separate models for smokers and non-smokers. According to the criteria for model reduction, the final model included PA, OS, and atopy (Table III). These analyses showed that in non-smokers the mean eosinophil count was 177×10^6/L higher among workers with PA than asymptomatic workers ($P < 0.001$), whereas the corresponding difference among current smokers was only 63×10^6/L ($P = 0.03$). Similarly, the difference in eosinophil count between workers reporting OS and asymptomatic workers was 155×10^6/L ($P < 0.001$), and 17×10^6/L ($P = 0.5$) among non-smokers and current smokers, respectively. It also appeared that the difference in eosinophil cell count between atopics and non-atopics was greater in non-smokers than in smokers.

In the multivariate logistic regression, the product term between current smoking and eosinophils regarding PA and OS had a corresponding P-value of 0.08 and 0.1, respectively. Thus, the final model did not include these product terms in either of the two models. Model reduction was performed as outlined previously. Eosinophil cell count was divided into three categories. The final models for PA and OS included eosinophil count and bronchial obstruction. The relationship between PA and OS, respectively, and eosinophils is presented in Table IV. The table suggests that the prevalence of PA and OS increased with the number of blood eosinophils, and the association appeared to be strongest for PA. The odds ratios (95% confidence interval) for PA and OS were 3.4 (1.2–9.7) and 2.6 (1.0–7.1), respectively, in obstructive workers compared to non-obstructive workers.

TABLE III. The Difference in Eosinophil Cell Counts (10^6/L) Between Categories of Respiratory Symptoms and Atopic Status in 338 Potroom Workers at Årdal Aluminum Smelter in Western Norway, Using Multiple Regression Analyses Grouped by Smoking Status

	Non-smokers	Smokers
Potroom asthma versus no symptoms	177 (84–270)	63 (7–118)
Other symptoms versus no symptoms	155 (71–238)	17 (−33–67)
Atopy present: yes versus no	106 (35–177)	10 (−36–56)

95% confidence intervals in parentheses.

DISCUSSION

The main findings of this study were a positive association between the number of peripheral blood eosinophils and respiratory symptoms, and this association was stronger in workers with PA than in operators reporting OS. Further, this association was weaker in smokers than in non-smokers, even among the atopic subjects. Eosinophils have traditionally been associated with asthma and allergic disease, and eosinophilia of the blood and lung is common in both atopic and non-atopic asthma [Robinson et al., 2002]. In population-based studies, the number of blood eosinophils has been positively correlated to respiratory symptoms [Lewis et al., 2001]. In occupational asthma, an increased number of peripheral blood eosinophils has been found in subjects with occupational asthma due to low molecular weight agents [Lemiere et al., 1999]. There is, however, an increasing recognition of non-eosinophilic forms of asthma [Douwes et al., 2002], which is also observed in occupational asthma [Anees et al., 2002].

An association between blood eosinophils and PA has been demonstrated during PA attacks [Midttun, 1960;

TABLE IV. Odds Ratios for Potroom Asthma and Other Symptoms Controlled for Bronchial Obstruction in 338 Potroom Workers at Årdal Aluminum Smelter in Western Norway

	Eosinophils in peripheral blood (10^6/L)			
Symptom	<200	200–399	≥400	*P* for trend
Potroom asthma	1	2.9 (1.3–6.4)	4.2 (1.5–9.7)	0.002
Other symptoms	1	1.1 (0.6–2.2)	3.3 (1.4–7.7)	0.02

Sorgdrager et al., 1995]. However, blood eosinophils have not previously been investigated in an entire workforce of potroom workers. The present study confirms a positive association between PA and blood eosinophil counts, showing an increased prevalence of asthma in workers with blood eosinophilia. However, as the majority of workers with PA had no blood eosinophilia, this study also supports the view that PA is a heterogonous disorder with both eosinophilic and non-eosinophilic phenotypes. Although our study supports a role for the eosinophils in the pathogenesis of PA, it also suggests that an eosinophil-driven mechanism may not be the only underlying mechanism.

The reduced number of eosinophils in smokers compared to non-smokers with PA could in theory be explained by a lower prevalence of atopy in smokers. However, the prevalence of atopy was similar in smoking and non-smoking asthmatics, and atopy was included as a covariate in the analyses. Furthermore, we adjusted for atopy in the analyses, and a similar effect of smoking was found regarding atopy. There is no reason to believe that observer bias should have influenced the results. The samples were coded before eosinophil counting and the investigator had no information about smoking status, respiratory symptoms, or atopic status. Selective relocation of workers with PA can hardly explain why blood eosinophil counts were lower in smoking asthmatics than in non-smoking asthmatics. There is no apparent reason why smokers with PA and eosinophilia should leave the potrooms more frequently than non-smokers with PA and eosinophilia. The workers are relocated according to their respiratory symptoms only, without considering eosinophil counts. As none of the asthmatics was treated with corticosteroids, the reduced eosinophil counts in the smoking asthmatics could not be explained by anti-inflammatory treatment either. Only one of the smoking asthmatics had an $FEV_1 < 70\%$ of the predicted value (69%). Accordingly, it is not a major concern that subjects with chronic obstructive pulmonary disease (COPD) were misclassified as asthmatics.

In a general population, it has been shown that smoking raises peripheral blood leukocyte counts [Taylor et al., 1985]. Despite the fact that both respiratory symptoms and current smoking are separately positively associated with eosinophilia [Mensinga et al., 1990; Jansen et al., 1999], we found that the mean eosinophil count was lower in smokers than in non-smokers with respiratory symptoms.

These results might appear conflicting, but they agree with another study showing that smoking attenuates the increase in blood eosinophils in asthma, suggesting that smoking plays a different immunological role in asthmatics than in non-asthmatics [Sunyer et al., 2003]. Our results are further supported by the observation of reduced eosinophils in induced sputum in smoking asthmatics compared to non-smoking asthmatics [Chalmers et al., 2001]. Previously we have shown an immune-modulating effect of smoking on eosinophil density in the airway wall in potroom workers [Sjaheim et al., 2004], and the present study confirmed that this smoking effect is reflected in peripheral blood.

An anti-inflammatory effect of smoking is supported by several human and animal studies [Holt, 1987]. Nicotine has a potent inhibitory effect on the production of proinflammatory mediators [Ouyang et al., 2000; Wang et al., 2003]. In a mouse model of asthma, it has recently been demonstrated that a nicotinic agonist reduced the number of eosinophils in bronchoalveolar lavage fluid [Blanchet et al., 2005].

Previous studies have indicated that the level of exposure to potroom fumes and the duration of exposure are important determinants of PA [Kongerud and Samuelsen, 1991; Sorgdrager et al., 1998]. In contrast to these reports, we found no association between the number of blood eosinophils and the levels or duration of exposure. As a result of improvement in the aluminum production process, the exposure levels for both total fluorides and total dust were reduced by about 50% in the 1–2 years preceding this study [Soyseth et al., 1997]. We therefore believe that the difference between the highest and the lowest level of exposure was too small to give any meaningful contrast of exposure. In addition, several workers were relocated from the potrooms to clean working conditions due to respiratory symptoms. These factors may explain why we failed to reveal any association between exposure and eosinophil counts.

In summary, this study confirms a positive association between eosinophil numbers in peripheral blood and PA, indicating that aluminum potroom exposure may induce an eosinophilic airway inflammation in subjects with work-related dyspnea and wheezing. The data also support the existence of a non-eosinophilic phenotype of PA. The increase in blood eosinophils observed in non-smoking asthmatics appeared to be partly inhibited in the smoking asthmatics, suggesting an immune-modulating effect of cigarette smoking.

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REFERENCES

Abramson MJ, Wlodarczyk JH, Saunders NA, Hensley MJ. 1989. Does aluminium smelting cause lung disease? Am Rev Respir Dis 139:1042–1057.

Anees W, Huggins V, Pavord ID, Robertson AS, Burge PS. 2002. Occupational asthma due to low molecular weight agents: Eosinophilic and non-eosinophilic variants. Thorax 57:231–236.

Blanchet MR, Israël-Assayag E, Cormier Y. 2005. Modulation of airway inflammation and resistance in mice by a nicotinic receptor agonist. Eur Respir J 26:21–27.

Chalmers GW, MacLeod KJ, Thomson L, Little SA, McSharry C, Thomson NC. 2001. Smoking and airway inflammation in patients with mild asthma. Chest 120:1917–1922.

Dacie J. 1984. Eosinophil counts by counting chamber method. In: Dacie J, Lewis SM, editors. Practical haematology. Edinburgh: Livingstone, p 42–43.

Douwes J, Gibson P, Pekkanen J, Pearce N. 2002. Non-eosinophilic asthma: Importance and possible mechanisms. Thorax 57:643–648.

Gulsvik A. 1979. Obstructive lung disease in an urban population. Thesis. University of Oslo, Norway.

Holt PG. 1987. Immune and inflammatory function in cigarette smokers. Thorax 42:241–249.

Jansen DF, Schouten JP, Vonk JM, Rijcken B, Timens W, Kraan J, Weiss ST, Postma DS. 1999. Smoking and airway hyperresponsiveness especially in the presence of blood eosinophilia increase the risk to develop respiratory symptoms: A 25-year follow-up study in the general adult population. Am J Respir Crit Care Med 160:259–264.

Kongerud J, Samuelsen SO. 1991. A longitudinal study of respiratory symptoms in aluminium potroom workers. Am Rev Respir Dis 144:10–16.

Kongerud J, Soyseth V. 1991. Methacholine responsiveness, respiratory symptoms and pulmonary function in aluminium potroom workers. Eur Respir J 4:159–166.

Kongerud J, Vale JR, Aalen OO. 1989. Questionnaire reliability and validity for aluminium potroom workers. Scand J Work Environ Health 15:364–370.

Kongerud J, Gronnesby JK, Magnus P. 1990. Respiratory symptoms and lung function of aluminium potroom workers. Scand J Work Environ Health 16:270–277.

Kongerud J, Boe J, Soyseth V, Naalsund A, Magnus P. 1994. Aluminium potroom asthma: The Norwegian experience. Eur Respir J 7:165–172.

Lemiere C, Pizzichini MM, Balkissoon R, Clelland L, Efthimiadis A, O'Shaughnessy D, Dolovich J, Hargreave FE. 1999. Diagnosing occupational asthma: Use of induced sputum. Eur Respir J 13:482–488.

Lewis SA, Pavord ID, Stringer JR, Knox AJ, Weiss ST, Britton JR. 2001. The relation between peripheral blood leukocyte counts and respiratory symptoms, atopy, lung function, and airway responsiveness in adults. Chest 119:105–114.

Mensinga TT, Schouten JP, Rijcken B, Weiss ST, Speizer FE, van der LR. 1990. The relationship of eosinophilia and positive skin test reactivity to respiratory symptom prevalence in a community-based population study. J Allergy Clin Immunol 86:99–107.

Midtun O. 1960. Bronchial asthma in the aluminium industry. Acta Allergol 15:208–221.

Ouyang Y, Virasch N, Hao P, Aubrey MT, Mukerjee N, Bierer BE, Freed BM. 2000. Suppression of human IL-1beta, IL-2, IFN-gamma, and TNF-alpha production by cigarette smoke extracts. J Allergy Clin Immunol 106:280–287.

Robinson DS, Kay AB, Wardlaw AJ. 2002. Eosinophils. Clin Allergy Immunol 16:43–75.

Saric M, Godnic-Cvar J, Gomzi M, Stilinovic L. 1986. The role of atopy in potroom workers' asthma. Am J Ind Med 9:239–242.

Simonsson BG, Sjoberg A, Rolf C, Haeger-Aronsen B. 1985. Acute and long-term airway hyperreactivity in aluminium-salt exposed workers with nocturnal asthma. Eur J Respir Dis 66:105–118.

Sjaheim T, Halstensen TS, Lund MB, Bjortuft O, Drablos PA, Malterud D, Kongerud J. 2004. Airway inflammation in aluminium potroom asthma. Occup Environ Med 61:779–785.

Sorgdrager B, Pal TM, de Looff AJ, Dubois AE, de Monchy JG. 1995. Occupational asthma in aluminium potroom workers related to pre-employment eosinophil count. Eur Respir J 8:1520–1524.

Sorgdrager B, de Looff AJ, de Monchy JG, Pal TM, Dubois AE, Rijcken B. 1998. Occurrence of occupational asthma in aluminium potroom workers in relation to preventive measures. Int Arch Occup Environ Health 71:53–59.

Soyseth V, Kongerud J, Ekstrand J, Boe J. 1994. Relation between exposure to fluoride and bronchial responsiveness in aluminium potroom workers with work-related asthma-like symptoms. Thorax 49:984–989.

Soyseth V, Boe J, Kongerud J. 1997. Relation between decline in FEV1 and exposure to dust and tobacco smoke in aluminium potroom workers. Occup Environ Med 54:27–31.

Steinegger AF, Schlatter C. 1992. Evaluation of fluoride exposure in aluminium smelters: State of the art. Med Lav 83:489–498.

Sunyer J, Springer G, Jamieson B, Conover C, Detels R, Rinaldo C, Margolick J, Munoz A. 2003. Effects of asthma on cell components in peripheral blood among smokers and non-smokers. Clin Exp Allergy 33:1500–1505.

Taylor RG, Gross E, Joyce H, Holland F, Pride NB. 1985. Smoking, allergy, and the differential white blood cell count. Thorax 40:17–22.

Wang H, Yu M, Ochani M, Amella CA, Tanovic M, Susarla S, Li JH, Wang H, Yang H, Ulloa L, Al-Abed Y, Czura CJ, Tracey KJ. 2003. Nicotinic acetylcholine receptor alpha7 subunit is an essential regulator of inflammation. Nature 421:384–388.

II

ORIGINAL ARTICLE

Airway inflammation in aluminium potroom asthma

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Aims: To examine whether asthma induced by exposure to aluminium potroom emissions (potroom asthma) is associated with inflammatory changes in the airways.
Methods: Bronchial biopsy specimens from 20 asthmatic workers (8 non-smokers and 12 smokers), 15 healthy workers (8 non-smokers and 7 smokers), and 10 non-exposed controls (all non-smokers) were analysed. Immunohistofluorescent staining was performed to identify mucosal total leucocytes ($CD45^+$ leucocytes), neutrophils, and mast cells.
Results: Median RBM thickness was significantly increased in both asthmatic workers (8.2 μm) and healthy workers (7.4 μm) compared to non-exposed controls (6.7 μm). Non-smoking asthmatic workers had significantly increased median density of lamina propria $CD45^+$ leucocytes (1519 cells/mm^2 v 660 and 887 cells/mm^2) and eosinophils (27 cells/mm^2 v 10 and 3 cells/mm^2) and significantly increased concentrations of exhaled NO (18.1 ppb v 6.5 and 5.1 ppb) compared to non-smoking healthy workers and non-exposed controls. Leucocyte counts and exhaled NO concentrations varied with smoking habits and fewer leucocytes were observed in asthmatic smokers than in non-smokers Asthmatic smokers had significantly increased numbers of eosinophils in lamina propria compared to non-exposed controls (10 v 3 cells/mm^2). Both eosinophilic and non-eosinophilic phenotypes of asthma were recognised in the potroom workers and signs of airway inflammation were also observed in healthy workers.
Conclusions: Airway inflammation is a central feature of potroom asthma and exposure to potroom emissions induces pathological alterations similar to those described in other types of asthma. Cigarette smoking seems to affect the underlying mechanisms involved in asthma, as the cellular composition of airway mucosa appears different in asthmatic smokers and non-smokers.

The occurrence of work related asthmatic symptoms with airflow limitation in aluminium potroom workers, referred to as potroom asthma, has been documented in cross-sectional[1 2] and longitudinal studies.[1 3] Potroom asthma has been objectively confirmed by characteristic work related decreases in peak expiratory flow (PEF) measurements,[4 5] and late asthmatic responses assessed by serial recording of forced expiratory volume in one second (FEV_1).[3] The reported incidence of potroom asthma varies from 0.06% to 4% of exposed workers per year. The variation in occurrence may partly be attributed to different definitions of the condition.[1] The annual incidence in Norway has been about 1.5% in recent years and potroom asthma continues to represent an important health problem in Norwegian aluminium production.

Potroom workers are exposed to a complex mixture of particulates and gases. The respirable particles of the pot fume emissions are mainly composed of aluminium oxide, carbon dust, and cryolit (a fluorinated compound of sodium and aluminium), to which gases such as hydrogen fluoride and sulphur dioxide are absorbed. As the concentrations of several pollutants are correlated to each other, it has been difficult to identify the causal agent of potroom asthma, although a number of authors have suggested fluoride compounds to be the major candidate.[1 6 7]

Potroom asthma develops after a symptom-free period (latency) varying from weeks to years after first exposure and the most common clinical presentation is a late asthmatic reaction occurring a few hours after work. The pathogenetic mechanisms in potroom asthma remain unknown. Specific immunological reactions or an irritant effect have been suggested and there is evidence for the presence of both.[8 9]

A potential inflammatory response caused by exposure to potroom contaminants has been examined in a few studies; blood eosinophil counts increase during asthmatic attacks in potroom workers[10] and increased pre-employment blood eosinophil levels are related to the occurrence of potroom asthma.[11] Although bronchial responsiveness is positively associated with potroom exposure,[12 13] the predicting value for potroom asthma is low.[14] Healthy subjects exposed to hydrogen fluorides in concentrations similar to those regularly measured in aluminium smelters have increased numbers of lymphocytes in bronchoalveolar lavage fluid.[15] In addition, both asthmatic and symptom-free potroom workers have increased concentrations of exhaled NO.[16]

These previous studies suggest that exposure to potroom contamination may induce airway inflammation and support the hypothesis that potroom asthma is an inflammatory disorder. However, examination of bronchial biopsy specimens, which is regarded as gold standard to assess airway inflammation, is lacking. We aimed to examine the presence of airway inflammation in asthmatic potroom workers by investigating bronchial biopsies and exhaled NO in subjects diagnosed as having occupational potroom asthma.

METHODS

Subjects

Potroom workers with occupational asthma (eight non-smokers and 12 smokers), healthy potroom workers (eight non-smokers and seven smokers), and 10 healthy non-exposed controls (all non-smokers) were included. All asthmatic workers who had not left the plant were identified by the company physicians and were examined by the main investigator (TS) who confirmed the diagnosis of potroom asthma. From a total asthmatic population of 39 workers, 18 subjects were excluded for different reasons (lack of

Abbreviations: DIC, differential interference contrast; FEV_1, forced expiratory volume in one second; FVC, forced vital capacity; NO, nitric oxide; RBM, reticular basement membrane; PEF, peak expiratory flow

Main messages

- Workers with potroom asthma develop chronic bronchial inflammation.
- Non-smoking potroom asthmatics have similar inflammatory changes in airway mucosa as conventional asthma, shown by thickened RBM, increased leucocyte and eosinophil influx, and increased exhaled NO.
- Smoking potroom asthmatics have thickened RBM but lower leucocyte density than asthmatic non-smokers, suggesting an immunomodulating effect of tobacco smoking.

Policy implications

- Early diagnosis of potroom asthma and relocation is essential to prevent irreversible histopathological changes.

reversible airway obstruction, atopy, childhood asthma, exercise induced asthma, frequent airway infections, FEV_1 <70% of predicted value, cardiac disease).

Of the 21 asthmatic workers fitting the inclusion criteria, only one was not included because he did not want to undergo a bronchoscopic examination.

The diagnosis of potroom asthma was made by confirming the diagnosis of bronchial asthma according to international guidelines[17] and by establishing a causal relation between asthma and the working environment, meeting the criteria for potroom asthma as previously defined.[1 11] Serial monitoring of PEF was performed at work and away from work. The clinical criteria were: initially asymptomatic exposure period (latency), symptoms of airway obstruction (dyspnoea, wheezing, and cough), usually occurring several hours after exposure or during sleep, and improvement of the symptoms after absence from work for several days or longer. Reversible airway obstruction defined as >15% increase in FEV_1 after inhalation of β_2 agonist and diurnal PEF variability >15% was documented in all asthmatic subjects. If work aggravation of underlying asthma was suspected, the subject was excluded. About half of the asthmatic workers were still working in the potrooms, and those relocated were working in non-polluted environments at the smelter. The relocated workers had persistent asthmatic symptoms, although all subjects reported gradual improvement of symptoms after withdrawal from exposure. The healthy workers and non-exposed controls had no symptoms from upper or lower airways and their lung function was normal. The 15 healthy workers were recruited by advertising the need for volunteers at one smelter employing about 440 pot operators. They were included successively as they responded and were comparable in age and smoking habits to the asthmatic workers. The 10 non-exposed controls were recruited from a normal healthy never-smoking population, responding to a request for volunteers at the University of Oslo. None of the participants had a history of allergy, familiar asthma, or childhood asthma and their total IgE levels were within the normal range. They had been free from respiratory tract infections for at least six weeks before investigations.

Table 1 shows the characteristics of the study population. Ex-smokers (quitting >1 year) and never-smokers were all classified as non-smokers. Five of the workers (two of the asthmatics and three of the healthy workers) were ex-smokers. Median smoking load was 13 pack-years for the asthmatics and 17 pack-years for the healthy workers. The Regional Committee for Medical Research Ethics approved the study and informed written consent was obtained from each subject.

Study design

The workers were recruited from five Norwegian aluminium smelters during the period 1998–2002. Clinical examination and recording of medical and occupational history was performed by a physician (TS); the subjects underwent spirometry, chest radiography, electrocardiography, blood tests, and measurement of exhaled NO the day before bronchoscopy with bronchial biopsy.

Exposure

In the Norwegian aluminium industry measurements of total airborne fluorides and dust are regularly performed from personal samplers. The mean levels of exposure are mostly far below the Norwegian threshold limit value (total fluorides 0.6 mg/m^3 and total particulates 5 mg/m^3), but peak exposures frequently occur. About 40% of the measurements for fluorides and 10% of the measurements for particulates are reported to exceed the hygienic limit values. In the present study, levels of exposure are not estimated for the individual worker, and only duration of exposure is recorded.

Measurement of lung function and exhaled NO

Spirometry was performed with a pneumotachograph (Vitalograph, Birmingham, UK) in accordance with the guidelines recommended by European Respiratory Society;[18] the reference values of European Coal and Steel Community were used.[18] Exhaled NO was measured by a chemiluminescence analyser (LR 2000, Logan Research, UK) at a sampling rate of 250 ml/min as previously described.[16]

Bronchoscopy and processing of bronchial biopsy specimens

Fibreoptic bronchoscopy and biopsy sampling was performed following the guidelines from the European Society of Pneumonology.[19] All subjects were premedicated with atropine 0.6 mg subcutaneously, 5 mg diazepam orally, and alfentanil intravenously as needed (0–2 mg) for mild sedation and analgesic. Under local anaesthesia with lidocaine, a bronchoscope with working channel 2.8 mm (Pentax FB-19H or Olympus 20D IT) was used to obtain a maximum of six bronchial biopsy specimens from the second and third generation carinae of the right lung by a single use forceps (Microvasive 1267, Radial Jaw, Boston Scientific). The biopsy specimens were immediately embedded in Tissue-Tek Optimal Cutting Temperature Compound (Miles Laboratories, IN), snap frozen in isophentane precooled in liquid nitrogen, and stored at −70°C. Cryo sections were cut serially at 4 µm, dried overnight, and stored at −20°C until use.

Immunohistochemistry

From a lower lobe biopsy, two sections of 100 µm interval were prepared for immunohistofluorescence examination using antibodies (all Dako A/S, Denmark) against total common leucocytes CD45 (catalogue number M701), mast cell tryptase (M7052), and neutrophil elastase (M752). The primary antibodies were applied at optimal dilutions and incubated one hour at room temperature. The sections were prefixed for 10 minutes at 4°C in 2% paraformaldehyde for CD45 staining and in 4% paraformaldehyde for mast cell and neutrophil staining. Goat serum 5% was then applied for 15 minutes to block unspecific binding sites. The secondary layer was biotinylated goat anti-mouse

Table 1 Characteristics of the study population

	Asthmatic workers (n=20)		Healthy workers (n=15)		Non-exposed controls (n=10)
	Non-smokers (n=8)	Smokers (n=12)	Non-smokers (n=8)	Smokers (n=7)	Non-smokers (n=10)
Age (years)*	35 (27–49)	40 (32–59)	36 (31–58)	43 (32–49)	24 (21–44)
FEV_1 % pred*	91 (75–120)	90 (73–111)	108 (90–135)	105 (85–124)	112 (81–124)
FEV_1/FVC %*	78 (61–82)	67 (59–79)	80 (71–87)	75 (71–84)	81 (74–90)
Inhaled corticosteroids (n)	1	3	0	0	0
Inhaled β_2 agonist (n)	4	6	0	0	0
Duration of exposure (years)*	14 (4–28)	11 (8–25)	14 (13–25)	19 (13–24)	0
Duration of symptoms (years)*	10 (1–22)	11 (1–18)	–	–	–
Latency period (years)*	7 (1–13)	7 (0.5–13)	–	–	–
Relocated subjects (n)	3	7	–	–	–

*Data are presented as median (range).
FEV_1, forced expiratory volume in 1 second; % pred, percentage of predicted value; FVC, forced vital capacity.

immunoglobulin (Dako A/S) incubated for 1.5 hours followed by 0.5 hour incubation with Streptavidin Alexa 594 conjugate (Molecular Probes, Nederlands) mixed with DNA staining to visualise the nucleus (4,6-diamino-2-phenylinodole (DAPI) Molecular Probes). Figure 1 shows an example of immunohistofluorescent staining of CD45$^+$ leucocytes. Methodological controls included sections stained without primary antibody and sections incubated with non-immune mouse immunoglobulin. The adjacent slides were stained with haematoxylin and eosin for examination of eosinophils.

Figure 1 Immunofluorescent staining of CD45$^+$ leucocytes (red) combined with 2,6-diamino-2-phenylinodole dihydrochloride (DAPI) to visualise the nucleus (blue) in a bronchial biopsy specimen from a non-smoking subject with potroom asthma. Positively stained cells are seen in both the epithelium and lamina propria. Original magnification × 630. Internal scale bar = 50μm. (Pseudocolour multilayer image superimposed on differential interference contrast image)

Quantitation of leucocytes

All slides were analysed blind by one observer (TS) using a Zeiss Axioplane2 microscope at 630× magnification. Eosinophils were identified on haematoxylin and eosin stained slides examined by light microscopy in combination with differential interference contrast (DIC) microscopy. This method for identification of eosinophils has been shown to be more reliable in cryo sections than immunohistochemical labelling of eosinophil granule proteins.[20] In cases of doubt, we additionally used the eosin fluorescence to localise the cell. Positively stained cells were counted in intact epithelium (defined as the presence of both basal and columnar cells) and in a tissue zone 114 μm beneath the RBM, referred to as lamina propria. All available area was analysed. The final result, expressed as number of intraepithelial cells per millimetre of intact epithelium or number of cells per square millimetre of lamina propria, was calculated as the average of all the measurements performed of each section. The median length of intact epithelium examined in a subject was 5 mm (range 2–16 mm) and the median area of lamina propria examined was 0.87 mm^2 (range 0.36–2.24 mm^2), corresponding to a basement membrane length of 6.2 mm (2.4–16.0 mm), as recommended.[21]

Measurements of RBM

By use of DIC microscopy combined with light microscopy, a digital captured high power image was used to measure the distance from the base of the bronchial epithelium to the outer limit of the RBM by AnalySIS Soft Imaging System, as illustrated in fig 2. Only perpendicular cut sections were examined and a median of 68 measurements (range 37–161) were performed on each section at approximately 20 μm intervals, as recommended.[21]

Epithelial integrity

The degree of epithelial damage was expressed as epithelial integrity, defined as the length of basal membrane covered with intact epithelium divided by the total length of the membrane. By light microscopy examination (haematoxylin and eosin stained slides and 630× magnification), a test grid (eyepiece reticule) was superimposed on the section, and the length of basal membrane with and without intact epithelium was recorded. A median of 31 grids were analysed for each subject, corresponding to a median length of basement membrane of 6.2 mm.

Statistics

Results are presented as median (range) values. Differences between two groups were compared by the Mann-Whitney U test and correlation coefficients were calculated using Spearman's rank method. To control for potential confounders

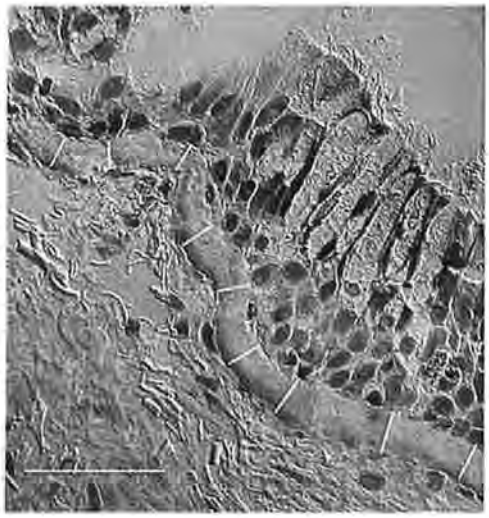

Figure 2 Examination of reticular basement membrane thickness performed on a haematoxylin and eosin stained slide combined with differential interference microscopy in a bronchial biopsy specimen from a patient with potroom asthma. Measurements are shown as white lines perpendicular to the reticular basement membrane. Original magnification × 630. Internal scale bar = 50 μm.

(age and smoking habits), analysis of variance was performed for all results. A p value <0.05 was regarded as significant.

RESULTS

Leucocyte density in lamina propria, RBM thickness, epithelial integrity, and exhaled NO concentrations are shown in table 2 and the variables are shown as individual scatter plots in fig 3. The results varied with smoking habits and are presented for non-smokers and smokers separately. The non-exposed control group was younger than the other two groups, and the results were controlled for age. The only outcome parameter that varied with age was neutrophils, and a reduction in lamina propria neutrophils was seen with increasing age for all groups ($p = 0.01$).

Leucocyte density in lamina propria

Asthmatic non-smokers had significantly higher density of $CD45^+$ leucocytes (fig 3A) and eosinophils (fig 3B) compared to both non-smoking healthy workers and non-exposed controls (table 2). In contrast, a rather low density of leucocytes was observed in asthmatic smokers, in whom the densities of $CD45^+$ leucocytes, eosinophils, and neutrophils were significantly reduced compared to asthmatic non-smokers ($p < 0.001$, $p = 0.05$, and $p = 0.04$ respectively). The difference in mast cells did not reach the level of significance. Healthy workers (both non-smoking and smoking subgroup) had a significantly increased density of eosinophils compared to non-exposed controls ($p = 0.04$ and $p = 0.01$).

Classifying subjects with eosinophil density above the upper range seen in non-exposed controls (0–15 cells/mm^2) as eosinophil(+), 50% of the asthmatics and 40% of the healthy workers were eosinophil(+) subjects. This cut-off value coincides with two standard deviations of the mean value in non-exposed controls.

Intraepithelial cell counts

The number of intraepithelial $CD45^+$ leucocytes was significantly increased in both asthmatic (22 cells/mm epithelium) and healthy smokers (35 cells/mm epithelium) compared to the non-smoking groups. Within the non-smoking groups there were no difference (varying from 8 to 11 cells/mm epithelium). Intraepithelial mast cells were significantly increased in asthmatic non-smokers compared to non-exposed controls (0.9 *v* 0.2 cells/mm epithelium, $p = 0.03$).

RBM

RBM thickness did not vary with smoking habits. The RBM was significantly thickened in both asthmatics (median 8.2 μm, range 5.9–12.5 μm) and healthy workers (median 7.4 μm, range 5.6–9.0 μm) compared to non-exposed controls (median 6.7 μm, range 6.0–7.6 μm) ($p = 0.002$ and $p = 0.04$ respectively, fig 3C). The difference in RBM thickness between asthmatic and healthy workers was 0.8 μm ($p = 0.055$).

Epithelial integrity

In non-smokers, we found no significant difference in epithelial integrity between asthmatics and controls (table 2). Epithelial integrity was significantly increased in asthmatic smokers compared to asthmatic non-smokers ($p = 0.03$), but no difference was found between asthmatic

Table 2 Leucocyte density in lamina propria, reticular basement membrane thickness, epithelial integrity, and exhaled nitric oxide*

	Asthmatic workers (n = 20)		Healthy workers (n = 15)		Non-exposed controls (n = 10)
	Non-smokers (n = 8)	Smokers (n = 12)	Non-smokers (n = 8)	Smokers (n = 7)	Non-smokers (n = 10)
$CD45^+$ leucocytes	1519 (720–1917)†§	675 (452–914)	660 (415–1289)	897 (503–1871)	887 (566–1595)
Eosinophils	27 (6–164)†§	10 (0–41)	10 (3–24)‡	33 (2–193)	3 (0–15)
Mast cells	116 (66–161)	78 (29–141)	93 (43–169)	105 (34–199)	89 (30–118)
Neutrophils	83 (46–162)§	42 (13–93)	45 (18–110)	56 (23–98)	84 (29–175)
RBM (μm)	8.2 (7.5–10.2)†	8.0 (5.9–12.5)	7.2 (5.6–8.7)	7.6 (6.8–9.0)‡	6.7 (6.0–7.6)
Epithelial integrity (%)	52 (0–90)§	70 (51–96)	41 (18–93)	67 (38–97)	42 (18–70)
Exhaled NO (ppb)	18.1 (6.3–91.1)†§	4.4 (1.2–80.0)	6.5 (3.1–22.8)	4.1 (1.6–20.3)	5.1 (3.5–8.4)

RBM, reticular basement membrane; ppb, parts per billion.
Cell counts are expressed as cells/mm^2.
*Data are expressed as median (range).
†$p < 0.05$ *v* non-smoking healthy workers and non-exposed controls.
‡$p < 0.05$ *v* non-exposed controls.
§$p < 0.05$ *v* smoking asthmatic workers.

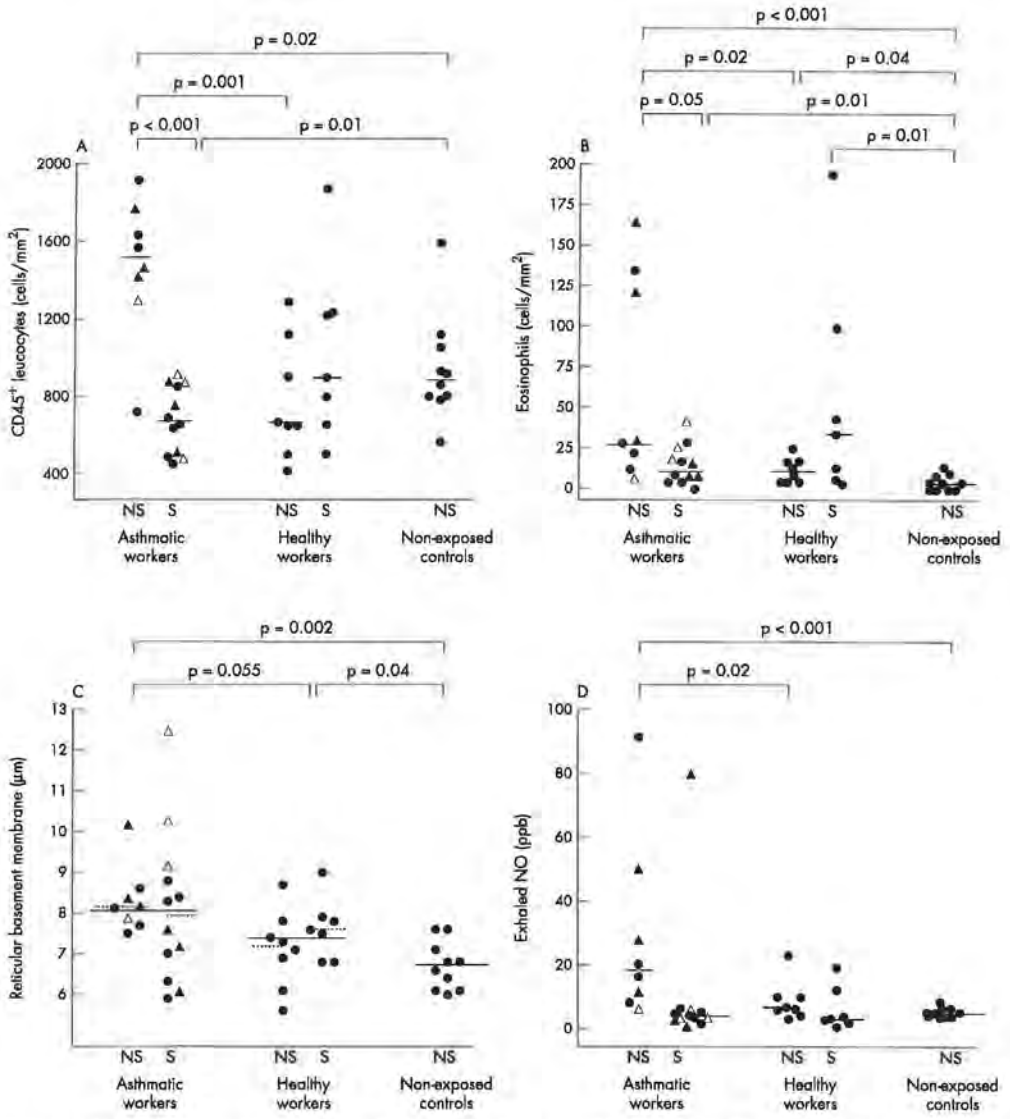

Figure 3 Individual leucocyte counts in lamina propria (A: CD45+ leucocytes; B: eosinophils), RBM thickness (C), and exhaled nitric oxide (D) in non-smoking (NS) and smoking (S) asthmatic potroom workers, healthy potroom workers, and non-exposed controls. Data are expressed as number of cells per square millimetre of tissue. Horizontal bars represent median values. Dark triangles indicate treatment with β_2 agonists and open triangles indicate treatment with inhaled corticosteroids.

smokers and the smoking healthy workers. In the damaged area, the ciliated epithelial cells were often absent while the layer of cuboidal basal cells was intact.

Exhaled NO

In non-smoking subjects, the concentrations of exhaled NO were significantly higher in asthmatic workers than in both healthy workers and non-exposed controls (fig 3D). Exhaled NO was, as expected, low in smokers, and NO values in asthmatic smokers did not differ from those in control smokers. In non-smoking asthmatic workers, exhaled NO correlated to the density of lamina propria CD45+ leucocytes ($r = 0.81$, $p = 0.02$) and eosinophils ($r = 0.79$, $p = 0.02$).

Exposure and outcome variables

We found no association between number of years employed in the potrooms and any of the outcome parameters. There was no difference between asthmatic workers who were still exposed in the potrooms and those who were relocated to non-polluted working environments.

DISCUSSION

This study reveals the presence of airway inflammation in subjects with potroom asthma, shown by significantly increased density of lamina propria CD45$^+$ leucocytes and eosinophils, significantly increased numbers of intraepithelial mast cells, thickening of RBM, and increased exhaled NO in asthmatic non-smokers. In addition, a subclinical inflammation, shown by significantly increased lamina propria eosinophils and RBM thickening, was observed in healthy potroom workers when compared to non-exposed controls.

Similar results have been reported in earlier studies of non-smokers with occupational asthma induced by low-molecular weight compounds such as isocyanates[23] and plicatic acid in western red cedar[25] as well as non-occupational asthma.[24 25] Earlier studies in aluminium smelters indicate that eosinophils contribute to the pathophysiology of potroom asthma and we could confirm the presence of airway mucosal eosinophilia. However, about half of the asthmatic workers had eosinophil counts in the range observed for non-exposed controls. These findings are in line with the increasing recognition of non-eosinophilic forms of asthma,[26 27] also observed in other forms of occupational asthma.[28] Our data did not support the hypothesis that non-eosinophilic asthma is a neutrophil mediated inflammation[27 29] as we found no difference in neutrophil density between eosinophilic and non-eosinophilic groups. Neutrophil involvement in the pathogenesis of occupational asthma has been documented in asthma induced by isocyanates[30] and grain-dust,[31] but in contrast to these studies, we were unable to show increased density of neutrophils in the airway wall in our asthmatics.

The number of intraepithelial mast cells was significantly increased in non-smoking asthmatics, but no difference in lamina propria mast cells was found between the groups. This is somewhat surprising, since mast cells are regarded as an important effectors cell of the asthmatic inflammation.[32] However, in previous studies the number of mast cells tended to vary.[32-34] Our results are in line with a study by Saetta *et al* who found mast cells numbers to be increased only in the epithelium.[32] In another study, increased numbers of mast cells were found in both the epithelium and lamina propria in subjects who developed asthma after a short time (median 2 years) of exposure to isocyanates.[33] The latency period in our study was longer (median 7 years, range 0.5-13 years), suggesting that mast cells might be associated with individual susceptibility to exposure.

Loss of epithelial integrity is commonly found in asthmatics, although this finding is inconsistently reported.[35 36] Our results in non-smoking asthmatics agree with previous studies.[36] We found, as have other researchers[37] that it is difficult to obtain biopsy samples from normal subjects. Hence, we hypothesise that mechanical biopsy induced damage to the bronchial epithelium is more likely to occur in healthy subjects and can explain the higher epithelial damage found in our controls. Thus, in vivo damage can possibly not be distinguished from biopsy induced, artifactual damage in our study. We also found that biopsy specimens were most easily taken from smokers.

Wide variations exist in the reported ranges of RBM thickness, in both asthmatic and control airways,[38] and may partly be due to different tissue handling and measurement techniques. Compared to a study with similar biopsy processing,[39] RBM in the present study appear thinner in the asthmatics and thicker in the controls. Although the difference between the groups appears to be smaller, it is significant. We have performed a higher number of measurements than in most other studies and found that the RBM thickness shows great variations within one section, even in perpendicular cut areas.

The finding of increased exhaled NO concentrations in non-smoking workers with potroom asthma is in accordance with a preceding study by Lund and colleagues.[16] In addition we found a positive correlation between exhaled NO concentrations and the density of lamina propria CD45$^+$ leucocytes and eosinophils in subjects with potroom asthma. Similar correlations were not observed in the healthy workers.

The observed significant increase in some inflammatory markers in healthy potroom workers is an interesting finding. Compared to non-exposed controls, the healthy potroom workers had a higher density of lamina propria eosinophils and thicker RBM, and their exhaled NO tended to be higher. These findings suggest that exposure to pollutants in aluminium smelters may affect the bronchial mucosa and induce a subclinical airway inflammation, even in healthy asymptomatic workers.

An unexpected finding was the impact of smoking in asthmatics. Asthmatic smokers had lower leucocyte density than asthmatic non-smokers. A higher proportion of asthmatic smokers were treated with inhaled steroids (3/12 of asthmatic smokers *v* 1/8 of asthmatic non-smokers). However, the difference in leucocyte density and exhaled NO values between the two asthma groups cannot be explained by anti-inflammatory treatment, as excluding the four subjects on inhaled corticosteroids from our analyses did not change the overall results.

A misclassification of asthma also seems unlikely. The smokers who were diagnosed as asthmatics had symptoms, PEF measurements, and reversibility consistent with potroom asthma and not chronic obstructive lung disease, and their median FEV_1 was comparable to that in non-smoking asthmatics (90% *v* 91% of predicted value). Two of the asthmatic workers and three of the healthy workers were ex-smokers. (The asthmatics had smoked respectively 8 and 20 pack-years and had stopped smoking 3 and 13 years ago. The healthy workers had smoked 2, 5, and 11 pack-years and had stopped respectively 14, 15, and 6 years ago.) No studies are available to clarify a potential effect of cigarette smoking on inflammatory parameters in the airways of previous smokers. Excluding the five ex-smokers from the analyses did not change the main results, but the sample size might be too small to show a hangover effect of smoking.

Studies of asthmatic inflammation in humans have been limited to non-smokers, and to our knowledge there is no published biopsy study including asthmatic smokers.

Hence, an immunomodulating effect of smoking on airway mucosa in asthmatics has not previously been shown, but recent publications from peripheral blood[40] and induced sputum[41] of smoking asthmatics support our data. In agreement with other studies showing smoking to induce an inflammatory airway reaction in non-asthmatics,[42] the smoking healthy workers tended to have higher leucocyte density compared to non-smoking healthy workers.

Smoking in itself may be immunomodulating rather than proinflammatory. This is supported by several human and animal studies.[43] Nicotine was recently shown to have a direct inhibitory effect on the production of proinflammatory mediators by stimulating the nicotinic acetylcholine receptor.[44] Moreover, in a recent study a smoke induced reduction in the number of dendritic cells in the murine lung was reported.[45] The finding that smoke exposure influences the antigen presenting cells may have profound effects on immune responsiveness.

According to this, it is likely that the relatively low number of leucocytes in the airway wall found in our asthmatic smokers was induced by smoking and not by occupational exposure or interaction effects. However, at present we have no explanation for why smoking seems to have a

pro-inflammatory effect in non-asthmatics but an anti-inflammatory effect in asthmatics.

In conclusion, in non-smoking workers with potroom asthma characteristic immunopathological features of asthma such as inflammatory cell infiltrate, thickening of RBM, and increased levels of exhaled NO were shown. In asthmatic smokers, only thickening of RBM was observed, suggesting a different pathophysiological process for potroom asthma in smokers than in non-smokers. In healthy workers, exposure to pot fume emissions may induce a subclinical airway inflammation.

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REFERENCES

1 **Abramson MJ**, Wlodarczyk JH, Saunders NA, *et al*. Does aluminium smelting cause lung disease? *Am Rev Respir Dis* 1989;**139**:1042–57.
2 **Kongerud J**, Gronnesby JK, Magnus P. Respiratory symptoms and lung function of aluminium potroom workers. *Scand J Work Environ Health* 1990;**16**:270–7.
3 **Kongerud J**, Samuelsen SO. A longitudinal study of respiratory symptoms in aluminium potroom workers. *Am Rev Respir Dis* 1991;**144**:10–16.
4 **Kongerud J**, Soyseth V, Burge S. Serial measurements of peak expiratory flow and responsiveness to methacholine in the diagnosis of aluminium potroom asthma. *Thorax* 1992;**47**:292–7.
5 **Desjardins A**, Bergeron JP, Ghezzo H, *et al*. Aluminium potroom asthma confirmed by monitoring of forced expiratory volume in one second. *Am J Respir Crit Care Med* 1994;**150**:1714–17.
6 **Soyseth V**, Kongerud J. Prevalence of respiratory disorders among aluminium potroom workers in relation to exposure to fluoride. *Br J Ind Med* 1992;**49**:125–30.
7 **Steinegger AF**, Schlatter C. Evaluation of fluoride exposure in aluminium smelters: state of the art. *Med Lav* 1992;**83**:489–98.
8 **Saric M**, Ziskin E, Gomzi M. Bronchoconstriction in potroom workers. *Br J Ind Med* 1979;**36**:211–15.
9 **Chan-Yeung M**, Lam S. Occupational asthma. *Am Rev Respir Dis* 1986;**133**:686–703.
10 **Miditun O**. Bronchial asthma in the aluminium industry. *Acta Allergol* 1960;**15**:208–21.
11 **Sorgdrager B**, Pal TM, de Looff AJ, *et al*. Occupational asthma in aluminium potroom workers related to pre-employment eosinophil count. *Eur Respir J* 1995;**8**:1520–4.
12 **Soyseth V**, Kongerud J, Ekstrand J, *et al*. Relation between exposure to fluoride and bronchial responsiveness in aluminium potroom workers with work-related asthma-like symptoms. *Thorax* 1994;**49**:984–9.
13 **Soyseth V**, Kongerud J, Aalen OO, *et al*. Bronchial responsiveness decreases in relocated aluminium potroom workers compared with workers who continue their potroom exposure. *Int Arch Occup Environ Health* 1995;**67**:53–7.
14 **Kongerud J**, Soyseth V. Methacholine responsiveness, respiratory symptoms and pulmonary function in aluminium potroom workers. *Eur Respir J* 1991;**4**:159–66.
15 **Lund K**, Refsnes M, Sandstrom T, *et al*. Increased CD3 positive cells in bronchoalveolar lavage fluid after hydrogen fluoride inhalation. *Scand J Work Environ Health* 1999;**25**:326–34.
16 **Lund MB**, Oksne PI, Hamre R, *et al*. Increased nitric oxide in exhaled air: an early marker of asthma in non-smoking aluminium potroom workers? *Occup Environ Med* 2000;**57**:274–8.
17 **Anon**. International consensus report on diagnosis and treatment of asthma. National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892. Publication no. 92-3091, March 1992. *Eur Respir J* 1992;**5**:601–41.
18 **Quanjer PH**, Tammeling GJ, Cotes JE, *et al*. Lung volumes and forced ventilatory flows. Report Working Party Standardization of Lung Function Tests, European Community for Steel and Coal. Official Statement of the European Respiratory Society. *Eur Respir J Suppl* 1993;**16**:5–40.
19 **Anon**. Investigative use of bronchoscopy, lavage and bronchial biopsies in asthma and other airways diseases. Workshop summary and guidelines. *Eur Respir J* 1992;**5**:115–21.
20 **Jahnsen FL**, Haraldsen G, Rugtveit J, *et al*. Differential interference contrast microscopy combined with immunofluorescence: a new method to phenotype eosinophils in situ. *J Immunol Methods* 1994;**173**:77–91.
21 **Sullivan P**, Stephens D, Ansari T, *et al*. Variation in the measurements of basement membrane thickness and inflammatory cell number in bronchial biopsies. *Eur Respir J* 1998;**12**:811–15.
22 **Saetta M**, Di Stefano A, Maestrelli P, *et al*. Airway mucosal inflammation in occupational asthma induced by toluene diisocyanate. *Am Rev Respir Dis* 1992;**145**:160–8.
23 **Frew AJ**, Chan H, Lam S, *et al*. Bronchial inflammation in occupational asthma due to western red cedar. *Am J Respir Crit Care Med* 1995;**151**:340–4.
24 **Busse WW**, Lemanske RF, Jr Asthma. *N Engl J Med* 2001;**344**:350–62.
25 **Kharitonov SA**, Yates D, Robbins RA, *et al*. Increased nitric oxide in exhaled air of asthmatic patients. *Lancet* 1994;**343**:133–5.
26 **Wenzel SE**, Schwartz LB, Langmack EL, *et al*. Evidence that severe asthma can be divided pathologically into two inflammatory subtypes with distinct physiologic and clinical characteristics. *Am J Respir Crit Care Med* 1999;**160**:1001–8.
27 **Douwes J**, Gibson P, Pekkanen J, *et al*. Non-eosinophilic asthma: importance and possible mechanisms. *Thorax* 2002;**57**:643–8.
28 **Anees W**, Huggins V, Pavord ID, *et al*. Occupational asthma due to low molecular weight agents: eosinophilic and non-eosinophilic variants. *Thorax* 2002;**57**:231–6.
29 **Gibson PG**, Simpson JL, Saltos N. Heterogeneity of airway inflammation in persistent asthma: evidence of neutrophilic inflammation and increased sputum interleukin-8. *Chest* 2001;**119**:1329–36.
30 **Park HS**, Hwang SC, Nahm DH, *et al*. Immunohistochemical characterization of the cellular infiltrate in airway mucosa of toluene diisocyanate (TDI)-induced asthma: comparison with allergic asthma. *Journal of Korean Medical Science* 1998;**13**:21–6.
31 **Park HS**, Jung KS, Hwang SC, *et al*. Neutrophil infiltration and release of IL-8 in airway mucosa from subjects with grain dust-induced occupational asthma. *Clin Exp Allergy* 1998;**28**:724–30.
32 **Djukanovi R**, Wilson JW, Britten KM, *et al*. Quantitation of mast cells and eosinophils in the bronchial mucosa of symptomatic atopic asthmatics and healthy control subjects using immunohistochemistry. *Am Rev Respir Dis* 1990;**142**:863–71.
33 **Di Stefano A**, Saetta M, Maestrelli P, *et al*. Mast cells in the airway mucosa and rapid development of occupational asthma induced by toluene diisocyanate. *Am Rev Respir Dis* 1993;**147**:1005–9.
34 **Saetta M**, Maestrelli P, Di Stefano A, *et al*. Effect of cessation of exposure to toluene diisocyanate (TDI) on bronchial mucosa of subjects with TDI-induced asthma. *Am Rev Respir Dis* 1992;**145**:169–74.
35 **Jeffery PK**. Remodeling in asthma and chronic obstructive lung disease. *Am J Respir Crit Care Med* 2001;**164**:S28–38.
36 **Boulet LP**, Laviolette M, Turcotte H, *et al*. Bronchial subepithelial fibrosis correlates with airway responsiveness to methacholine. *Chest* 1997;**112**:45–52.
37 **Ordonez C**, Ferrando R, Hyde DM, *et al*. Epithelial desquamation in asthma: artifact or pathology? *Am J Respir Crit Care Med* 2000;**162**:2324–9.
38 **Wilson JW**, Li X. The measurement of reticular basement membrane and submucosal collagen in the asthmatic airway. *Clin Exp Allergy* 1997;**27**:363–71.
39 **Van Den Toorn LM**, Overbeek SE, De Jongste JC, *et al*. Airway inflammation is present during clinical remission of atopic asthma. *Am J Respir Crit Care Med* 2001;**164**:2107–13.
40 **Sunyer J**, Springer G, Jamieson B, *et al*. Effects of asthma on cell components in peripheral blood among smokers and non-smokers. *Clin Exp Allergy* 2003;**33**:1500–5.
41 **Chalmers GW**, MacLeod KJ, Thomson L, *et al*. Smoking and airway inflammation in patients with mild asthma. *Chest* 2001;**120**:1917–22.
42 **Amin K**, Ekeberg-Jansson A, Lotfdahl C, *et al*. Relationship between inflammatory cells and structural changes in the lungs of asymptomatic and never smokers: a biopsy study. *Thorax* 2003;**58**:135–42.
43 **Holt PG**. Immune and inflammatory function in cigarette smokers. *Thorax* 1987;**42**:241–9.
44 **Wang H**, Yu M, Ochani M, *et al*. Nicotinic acetylcholine receptor alpha7 subunit is an essential regulator of inflammation. *Nature* 2003;**421**:384–8.
45 **Robbins CS**, Dawe DE, Goncharova SI, *et al*. Cigarette smoke decreases pulmonary dendritic cells and impacts antiviral immune responsiveness. *Am J Respir Cell Mol Biol* 2004;**30**:202–11.

III

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Reduced bronchial CD4+ T-cell density in smokers with occupational asthma

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ABSTRACT: Cigarette smoking may alter bronchial inflammation in asthma. Multicolour immunohistofluorescent examination on bronchial cryosections was used to examine bronchial inflammatory cell infiltrate in patients with occupational asthma. Monoclonal antibodies to CD3, CD4, CD8, T-cell receptor-δ1, CD68 and human leukocyte antigen-DR were combined to identify T-cell subsets and macrophages in bronchial biopsies from 20 workers with occupational asthma (12 smokers and eight nonsmokers), 15 healthy workers (seven smokers and eight nonsmokers) and 10 nonsmoking, nonexposed controls.

The increased subepithelial CD4+ T-cell density in nonsmoking asthmatics was not present in smoking asthmatics, who had the lowest CD4+ T-cell density of all groups. The decreased subepithelial CD4+ and CD8+ T-cell density correlated with a reduction in lung function, as measured by percentage predicted forced expiratory volume in one second, in smoking asthmatics only. Although smoking asthmatics had a significantly increased number of intraepithelial CD8+ T-cells and macrophages compared with nonsmoking asthmatics, the proportion of γδ-T-cells was significantly decreased in both asthmatic groups.

Smoking asthmatics had a distinctly different distribution of T-cell subsets compared with nonsmoking asthmatics. The accumulation of subepithelial CD4+ T-cells, which was observed in nonsmoking asthmatics, appeared to be inhibited in smoking asthmatics, suggesting a smoking-induced bronchial immune modulation, at least in occupational asthma in the aluminium industry.

KEYWORDS: Asthma, inflammation, smoking, T-lymphocyte subsets

Asthmatic airway inflammation has mostly been examined in atopic, nonsmoking subjects, where the increased bronchial density of CD4+ T-cells appears central [1, 2]. Although nonatopic and occupational asthma seem to have different immunopathological mechanisms from atopic asthma, mucosal T-cells are prominent in all conditions [3–5]. Little is known regarding the influence of smoking on the bronchial inflammatory response in asthma. Only few data are available on airway pathology in smoking asthmatics, as reviewed by THOMSON *et al.* [6]. The main findings are reduced eosinophils in both induced sputum and peripheral blood, while neutrophils are increased in induced sputum samples.

Bronchial biopsy studies in smoking asthmatics are lacking, apart from the current authors' previous study of occupational nonatopic asthma in aluminium potroom workers [7], who were exposed to a complex mixture of particulates and gases [8]. Even though smoking and nonsmoking asthmatics had parallel increases in reticular basement membrane thickness, the smoking asthmatics had no increase in subepithelial total leukocyte density (CD45+ cells). This was in contrast to the nonsmoking asthmatics, who had increased total leukocyte density and displayed similar mucosal-airway inflammation as previously shown in "traditional", nonoccupational asthma [1]. Accordingly, the aim of the present study was to examine bronchial T-cell subsets and macrophages in smokers and nonsmokers with occupational asthma. Multicolour immunohistofluorescence staining was performed to identify macrophages and T-lymphocyte subsets (CD3+, CD4+, CD8+, γδ-T-cells) in bronchial biopsies. The results revealed that the increase in subepithelial CD4+ T-cells found in nonsmoking asthmatics was not observed in smoking asthmatics.

METHODS

Subjects

Potroom workers in aluminium smelters with occupational asthma (n=20; eight nonsmokers and 12 smokers) were included in the present study. The controls consisted of 15 healthy potroom workers (eight nonsmokers and seven smokers) and 10 healthy, nonexposed subjects (all nonsmokers). The diagnosis of occupational

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asthma was set by confirming the diagnosis of bronchial asthma according to international guidelines [9] and by establishing a causal link between asthma and the working environment, in line with previously defined criteria for occupational asthma in the aluminium industry [10]. Serial monitoring of peak expiratory flow was performed both at work and away from work. The inclusion criteria were: initially asymptomatic exposure period (latency); symptoms of airway obstruction (*i.e.* dyspnoea, wheezing and cough), usually occurring several hours after exposure or during sleep; and improvement of the symptoms after absence from work for several days or longer. Reversible airway obstruction, defined as >15% increase in forced expiratory volume in one second (FEV_1) after inhalation of β_2-agonist and diurnal peak expiratory flow variability >15%, was documented in all asthmatic subjects. All patients with suspected work aggravation of underlying asthma or a history of allergy or childhood asthma were excluded. Characteristics of the study population are described in table 1 and further detailed elsewhere [7]. Half of the asthmatics (three out of eight of the nonsmokers and seven out of 12 of the smokers) had been relocated to nonpolluted working environments. Ex-smokers (who had ceased smoking >1 yr previously) and never-smokers were all classified as nonsmokers. Two of the asthmatics and three of the healthy workers were ex-smokers. Median smoking load was 13 pack-yrs (range 8–31) for the asthmatics and 17 pack-yrs (range 13–32) for the healthy workers. None of the participants had a history of allergy, familial asthma or childhood asthma and their total immunoglobulin (Ig)E levels were within the normal range. The controls had no symptoms from upper or lower airways. Two of the asthmatic subjects were females (one nonsmoker and one smoker), whereas the rest of the participants were males. The study was approved by the Regional Ethics Committee and written informed consent was obtained from all subjects.

Spirometry and flexible bronchoscopy were performed in accordance with international guidelines as previously described [7]. Bronchial biopsies (maximum six from each subject) were taken from the second- and third-generation carina of the right lung and processed for cryosectioning (snap frozen).

Immunohistochemistry

As both macrophages and mast cells express the CD68 epitope detected with the monoclonal antibody (mAb) KP1, double-labelling for CD68 and human leukocyte antigen (HLA)-DR was needed to identify macrophages (CD68+HLA-DR+ cells), as mast cells are HLA-DR negative (fig. 1a). Similarly, as macrophages, eosinophils and T-cells may all express CD4, double-staining with CD3 and CD4 was essential to identify CD4+ T-cells (fig. 1b). From a lower lobe biopsy, 4-µm cryosections were prefixed for 10 min in 1% paraformaldehyde. Macrophages were identified by a combination of mAb directed against CD68 (clone KP1, MO814, IgG_1; DAKO A/S, Glostrup, Denmark) and HLA-DR (clone L123, IgG_{2a}; Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) followed by an appropriate mixture of fluorochrome-conjugated goat anti-mouse IgG-subclass specific reagents, combined with 4',6-diamino-2-phenylindole to visualise the nucleus (all from Molecular Probes, Eugene, OR, USA). T-cells and their subsets were identified by anti-CD3 (clone RIV9, IgG_3; Monosan, Am Uden, The Netherlands) combined with anti-CD4 (Leu 3, IgG_1; Becton Dickinson), anti-CD8 (clone DK25, IgG_1; DAKO A/S) or mAb to the δ chain of the T-cell receptor (TCR)-γδ (TCRδ1, IgG_1; T Cell Sciences, Cambridge, MA, USA), followed by Alexa-conjugated goat anti-mouse IgG subclass specific reagents (Molecular Probes). mAb TCR-γδ was also combined with anti-CD8 (IgG_{2b}; Serotec Ltd., Oxford, UK). Negative controls included omitting primary antibodies and using nonimmune mouse Ig in similar concentrations. A jejunal section from a patient with coeliac disease was used as a positive control for γδ-T-cells. The primary antibodies were incubated for 20 h at 4°C and the secondary antibodies were applied for 1 h at room temperature.

Quantification of leukocytes

All slides were coded and analysed using a Zeiss Axioplane2 microscope (Carl Zeiss, Oberkochen, Germany) at 630× magnification. Positively stained cells were counted in intact epithelium (defined as presence of both basal and columnar cells) and in a subepithelial zone 114 µm beneath the reticular basement membrane. Excluding submucosal glands and vessels, all available area was analysed. To gain sufficient tissue to obtain representative cell counts [11], 2–4 sections

TABLE 1 Characteristics of the study population

	Asthmatic workers		Healthy workers		Nonexposed controls
	Nonsmokers	Smokers	Nonsmokers	Smokers	Nonsmokers
Subjects	8	12	8	7	10
Age yrs	35 (27–49)	40 (32–59)	36 (31–58)	43 (32–49)	24 (21–44)
FEV_1 % pred	91 (75–120)	90 (73–111)	108 (90–135)	105 (85–124)	112 (81–124)
FEV_1/FVC %	78 (61–82)	67 (59–79)	80 (71–87)	75 (71–84)	81 (74–90)
Inhaled corticosteroids	1#	3¶	0	0	0
Inhaled β_2-agonist	4	6	0	0	0

Data are presented as n or median (range). FEV_1: forced expiratory volume in one second; % pred: % predicted; FVC: forced vital capacity. # fluticasone 500 µg; ¶: budesonid 400 µg (two subjects) and budesonid 800 µg.

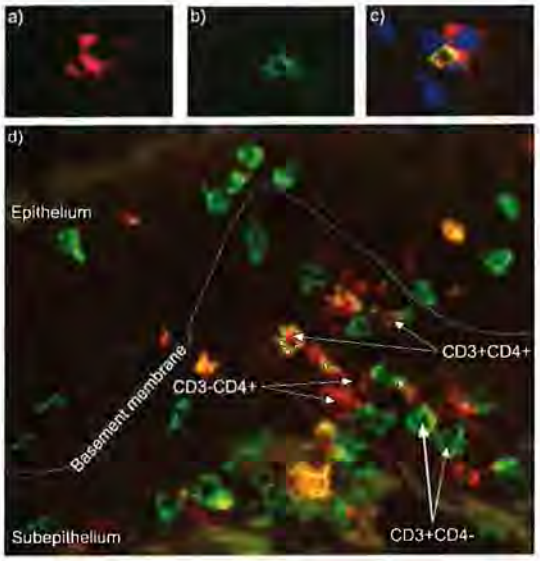

FIGURE 1. a–c) Immunohistofluorescence triple-staining for identification of subepithelial macrophages. a) The monoclonal antibody KP1 to CD68 reacts with mast cells and macrophages, both of which are stained red. b) However, macrophages (but not mast cells) express human leukocyte antigen (HLA)-DR, which is stained green. c) Accordingly, the double-positive cell (CD68+HLA-DR+) represents a macrophage and appears yellow in the overlay image. The CD68+HLA-DR- cell appears red and probably represents a mast cell. In c) the nuclei are visualised blue (4',6-diamino-2-phenylindole). d) Immunohistofluorescence identification of T-cells and T-cell subsets in bronchial mucosa from a nonsmoking asthmatic patient, showing increased density of subepithelial T-cells. The T-cell marker (CD3) is stained green. The CD4+ cells are stained red. The CD3+ (green) T-cells that also express CD4+ (red) appear yellow in this double exposed image (CD3+CD4+ cells). Note that macrophages (CD3-CD4+) appear red, as they do not express the T-cell marker CD3. This particular field contained many CD4- T-cells (CD3+CD4-, green) both in the subepithelium and in the epithelium (*i.e.* CD8 T-cell subset).

were analysed for each subject and all analysed sections were included. Median (range) 5.7 (1.8–17.2) mm of intact epithelium and 1.2 (0.4–2.8) mm subepithelial area, which corresponded to a basement membrane length of 8.2 (2.6–19.8) mm were examined per subject. The final result, expressed as number of intraepithelial cells·mm^{-1} of intact epithelium or number of cells·mm^{-2} of lamina propria, was calculated as the average of all the measurements performed on each section. In order to directly compare intraepithelial and subepithelial cell numbers and evaluate a possible reallocation of cells between the two compartments, subepithelial leukocytes were also expressed as number of cells·mm^{-1} of lamina propria. γδ-T-cells were expressed as a percentage of the total number of T-cells (CD3+ cells) counted.

Computer-assisted digital image analysis

Single-colour images were captured with a MicroMax charge-coupled device digital camera system (Princeton Instruments, Roper Scientific Inc., Princeton, NJ, USA) at 630 × magnification and AnalySIS software (Soft Imaging System GmbH, Münster, Germany; fig. 1a) or the imaging software package MetaMorph 3.0 (Universal Imaging Corporation, Downingtown, PA, USA; fig. 1b).

Statistics

Results are presented as median (range). Differences between two groups were compared by Mann–Whitney U-test. Instead of Bonferroni adjustments (which have limited applications in biomedical research [12]), the level of significance was set to 1% to correct for multiple comparisons. Accordingly, when comparing multiple groups, only $p \leq 0.01$ was considered statistically significant. The nonparametric statistical analysis used in the current study is rather sensitive to low sample size, making it more difficult to achieve statistically significant differences. However, p-values between 0.05–0.01 have been taken into consideration in order not to exclude real differences that do not reach the significance level owing to the low sample size (type II error). The term "significantly increased" is used for p-values ≤ 0.01, while the term "increased or decreased" is used for differences with p-values 0.05–0.01. Multiple linear regression analysis was performed in asthmatics and healthy subjects separately by using exposure, lung function, age and smoking as explanatory variables. Chi-squared tests were used to calculate differences in the proportion of γδ-T-cells. Correlation coefficients were calculated using Spearman's rank method.

RESULTS

Subepithelial cell counts

Nonsmoking asthmatics had significantly increased T-cell (CD3+) density compared with smoking asthmatics ($p=0.001$), but compared with the control groups the increase was smaller ($p=0.04$). The increase was predominantly made up by rise in the CD4+ T-cell subpopulation, which was significantly increased ($p \leq 0.01$) in nonsmoking asthmatics compared with all groups, while there were no difference in CD8+ T-cell densities (table 2; fig. 2a, b). A corresponding CD4+ T-cell density was not observed in smoking asthmatics, who actually had the lowest density of all groups. If asthmatic ex-smokers and steroid-treated asthmatics were excluded from the analysis, the difference in subepithelial CD4+ T-cells between the two asthmatic groups was still highly significant ($p<0.001$). Multiple linear regression analysis using exposure, lung function, age and smoking as explanatory variables confirmed that apart from smoking in the asthmatic group, none of the variables influenced the results.

The macrophage (CD68+HLA-DR+) density was increased in nonsmoking asthmatics compared with nonsmoking exposed and nonexposed controls ($p=0.02$ and $p=0.03$, respectively), but such an increase was not observed in smoking asthmatics (table 2; fig. 2c). Both relocated smoking asthmatics (228 (106–407) cells·mm^{-2}) and relocated nonsmoking asthmatics (788 (556–989) cells·mm^{-2}), had similar subepithelial CD4+ T-cell densities to their corresponding exposed asthmatic colleagues (smoking asthmatics 225 (88–317) cells·mm^{-2}; nonsmoking asthmatics 737 (331–1,131) cells·mm^{-2}).

No mucosal γδ-T-cells were observed in seven out of the 45 subjects, and the overall amount of γδ-T-cells was too low to

TABLE 2 Subepithelial leukocyte subsets

	Asthmatic workers		Healthy workers		Nonexposed controls
	Nonsmokers	Smokers	Nonsmokers	Smokers	Nonsmokers
Subjects n	8	12	8	7	10
CD3+ T-cells	1374# (585–1797)	588 (246–935	732 (502–1496)	665 (513–1243)	757 (409–1295)
CD4+ T-cells	738#¶ (331–1131)	226 (88–407)	352# (231–764)	288 (182–660)	424# (205–626)
CD8+ T-cells	595 (240–927)	359 (158–550)	329 (174–732)	396 (331–780)	362 (158–868)
Macrophages	174 (53–366)	65 (36–210)	70 (22–183)	138 (57–237)	88 (38–222)
CD4+/CD8+ T-cell ratio	1.3# (0.8–2.3)	0.6 (0.4–0.8)	1.1 (0.7–1.9)	0.8 (0.2–1.1)	1.1 (0.5–1.9)
γδ -T-cells	0.4# (13/3142)	0.7# (16/2356)	0.9 (13/1451)	0.6 (7/1157)	1.9 (40/2119)

Data for CD3+, CD4+, CD8+ T-cells and macrophages are expressed as median cells·mm^{-2} (range). γδ-T-cells are expressed as percentage of all subepithelial CD3+ T-cells (grouped cell count) in each group. #: $p \leq 0.01$ *versus* smoking asthmatics, ¶: $p \leq 0.01$ *versus* nonsmoking healthy workers, smoking healthy workers and nonexposed controls. +: $p \leq 0.01$ *versus* nonexposed controls.

justify interindividual group evaluation. The individual γδ+ T-cell and CD3+ T-cell numbers were therefore summarised separately within each of the five groups. Such group-based data showed that the proportion of subepithelial γδ-T-cells was significantly reduced in both smoking and nonsmoking asthmatics compared with the nonexposed controls (Chi-squared test; table 2). When all subjects were grouped together, 89 out of 10,225 subepithelial CD3+ T-cells (0.9%) expressed γ and δ chains (TCRγδ), and double-labelling for TCRγδ and CD8 revealed that 23% of these γδ-T-cells co-expressed CD8.

Intraepithelial cell counts

The number of intraepithelial macrophages and CD8+ T-cells·mm^{-1} intact epithelium was increased in both asthmatic and healthy smokers compared with their corresponding nonsmoking groups (table 3). While intraepithelial CD4+ T-cell counts were significantly increased ($p<0.001$) in the healthy smokers, no such increase was observed in smoking asthmatics compared with nonsmoking asthmatics (table 3). The proportion of intraepithelial γδ-T-cells was significantly reduced in both smoking and nonsmoking asthmatics ($p<0.001$) compared with nonexposed controls (table 3). When all subjects were grouped together, 172 out of 6,492 intraepithelial CD3+ T-cells (2.6%) expressed TCRγδ. Double-labelling for TCRγδ and CD8 revealed that 53% of the intraepithelial γδ-T-cells co-expressed CD8.

T-cell distribution

In order to directly compare intraepithelial and subepithelial T-cell numbers, and evaluate a possible reallocation of cells between the two compartments, subepithelial T-cells were additionally expressed as number of cells·mm^{-1} of lamina propria. When subepithelial and intraepithelial T-cells were summarised, the number of CD4+ T-cells was still significantly reduced in smoking asthmatics (39 (20–70) cells·mm^{-1}) compared with nonsmoking asthmatics (108 (50–174) cells·mm^{-1}; $p<0.001$). However, the initial difference in lamina propria CD8+ T-cells between smoking and nonsmoking asthmatics was no longer significant (75 (47–128) cells·mm^{-1} in smoking asthmatics *versus* 94 (41–149) cells·mm^{-1} in nonsmoking asthmatics).

Correlation

FEV1 was positively correlated to the subepithelial T-cell (CD3+) density ($r=0.69$, $p=0.01$) in smoking asthmatics, with similar contribution from the CD4+ (fig. 3) and CD8+ T-cell subsets, as both were correlated equally to FEV1 (both $r=0.57$, $p=0.05$). No such correlation was found in nonsmoking asthmatics or in the control groups.

DISCUSSION

The present increase in subepithelial CD4+ T-cell density in nonsmokers with occupational asthma is comparable to what has previously been shown in "traditional" asthma [1, 2], and illustrates the immunopathological similarity between occupational and nonoccupational asthma [13, 14]. The lack of a similar increase in subepithelial CD4+ T-cell density in the smoking asthmatics was rather surprising, and apparently disagrees with the current concept of asthma immunopathology. There are no other comparable human studies of bronchial inflammation in smoking asthmatics, except a previous report by the current authors of inhibited increase of total leukocytes (CD45+ cells) and eosinophils in smokers with occupational asthma [7]. The current results identify the CD4+ T-cells as the major leukocyte subgroup responsible for the reduction in CD45+ cells in smoking asthmatics. As CD4+ T-cells were reduced to a greater extent than CD8+ T-cells, the CD4+/CD8+ T-cell ratio was reduced in smoking asthmatics.

In general, cigarette smoking seems to induce a bronchial inflammatory reaction, even in asymptomatic subjects [15, 16]. Increased bronchial CD8+ T-cell and macrophage density and reduced subepithelial CD4/CD8 ratio are consistent findings in smokers [17, 18], a phenomenon which was also observed in the current healthy smokers. Thus, a smoking-induced inhibited increase in subepithelial CD4+ T-cell density in asthmatics appears to disagree with the generally accepted hypothesis that smoking amplifies asthmatic airway inflammation [19]. The present smoking asthmatics do not resemble chronic obstructive pulmonary disease (COPD) patients, as they all had history, symptoms and reversible airflow obstruction consistent with asthma and not COPD. Moreover, the bronchial cell infiltrate in these smoking asthmatics differed markedly from that in COPD patients as

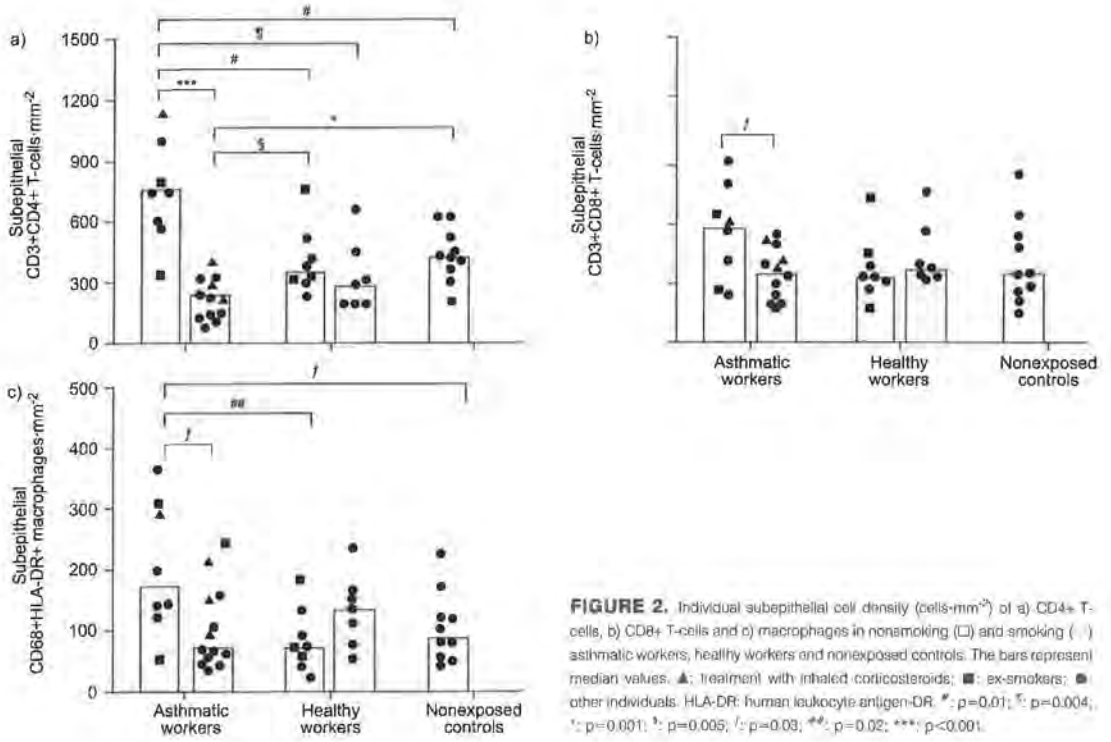

FIGURE 2. Individual subepithelial cell density (cells·mm^{-2}) of a) CD4+ T-cells, b) CD8+ T-cells and c) macrophages in nonsmoking (□) and smoking (■) asthmatic workers, healthy workers and nonexposed controls. The bars represent median values. ▲: treatment with inhaled corticosteroids; ■: ex-smokers; ●: other individuals. HLA-DR: human leukocyte antigen-DR. #: p=0.01; ¶: p=0.004; +: p=0.001; §: p=0.005; ƒ: p=0.03; ##: p=0.02; ***: p<0.001.

they had no increase in subepithelial macrophages or CD8+ T-cells as previously reported in COPD [20, 21].

The majority of T-cells express the α and β chains of the TCR, while a small fraction (~5%) expresses the γ and δ chains (γδ-T-cells). The function of γδ-T-cells in normal and asthmatic airways is still unknown, and they predominantly localise to mucosal surfaces. It is debated whether γδ-T-cells protect the bronchial mucosa or promote airway inflammation [22]. While increased γδ-T-cell counts have been reported in bronchoalveolar lavage [23] and induced sputum from asthmatics with acute exacerbations [24], other studies report no difference between asthmatic and control subjects in bronchial biopsies [25] and bronchoalveolar lavage [22, 26]. The current observation of depleted intraepithelial γδ-T-cell levels in asthmatics supports the theory that these cells are involved in maintaining mucosal homeostasis.

Both smoking asthmatics and healthy smokers had increased numbers of intraepithelial macrophages and CD8+ T-cells, but in contrast to healthy smokers, there was no increase in intraepithelial CD4+ T-cells in smoking asthmatics. Thus, smoking may predominantly affect CD4+ T-cell recruitment. Whereas decreased subepithelial CD8+ T-cell density in smoking asthmatics could partly be explained by migration from the lamina propria to the epithelium, intraepithelial migration could not explain the reduced CD4+ T-cell density.

Smoking may have profound immunosuppressive effects, as shown by the smoking-induced inhibition of pulmonary T-cell responses in mice [27, 28] and humans [29]. Although short-term smoke exposure may induce airway inflammation in murine asthma models [30], more chronic antigen stimulation and smoke exposure may actually inhibit an asthma-induced airway influx of macrophages, CD4+ T-cells and eosinophils [31] as well as dendritic cells [32]. Moreover, tobacco smoke prevented the activation and expansion of pulmonary CD4+ T-cells in a murine adenovirus infection model [32]. Thus, smoking may modify airway inflammation in a dose-dependent and time-related manner. Interestingly, one third of smoking asthmatics reported worsening of their asthmatic symptoms after smoking cessation [33]. It is, however, rather challenging to explain why subepithelial CD4+ and CD8+ T-cell density correlated to FEV1 in smoking asthmatics, while no such correlation was found in nonsmoking asthmatics or in the control groups.

A rather limited number of subjects was included in the present study, a weakness shared with many published bronchial biopsy studies. However, the lack of an asthma-associated increase in subepithelial CD4+ T-cells in smokers was observed in a group of 12 patients and compared with a smaller group of nonsmoking asthmatics (n=8), where the expected increase in subepithelial CD4+ T-cells was observed. The difference between the two asthmatic groups was highly significant when nonparametric statistics that are sensitive to

TABLE 3 Intraepithelial leukocyte subsets

	Asthmatic workers		Healthy workers		Nonexposed controls
	Nonsmokers	Smokers	Nonsmokers	Smokers	Nonsmokers
Subjects n	8	12	8	7	10
CD3+ T-cells	15# (7–34)	33 (19–74)	9¶ (6–29)	32 (22–50)	26 (8–30)
CD4+ T-cells	5 (0–16)	6 (2–17)	2¶ (1–4)	8 (5–16)	6 (3–7)
CD8+ T-cells	10# (8–20)	22 (17–61)	8 (3–25)	21 (15–42)	16 (6–25)
Macrophages	11# (4–18)	16 (10–26)	7¶ (3–13)	17 (8–33)	7 (1–13)
CD4+/CD8+ T-cell ratio	0.4 (0.0–1.2)	0.3 (0.1–0.7)	0.1 (0.0–1.1)	0.4 (0.2–0.7)	0.4 (0.1–0.8)
γδ-T-cells	2.5+ (23/925)	1.7+ (57/3357)	4.1 (19/467)	3.3 (30/916)	5.3 (43/812)

Data for CD3+, CD4+, CD8+ T-cells and macrophages are expressed as median (range) cells·mm^{-1} epithelium. γδ-T-cells are expressed as percentage of all intraepithelial CD3+ T-cells (grouped count number) in each group. #: $p \leq 0.01$ *versus* smoking asthmatics; ¶: $p \leq 0.01$ *versus* smoking healthy workers; +: $p \leq 0.01$ *versus* nonexposed controls.

small samples were used. The difference could not be ascribed to the inclusion of ex-smokers or steroid treated patients, as excluding these subjects did not change the statistical differences.

The study was performed in subjects with occupational asthma who had been exposed to aluminium potroom fume emissions. Although it can be argued that the decrease in bronchial CD4+ T-cells in smoking asthmatics was due to occupational exposure in combination with smoking, there was no difference in the CD4+ T-cell density between smoking and nonsmoking healthy workers who were all exposed to the same working environment as the asthmatics. Moreover, seven of the smoking asthmatics had been relocated to unexposed environments for median 2 yrs prior to examination. These relocated smoking asthmatics had similar CD4+ T-cell densities to the smoking asthmatics who were still exposed.

Although the smoking-induced lack of increase in bronchial CD4+ T-cells was observed in asthmatics in an occupational setting, this phenomenon may not be restricted to occupational asthma. Rather, smoking asthmatics may represent a separate asthmatic phenotype without the characteristic asthma-associated increase in bronchial CD4+ T-cells, which challenges the current understanding of the pathogenetic mechanisms in asthma.

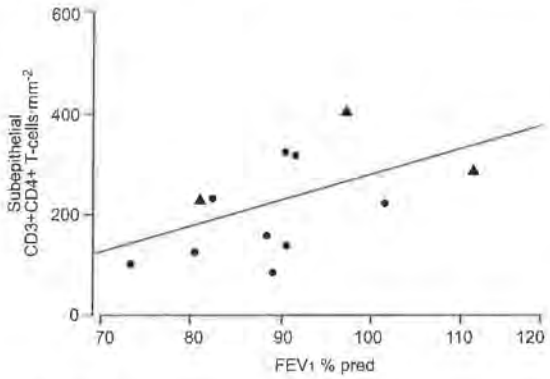

FIGURE 3. The subepithelial density of CD4+ T-cells was positively correlated to forced expiratory volume in one second (FEV1) % predicted (% pred) value in smoking asthmatics. r=0.57, p=0.05. ▲: treatment with inhaled corticosteroids; ●: other individuals.

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REFERENCES

1 Bousquet J, Jeffery PK, Busse WW, Johnson M, Vignola AM. Asthma. From bronchoconstriction to airways inflammation and remodeling. *Am J Respir Crit Care Med* 2000; 161: 1720–1745.
2 Eapen SS, Busse WW. Asthma. *Clin Allergy Immunol* 2002; 16: 325–353.
3 Humbert M, Menz G, Ying S, *et al*. The immunopathology of extrinsic (atopic) and intrinsic (non-atopic) asthma: more similarities than differences. *Immunol Today* 1999; 20: 528–533.
4 Karjalainen EM, Lindqvist A, Laitinen LA, *et al*. Airway inflammation and basement membrane tenascin in newly diagnosed atopic and nonatopic asthma. *Respir Med* 2003; 97: 1045–1051.
5 Mapp CE, Boschetto P, Maestrelli P, Fabbri LM. Occupational Asthma. *Am J Respir Crit Care Med* 2005; 172: 280–305.
6 Thomson NC, Chaudhuri R, Livingston E. Asthma and cigarette smoking. *Eur Respir J* 2004; 24: 822–833.
7 Sjaheim T, Halstensen TS, Lund MB, *et al*. Airway inflammation in aluminium potroom asthma. *Occup Environ Med* 2004; 61: 779–785.
8 Kongerud J, Boe J, Søyseth V, Naalsund A, Magnus P. Aluminium potroom asthma: the Norwegian experience. *Eur Respir J* 1994; 7: 165–172.
9 International consensus report on diagnosis and treatment of asthma. National Heart, Lung and Blood Institute, National Institutes of Health. Bethesda, Maryland 20892.

Publication no. 92–3091, March 1992. *Eur Respir J* 1992; 5: 601–641.

10 Abramson MJ, Wlodarczyk JH, Saunders NA, Hensley MJ. Does aluminum smelting cause lung disease? *Am Rev Respir Dis* 1989; 139: 1042–1057.

11 Sullivan P, Stephens D, Ansari T, Costello J, Jeffery P. Variation in the measurements of basement membrane thickness and inflammatory cell number in bronchial biopsies. *Eur Respir J* 1998; 12: 811–815.

12 Perneger TV. What's wrong with Bonferroni adjustments. *BMJ* 1998; 316: 1236–1238.

13 Frew AJ, Chan H, Lam S, Chan-Yeung M. Bronchial inflammation in occupational asthma due to western red cedar. *Am J Respir Crit Care Med* 1995; 151: 340–344.

14 Mapp CE, Saetta M, Maestrelli P, *et al.* Mechanisms and pathology of occupational asthma. *Eur Respir J* 1994; 7: 544–554.

15 Amin K, Ekberg-Jansson A, Lofdahl CG, Venge P. Relationship between inflammatory cells and structural changes in the lungs of asymptomatic and never smokers: a biopsy study. *Thorax* 2003; 58: 135–142.

16 Cosio MG, Majo J, Cosio MG. Inflammation of the airways and lung parenchyma in COPD: role of T cells. *Chest* 2002; 121: Suppl. 5, 160S–165S.

17 Di Stefano A, Caramori G, Ricciardolo FL, Capelli A, Adcock IM, Donner CF. Cellular and molecular mechanisms in chronic obstructive pulmonary disease: an overview. *Clin Exp Allergy* 2004; 34: 1156–1167.

18 Saetta M, Turato G, Maestrelli P, Mapp CE, Fabbri LM. Cellular and structural bases of chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2001; 163: 1304–1309.

19 Floreani AA, Rennard SI. The role of cigarette smoke in the pathogenesis of asthma and as a trigger for acute symptoms. *Curr Opin Pulm Med* 1999; 5: 38–46.

20 Saetta M, Turato G. Airway pathology in asthma. *Eur Respir J* 2001; 18: Suppl. 34, 18s–23s.

21 Sutherland ER, Martin RJ. Airway inflammation in chronic obstructive pulmonary disease: comparisons with asthma. *J Allergy Clin Immunol* 2003; 112: 819–827.

22 Krug N, Erpenbeck VJ, Balke K, *et al.* Cytokine profile of bronchoalveolar lavage-derived $CD4^+$, $CD8^+$, and γδ T cells in people with asthma after segmental allergen challenge. *Am J Respir Cell Mol Biol* 2001; 25: 125–131.

23 Spinozzi F, Agea E, Bistoni O, *et al.* Increased allergen-specific, steroid-sensitive γδ T cells in bronchoalveolar lavage fluid from patients with asthma. *Ann Intern Med* 1996; 124: 223–227.

24 Hamzaoui A, Kahan A, Ayed K, Hamzaoui K. T cells expressing the γδ receptor are essential for Th2-mediated inflammation in patients with acute exacerbation of asthma. *Mediators Inflamm* 2002; 11: 113–119.

25 Fajac I, Roisman GL, Lacronique J, Polla BS, Dusser DJ. Bronchial γδ T-lymphocytes and expression of heat shock proteins in mild asthma. *Eur Respir J* 1997; 10: 633–638.

26 Walker C, Kaegi MK, Braun P, Blaser K. Activated T cells and eosinophilia in bronchoalveolar lavages from subjects with asthma correlated with disease severity. *J Allergy Clin Immunol* 1991; 88: 935–942.

27 Holt PG. Immune and inflammatory function in cigarette smokers. *Thorax* 1987; 42: 241–249.

28 Lambert C, McCue J, Portas M, *et al.* Acrolein in cigarette smoke inhibits T-cell responses. *J Allergy Clin Immunol* 2005; 116: 916–922.

29 Pyatt DW, Stillman WS, Irons RD. Hydroquinone, a reactive metabolite of benzene, inhibits NF-kappa B in primary human CD4+ T lymphocytes. *Toxicol Appl Pharmacol* 1998; 149: 178–184.

30 Moerloose KB, Pauwels RA, Joos GF. Short-term cigarette smoke exposure enhances allergic airway inflammation in mice. *Am J Respir Crit Care Med* 2005; 172: 168–172.

31 Melgert BN, Postma DS, Geerlings M, *et al.* Short-term smoke exposure attenuates ovalbumin-induced airway inflammation in allergic mice. *Am J Respir Cell Mol Biol* 2004; 30: 880–885.

32 Robbins CS, Dawe DE, Goncharova SI, *et al.* Cigarette smoke decreases pulmonary dendritic cells and impacts antiviral immune responsiveness. *Am J Respir Cell Mol Biol* 2004; 30: 202–211.

33 Hillerdahl G, Rylander R. Asthma and cessation of smoking. *Clin Allergy* 1984; 14: 45–47.

IV

Increased bronchial density of CD25+ Foxp3+ regulatory T-cells in nonsmokers with occupational asthma

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ABSTRACT: This study aimed to identify activated bronchial T-cells in nonsmoking and smoking subjects with occupational asthma. Because activated (CD25+) T-helper (CD4+) cells include both regulatory (Foxp3+) and effector ($Foxp3^{neg}$) T-cells, we developed a triple colour immunohistofluoresence labelling technique to discriminate these subsets and to identify T-cell subtypes expressing additional activation markers like Ki-67 (proliferation) and HLA-DR. Monoclonal antibodies to CD3, CD4, CD8, CD25, Foxp3, HLA-DR and KI-67 were applied on bronchial biopsies from 20 workers with occupational asthma (12 smokers), 15 healthy workers (7 smokers) and 10 nonsmoking, nonexposed controls. Nonsmoking asthmatics had significantly higher subepithelial density of CD4+CD25+ T-cells, which predominantly expressed the regulatory T-cell marker Foxp3, and increased density of both proliferating (Ki-67+) T-cells (CD4+ and CD8+), and HLA-DR+ T-cells (predominantly CD8+). A corresponding increase in activated T-cells was not observed in smoking asthmatics, although a decreased percentage of the CD25+ T-cells expressed Foxp3, resulting in an altered effector/regulatory T-cell balance. The majority of the CD4+Foxp3+ T-cells did not express CD25, and may be a hereto undescribed T-cell-subtype in the bronchial mucosa with unknown function. Whereas asthmatic smokers were almost similar to controls, nonsmoking asthmatics had increased density of activated T-cells, despite increased CD25+Foxp3+ regulatory T-cell density.

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Keywords; Asthma, effector T-cells, regulatory T-cells, smoking, T-cell proliferation,

INTRODUCTION

Workers in aluminium smelters have increased risk of developing asthma, often referred to as potroom asthma [1]. The causal agent(s) and pathogenesis are unknown, but nonsmoking potroom workers have the same bronchial pathophysiological alterations as described for other types of asthma [2, 3]. The phenotype of the bronchial leukocyte infiltrate in conventional asthma has revealed the importance of CD4+ T-cells, in particular the T-helper 2 (Th2) subset [4]. An increased density of activated CD4+(CD25+) T-cells is demonstrated in atopic- [5], non-atopic- [6], and occupational asthma [7]. This asthma-associated increase in bronchial CD4+ T-cells was, however, not observed in tobacco smoking subjects with occupational asthma, in contrast to their nonsmoking asthmatic colleagues [3]. Although smoking appeared to inhibit bronchial influx of CD4+ T-cells in particular [3], also other leukocytes were affected [8]. Although CD8+ T-cells have predominantly been implicated in the immunopathology of smoking-induced chronic obstructive pulmonary disease (COPD) [9], they may also be involved in the immunopathology of asthma [10], in particular when fatal [11].

T-cell activation may induce *de novo* expression of the IL-2 receptor α–chain (CD25), human leukocyte antigen class-II (HLA-DR), or induce T-cell proliferation identified by expression of the nuclear Ki-67 antigen. Although the bronchial CD25+ T-cells in asthma have been assumed to be activated asthma-promoting effector T-cells, this subset may include regulatory T-cells (Tregs), identified by nuclear expression of the forkhead/winged-helix transcription factor Foxp3 (Foxp3) [12]. Tregs are important in maintaining immunological homoeostasis [13] and may inhibit both autoimmunity and allergic diseases through suppression of Th1 as well as Th2 responses [14]. It is hypothesized that impaired expansion or function of Tregs lead to the development of asthma [14, 15]. However, Tregs in asthmatics have to date only been studied in peripheral blood [14, 16-18] and broncheoalveolar lavage (BAL) [16].

Multicolour immunohistofluorescent labelling on bronchial biopsies from nonsmokers and smokers with occupational asthma was used to reveal which of the T-cell subsets (CD4, CD8) that in particular expressed the activation markers (CD25, HLA-DR, Ki-67) and their relationship to Foxp3-expressing T-cells. The results revealed that most CD4+CD25+ T-cells expressed Foxp3, presumably representing Tregs, and this subset was increased in nonsmoking asthmatics.

METHODS

Subjects

Twenty potroom workers in aluminium smelters with occupational asthma (eight nonsmokers and 12 smokers), 15 healthy potroom workers (eight nonsmokers and seven smokers) and 10 healthy nonexposed nonsmoking controls were included. The study population was identical as in foregoing studies [3, 8]. Asthma was diagnosed according to international guidelines, and the inclusion criteria were as detailed elsewhere [8]. Briefly, symptoms of airway obstruction (dyspnoea, wheezing, cough) and reversible airway obstruction defined as > 15 % increase in forced expiratory volume in one second (FEV_1) after inhalation of β_2-agonists, were documented in all asthmatics. Characteristics of the study population are described in table 1 and in further details previously [8]. Ex-smokers (who had ceased smoking > 1 year previously) and never-smokers were all classified as nonsmokers. Two of the asthmatics and three of the healthy workers were ex-smokers. Median smoking load

TABLE 1. Characteristics of the study population

	Asthmatic workers		Healthy workers		Nonexposed controls
	nonsmokers	smokers	nonsmokers	smokers	nonsmokers
Subjects n	8	12	8	7	10
Age	35 (27-49)	40 (32-59)	36 (31-58)	43 (32-49)	24 (21-44)
FEV_1 % pred	91 (75-120)	90 (73-111)	108 (90-135)	105 (85-124)	112 (81-124)
FEV_1/FVC %	78 (61-82)	67 (59-79)	80 (71-87)	75 (71-84)	81 (74-90)
Inhaled corticosteroids	1	3	0	0	0
Inhaled β_2-agonist	4	6	0	0	0

Data are presented as n or median (range). FEV_1: forced respiratory volume in one second; % pred: % predicted; FVC: forced vital capacity

was 13 pack-years for the asthmatics and 17 pack-years for the healthy workers. Half of the asthmatics had been relocated to non-polluted working environments. None of the participants had a history of allergy, familiar asthma or childhood asthma and their total IgE-levels were within the normal range. The controls had no symptoms from upper or lower airways. The study was approved by the Regional Ethics Committee and informed written consent was obtained from all subjects.

Spirometry and flexible bronchoscopy were performed as previously described [8]. Bronchial biopsies were taken from the second and third generation carina of the right lung, and snap frozen.

Multicolour immunohistofluorescent labelling

Mucosal T-cell subsets (CD4 and CD8) were examined for expression of the activation markers CD25, HLA-DR, and the proliferation marker Ki-67 and/or expression of the regulatory T-cell associated nuclear transcription factor Foxp3, by double-or triple-colour immunohistofluorescent labelling in situ, using various antibodies to CD3 (monoclonal antibody (mAb) IgG_1 κ, Diatec Monoclonals AS, Oslo, Norway, mAb clone RIV9, IgG_3; Monosan, Am Uden, The Netherlands; rabbit antiserum to a CD3ε-peptide; DAKO A/S, Glostrup, Denmark) in various combination with mAb against HLA-DR (clone L123, IgG_{2a}; Becton Dickinson, San Jose, CA, USA); Ki-67 (IgG_1, Zymed Invitrogen, Carlsbad, CA, USA); CD4 (clone MT310, IgG_1, DAKO); CD8 (clone DK25, IgG_1, DAKO or clone 4B11, IgG_{2b}, SeroTech Ltd. Oxford UK); CD25 (clone ACT-1, IgG_1, DAKO), or Foxp3 (IgG_3, Abcam plc, Cambridge, UK). The primary antibody combinations were applied for 20 h at 4°C, followed by peroxidise quenching in 3 % H_2O_2 for 30 minutes when needed. The following labelling were appropriate combinations of peroxidate-, biotin-, (Southern Biotechnology, Birgmingham, AL, USA), Alexa-488, or -594 conjugated mouse IgG-subclass specific goat antisera, (Molecular Probes, Eugene, OR, USA) and either Thyramide Signal Amplification (TSA)-Coumarin (PerkinElmer Life Science, Boston, MA, USA) or the DNA binding reagent 4',6-diamino-2-phenylindole (DAPI, Molecular Probes) when appropriate. The CD25 expression was in particular amplified with the TSA-system to ensure detection of even weak

CD25 expression. $CD8^{neg}CD3+$ T-cells were used to identify CD4+ T-cells when examining CD25 and Ki-67 expression, because the mAb to CD4 have the same murine Ig-subclass as mAb to CD25 and Ki-67. We have previously revealed that there are less than 2 % $CD4^{neg}CD8^{neg}$ T-cells (γδ T-cells) in this study population [3]. Methodological negative controls included sections incubated with non-immune mouse Ig and rabbit serum in similar concentrations.

Quantification of leukocytes

All slides were coded and analyzed using a Zeiss Axioplane2 microscope (Carl Zeiss, Oberkochen, Germany) at 630x magnification. Positively stained cells were counted in a subepithelial zone 114 µm beneath the reticular basement membrane, excluding cells in submucosal glands and vessels. Allover, 88 430 CD3+ T-cells (median 1423 cells per subject, range 690-5461) were individually examined for Foxp3 and CD25 expression in triple labeling, covering an area of median 1.8 mm^2 per subject (range 0.9-3.9 mm^2); ~ 13.0 mm basement membrane length (range 6.2-27.8 mm). Similarly, a total number of 14 620 CD3+ T-cells (median 208 cells per subject, range 101-1802) were individually examined for HLA-DR expression and a total number of 16 718 T-cells (median 282 per subject, range 103-1664) were examined for Ki-67 expression, corresponding to an area of median 0.9 mm^2 per subject, (range 0.3-2.4 mm^2); ~ median 6.6 mm basement membrane length (range 2.4-17.0 mm). The analyzed area was then above the recommended area to obtain representative cell counts (19). The final result, expressed as number of cells per square millimeter of lamina propria, was calculated as the sum of all cells divided by the total area examined for each subject. In the epithelium, only Foxp3 positive T-cells were counted and examined for CD25 co-expression.

Statistics

Results are presented as median (range) values. Differences between two groups were compared by Mann-Whitney U-test. Analysis of variance was performed to control for age as a potential confounder. A p value ≤ 0.05 was regarded as significant. Subtyping T-cells expressing some of the activation markers (in particular Ki-67, HLA-DR, and partly CD25) revealed often <10 positive cells per subject, making individual statistics difficult to justify. Although such individual data are shown in table 2, additional group based statistics were applied (figure 4 and 5). The density of T-cells expressing a combination of activation markers were calculated by adding the counted cells within each group, divided by the total area examined, to normalize group-based differences in total area examined. Chi-squared test was then used to examine any group-based differences in the proportion of T-cells that expressed a given activation marker(s).

RESULTS

Increased regulatory T-cell (CD25+ Foxp3+CD3+) density in nonsmoking asthmatics

The density of Foxp3+ T-cells was significantly increased in nonsmoking asthmatics (table 2, figure 1a). Foxp3+ was expressed on both CD25+ and $CD25^{neg}$ T-cells (figure 2a-d) and the majority of the Foxp3-expressing T-cells was actually $CD25^{neg}$ in all groups (table 2). Moreover, triple-colour immunohistofluorescent staining for CD4, Foxp3 and CD3 revealed that 99 % of the Foxp3+ T-cells co-stained CD4, which was similar in all groups. Further triple-labelling for CD25, Foxp3 and CD3 was performed to discriminate the two functional CD4+CD25+ T-cell subsets; Tregs (Foxp3+) and effector T-cells ($Foxp3^{neg}$) (figure 2a-d). The majority of the CD25+ T-cells was Foxp3+, and thus Tregs, (medians 54 - 83 %). The sub-

TABLE 2 Subepithelial leukocyte subsets

	Asthmatic workers		Healthy workers		Non-exposed controls
	nonsmokers	smokers	nonsmokers	smokers	nonsmokers
Subjects	8	12	8	7	10
Density of Foxp3+ T-cells	83# * (44-125)	22 (9-102)	41 (19-109)	44 (19-104)	35 (18-60)
% Foxp3+ T-cells of total T-cells	6.4 * (4.3-8.3)	2.7 (1.3-14.1)	4.4 (3.0-12.2)	5.0 (2.0-7.7)	3.8 (2.3-5.1)
Density of regulatory T-cells (CD25+Foxp3+CD3+)	22# (9-66)	4 (1-42)	6 (3-54)	4 (1-35)	7 (1-15)
% regulatory T-cells of total T-cells	1.9# (0.8-4.4)	0.6 (0.1-5.8)	0.7 (0.4-6.0)	0.5 (0.2-2.0)	0.7 (0.1-1.2)
Density of effector T-cells (CD25+Foxp3^{neg} CD3+)	10# * (2-37)	4 (1-12)	2¶ (1-5)	2 (1-15)	1 (1-3)
% effector T-cells of total T-cells	0.8# (0.2-2.6)	0.5 (0.2-1.5)	0.2 (0.1-0.5)	0.2 (0.1-0.7)	0.1 (0.1-0.2)
Density of CD25+ T-cells	31# * (13-99)	9 (3-53)	7 (4-59)	6 (3-49)	8 (2-16)
% CD25+ T-cells of total T-cells	2.6# * (1.0-7.0)	1.0 (0.4-7.3)	0.8 (0.6-6.5)	0.8 (0.4-2.4)	0.9 (0.2-1.3)
Density of CD25^{neg}Foxp3+ T-cells	45* (23-95)	16 (8-60)	33 (16-56)	42 (18-70)	29 (17-46)
% CD25^{neg}Foxp3+ T-cells of total Foxp3+ T-cells	65# (41-85)	77 (59-98)	85 (51-89)	89 (65-95)	81 (74-97)
Density of HLA-DR+ T-cells	37# (0-104)	15 (0-73)	0 (0-100)	15 (0-48)	8 (0-38)
% HLA-DR+ T cells of total T-cells	3.7 (0.0-9.3)	3.9 (0.0-12.7)	0 (0.0-6.7)	2.1 (0.0-5.7)	0.8 (0.0-3.8)
Density of Ki-67+ T-cells	14# * (0-186)	0 (0-4)	0 (0-0)	0 (0-145)	0 (0-16)
% Ki-67+ T-cells of total T-cells	1.2# * (0.0-14.2)	0.0 (0.0-0.9)	0.0 (0.0-0.0)	0.0 (0.0-17.1)	0.0 (0.0-1.5)

*The data are expressed as median cells·mm^{-2}. # $p \leq 0.05$ compared to nonsmoking healthy workers and nonexposed controls. * $p \leq 0.05$ compared to smoking asthmatics. ¶ $p \leq 0.05$ compared to nonexposed controls*

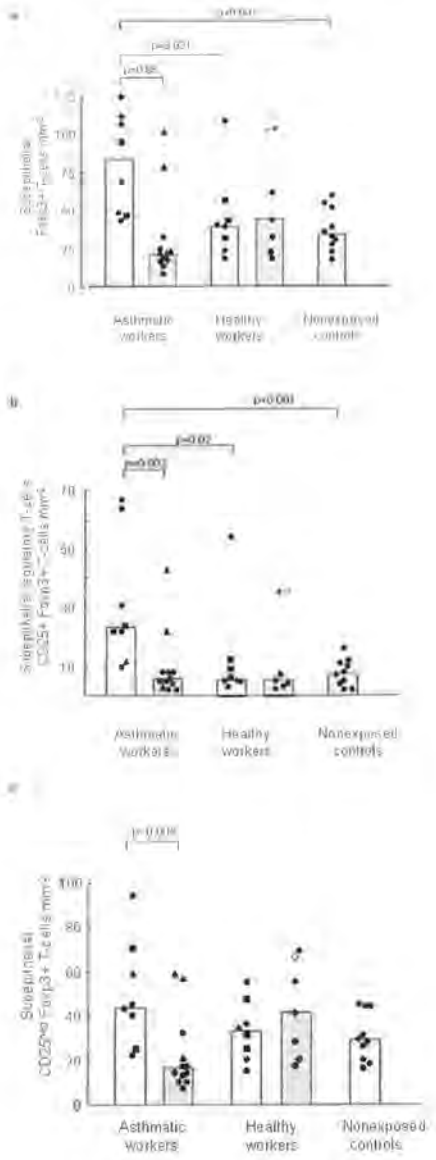

FIGURE 1. *Individual subepithelial cell density (cells·mm^{-2}) of a) Foxp3+ T-cells, b) CD25+Foxp3+ T-cells (regulatory T-cells), and c) CD25^{neg}Foxp3+ T-cells in nonsmoking (□) and smoking (▨) asthmatic workers, healthy workers and nonexposed controls. The bars represent median values. ▲: treatment with inhaled corticosteroids; ■ ex-smokers; ○ convalescent subject; ● : other individuals.*

epithelial density and percentage of putative Tregs (CD25+Foxp3+ T-cells) were significantly increased in nonsmoking asthmatics (table 2, figure 1b). Based on the known subepithelial CD4/CD8 ratio [3], it was revealed that median 3.4 % of the CD4+ T-cells were CD25+Foxp3+, putative Tregs in nonsmoking asthmatics compared to 1.7 % in smoking asthmatics (p=0.08). Intraepithelial Foxp3+ T-cells were only occasionally observed, but 24 % of the 354 intraepithelial Foxp3+ T-cells co-expressed CD25.

Increased CD25+ T-cell density in nonsmoking asthmatics

Both the density and the percentage of subepithelial CD25+ T-cells were significantly increased in nonsmoking asthmatics compared to all the other groups (table 2, figure 3a). Further subtyping of the CD25 expressing T-cells (CD3+) revealed that the vast majority, (medians 90-100 %), was CD8 negative and thus CD4+. Accordingly, as the bronchial CD4+ T-cell density was considerably higher in nonsmoking than in smoking asthmatics [3], subepithelial CD4/CD8 T-cell ratio was used to calculate the percentage of CD4+ T-cells that expressed CD25. Median 4.6 % of the CD4+ T-cells expressed CD25 in nonsmoking asthmatics compared to 3.0 % in smoking asthmatics (p=0.1).

Increased effector T-cells (CD25+ Foxp3^{neg}CD3+) in asthmatics

The nonsmoking asthmatics had a significantly higher density of putative effector T-cells (CD25+Foxp3^{neg}CD3+) compared to all groups (table 2, figure 3b). Although the effector T-cell density in smoking asthmatics was similar to smoking healthy workers group based data revealed that both asthmatic groups had an increased percentage of effector T-cells (of all CD25+ T-cells) compared to their respective control groups (figure 4).

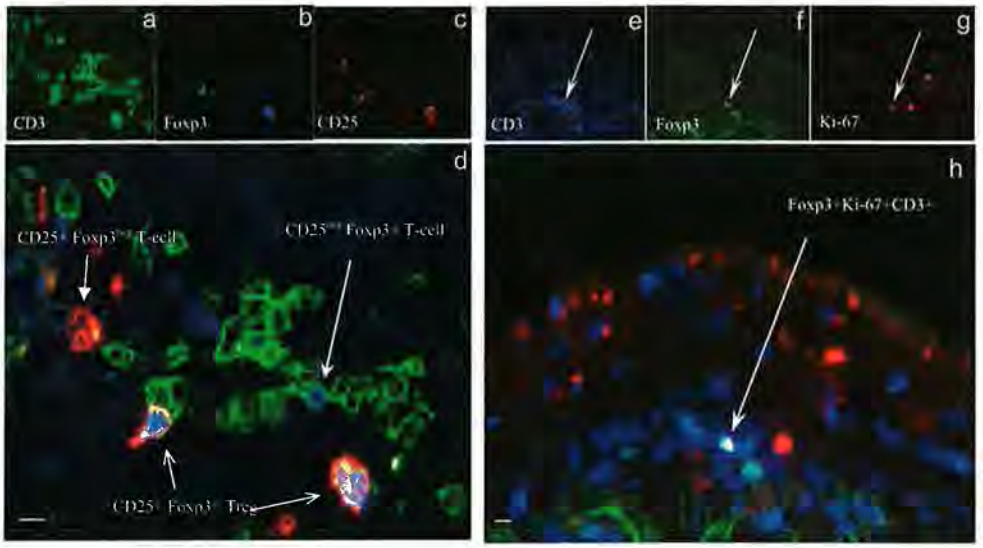

FIGURE 2. *a-d): Triple-colour immunohistofluorescent staining for CD3 (a, green), Foxp3 (b, blue nuclear staining) and CD25 (c, red). The triple, CD25+Foxp3+CD3+ labelled cells (d) represent putative regulatory T-cells and appear yellow (red and green fluorescence) with blue nuclear Foxp3-positivity. Blue nuclear Foxp3-positivity was also noted in several CD25negative green CD3+ T-cells (d). CD25-positivity on CD3+ T cells which did not express Foxp3 (CD25+Foxp3^{neg}CD3+), are yellow without blue nuclear positivity and represent putative effector T-cells (d). In e-h) is triple-colour immunohistofluorescent staining for CD3 (e, blue), Foxp3 (f, green) and Ki-67 (g, red nuclear staining) visualized. A proliferating (Ki-67+) Foxp3+CD3+ T-cell (arrow) is indicated in h. Note several proliferating (Ki-67+) epithelial cells. Multi-colour immunohistofluorescent staining of bronchial cryo-sections from a nonsmoking asthmatic patient. Single colour images were captured with a MicroMax CCD digital camera system and the AnalySIS Soft Imaging Systems produced the pseudo-coloured images. Internal scale bar = 10μm.*

The majority of CD25+ cells was non T-cells

It has previously been assumed that the majority of the increase in bronchial CD25+ cells in asthmatics reflected CD25+ T-cells. However, by using multi-colour immunohistofluorescence, the present study reveals that the majority of the bronchial CD25+ cells actually was non-T-cells (CD3^{neg}) in all groups (medians 71-92 %). The density of CD25+ non-T-cells was similar in nonsmoking (median 65 cells·mm^{-2}, range 51-115) and smoking asthmatics (median 53, range 21-136).

Increased HLA-DR+ T-cell density in asthmatics

HLA-DR+ T-cell density was increased in nonsmoking asthmatics compared to the nonsmoking controls (table 2). Triple-labelling revealed that HLA-DR was mainly expressed on CD8+ T-cells (medians 79-93 %). The distribution of HLA-DR on CD8+ or CD4+ T-cells was calculated by use of the CD4/CD8 ratio and group based data. Although the HLA-DR+CD8+ T-cell density was significantly higher in nonsmoking compared to smoking asthmatics, the density was also increased in smoking asthmatics compared to controls (figure 5). Even the density of

CD4+ HLA-DR+ T-cells was significantly increased in nonsmoking asthmatics compared to the other groups (figure 5).

Bronchial T-cells proliferate in asthma

Both the density and percentage of proliferating T-cells (Ki-67+CD3+) were increased in nonsmoking asthmatics (table 2). These proliferative T-cells were predominantly observed as scattered cells in the mucosa (figure 2e-h). Triple-labelling revealed that approximately half (48 %) of these subepithelial proliferative T-cells in nonsmoking asthma co-expressed CD8, the remaining being $CD8^{neg}$ and thus CD4+ T-cells. The distribution of Ki-67 on CD8+ or CD4+ T-cells was calculated by use of the CD4/CD8 ratio and group based data. The density of both CD4+Ki-67+ and CD8+Ki-67+ T-cells was significantly increased in nonsmoking asthmatics compared to all the other groups (figure 5).

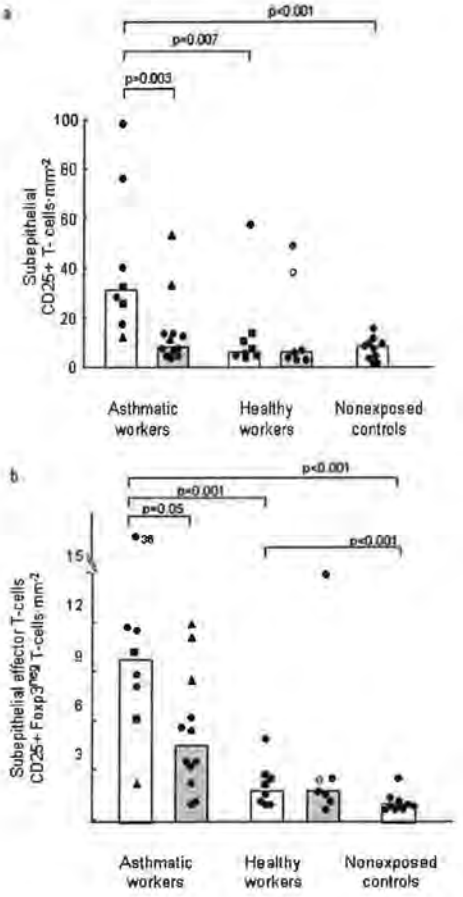

FIGURE 3. *Individual subepithelial cell density (cells·mm^{-2}) of a) CD25+ T-cells, and b) CD25+Foxp3^{neg} T-cells (effector T-cells) in nonsmoking (□) and smoking (■) asthmatic workers, healthy workers and nonexposed controls. The bars represent median values. ▲: treatment with inhaled corticosteroids; ■ ex-smokers; ○ convalescent subject; ● : other individuals.*

Bronchial proliferation of Foxp3+ T-cells

Ten samples from nine subjects with high T-cell proliferative response were selected for detailed triple immunohistofluorescent labelling to examine whether Foxp3+ T-cells proliferated in the bronchial mucosa. Distinct nuclear Ki-67+ labelling were observed on 24 % of 121 subepithelial Foxp3+ T-cells (figure 2e-h) and on nine out of 13 intraepithelial Foxp3+ T-cells.

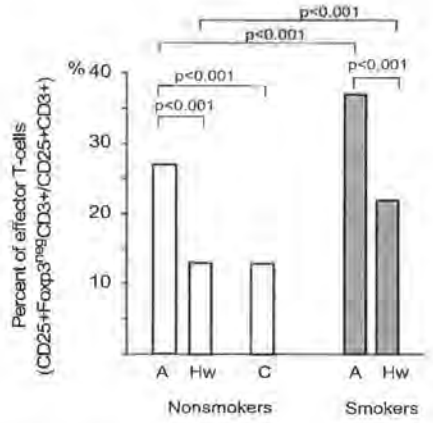

FIGURE 4. *Group based data (Chi-Square test). Percentage of effector T-cells (of CD25+ T-cells) in nonsmoking (□) and smoking (■) asthmatic workers (A), healthy workers (Hw) and nonexposed controls (C).*

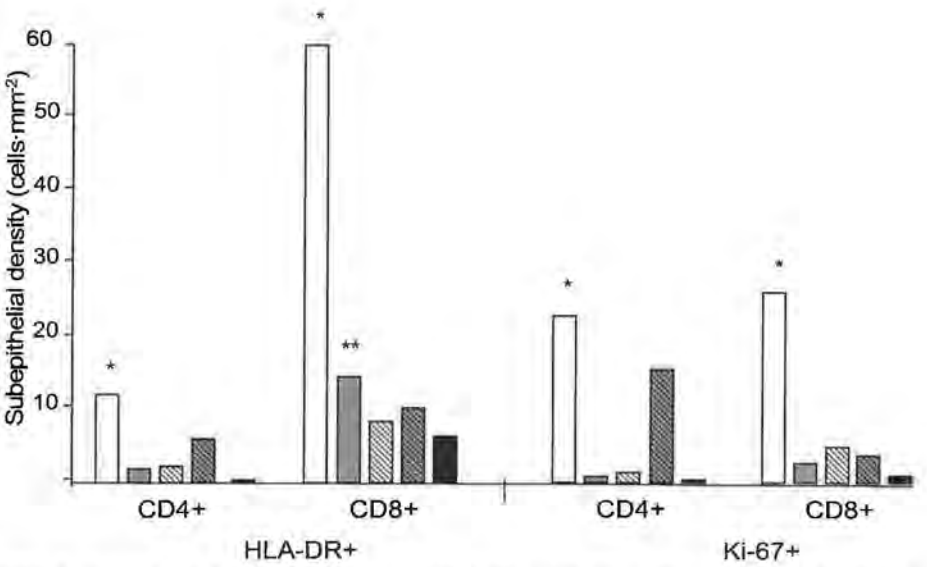

FIGURE 5. *Group based data of the expression of HLA-DR and Ki-67 on CD4+ and CD8+ T-cells in bronchial mucosa in nonsmoking asthmatics (□), smoking asthmatics (■), nonsmoking healthy workers (▨), smoking healthy workers (■) and nonsmoking nonexposed controls (■). * $p< 0.05$ compared to all groups ** $p< 0.05$ compared to the nonsmoking control groups. When the convalescent subject is excluded from the smoking healthy workers group, the density decreases from 10.3 to 6.1 cells·mm^{-2} in this group and the difference between smoking asthmatics and smoking healthy workers then became significant ($p=0.005$).*

There were few proliferative T-cells in the controls, except for one smoking healthy worker who had a strikingly high density of proliferative T-cells. By inclusion, he had no respiratory symptoms, normal blood samples, and denied any respiratory infection the last weeks. When asked later on, facing the present observation, he admitted having some cough and sore throat several days prior to the investigation. Thus, he was probably recovering from a respiratory tract infection which can explain the particular strong proliferative activity. All statistics were performed with and without this convalescent subject, but his inclusion did not change the main results. His data are identified in all scatter diagrams.

DISCUSSION

The study revealed several, actually novel phenotypic characteristics of the bronchial leukocyte infiltration in nonsmoking and smoking subjects with asthma. Primarily, the asthma-associated increase in subepithelial CD25+ T-cells observed in nonsmoking asthmatics was not observed in smoking asthmatics. The majority of the bronchial CD4+CD25+ T-cells co-expressed Foxp3, as described in peripheral blood [16, 18] and BAL [16], presumably reflecting Tregs. The increased Treg density in nonsmoking asthmatics was not only a consequence of the increased CD4+ T-cell density [3], as the percentage of Tregs was increased as well. This is somewhat in contrast to the reduced percentage of Treg in BAL from untreated asthmatic children, which was unable to inhibit Th2-immune responses, a features that returned to normal after inhaled corticosteroid treatment [16]. This was taken to reflect reduced number of bronchial Tregs [14], similar to atopic dermatitis [20]. However, the transepithelial migration of Tregs may also

have been suppressed during active inflammation, increasing the effector/ regulatory T-cell ratio in BAL.

Although several recent studies indicate that Tregs may inhibit Th2 responses and protect against allergy and asthma [15], Tregs may predominantly inhibit Th1 responses, perhaps depending on Foxp3 expression levels [21]. The predominance for Th1 inhibition may even facilitate Th2 type of inflammation, as it reduces Th1-induced Th2 inhibition. Interestingly, mouse CD4+CD25+Foxp3+ Tregs were shown to upregulate airway Th2 cell-mediated allergic inflammation [22]. Thus, Treg-induced T-cell inhibition appears to have a Th1>Th2 inhibitory hierarchy.

The majority of Foxp3+ T-cells did actually not express CD25. The functional property of the CD4+CD25^{neg} Foxp3+ T-cell phenotype has not been fully examined, but these cells appear not to be as inhibitory as CD25+ Tregs. Although there are some discrepancies concerning methodology [23], even newly activated T-cells may express some Foxp3 [24], but these CD25^{neg}Foxp3+ T-cells were unable to inhibit T-cell proliferation or cytokine production [24]. The Foxp3 expression level may actually regulate Treg function, as inhibited Foxp3 expression in a transgenetic mouse model, induced Tregs which only inhibited Th1, and not Th2 type of cytokine production [21]. Taken together, this may partly explain why the increased subepithelial Treg density in the present study was unable to prevent asthma-induced inflammation and mucosal T-cell proliferation in the nonsmoking asthmatics. Tregs are, moreover, generally considered to be anergic *in vitro*. However, a relatively large fraction (24 %) of the subepithelial Foxp3+ T-cells in the present study was in a proliferative state (Ki-67+), which is in accordance with the rather high proliferative indexes observed *in vivo* for murine [25, 26] and human peripheral blood [27] Tregs. Thus, there appears to be an ongoing activation of both the Treg and the effector T-cell system, where the balance between them may modulate bronchial inflammation. Although the T-cells may have been induced to proliferate locally in the bronchial mucosa, it can not be excluded that the Ki-67+ T-cells represented newly arrived cells, which had been induced to proliferate in organized lymphoid tissue 6-8 hours prior to biopsy sampling.

Although smoking asthmatics did not have a parallel increase in bronchial Treg density, the percentage of CD25+ T-cells that expressed Foxp3 was reduced in both asthmatic groups, resulting in an increased effector/regulatory T-cell ratio. However, it may be difficult to argue that such slightly increased effector/regulatory T-cell ratio could be the driving force inducing asthma in smoking asthmatics. It can therefore not be excluded that the predominant pathophysiology in asthmatic smokers may be different and less dependent on activated CD4+ T-cells. However, the ability to subdivide the CD4+CD25+ T-cell subset into Foxp3+ (Tregs) and Foxp3^{neg} (effector T- cells) revealed, nevertheless that subepithelial CD4+ T-cells may actually be involved in smoking asthmatics as well, even though the bronchial density of CD25+ T-cells was similar to controls [3].

Not only CD4+ T-cells, but also the CD8+ T-cell subset appeared activated in the nonsmoking asthmatics, reflected by the increased HLA-DR expression on subepithelial CD8+ T-cells. The activated HLA-DR+CD8+ T-cells (reviewed in [28]) may either have produced or responded to cytokines like TNF-α [29] or proliferated, as some expressed Ki-67.

Occupational exposure in the potroom may induce a bronchial inflammation even in asymptomatic workers, as this group had a slightly increased bronchial density and percentage of subepithelial effector T-cells.

This is in line with the previously reported increased reticular basement membrane thickness and increased subepithelial eosinophil density in these subjects [8].

In conclusion, the increased subepithelial density of Tregs (CD4+CD25+Foxp3+ CD3+) in nonsmoking asthmatics was apparently unable to fully inhibit the bronchial inflammation, as the density of proliferative (Ki-67+) T-cells, effector T-cells (CD4+CD25+FOXp3^{neg}CD3+) and activated (HLA-DR+) T-cells were increased. Although asthmatic smokers were almost devoided of the asthma-associated accumulation of activated bronchial T-cells, the decreased expression of Foxp3 in CD25+ T-cells, resulting in an increased effector/regulatory T-cell ratio, suggested that CD4+ T-cells participate in the immunopathology of asthma in smokers as well.

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REFERENCES

(1) Kongerud J, Boe J, Soyseth V, Naalsund A, Magnus P. Aluminium potroom asthma: the Norwegian experience. Eur Respir J 1994; 7:165-72.

(2) Mapp CE, Boschetto P, Maestrelli P, Fabbri LM. Occupational Asthma. Am J Respir Crit Care Med 2005; 172:280-305.

(3) Sjaheim T, Kongerud J, Bjortuft O, Drablos PA, Malterud D, Halstensen TS. Reduced bronchial CD4+ T-cell density in smokers with occupational asthma. Eur Respir J 2006; 28:1138-44.

(4) Cohn L, Elias JA, Chupp GL. Asthma: Mechanisms of Disease Persistence and Progression. Annual Review of Immunology 2004; 22:789-815.

(5) Azzawi M, Bradley B, Jeffery PK, Frew AJ, Wardlaw AJ, Knowles G, et al. Identification of activated T lymphocytes and eosinophils in bronchial biopsies in stable atopic asthma. Am Rev Respir Dis 1990; 142:1407-13.

(6) Bentley AM, Menz G, Storz C, Robinson DS, Bradley B, Jeffery PK, et al. Identification of T lymphocytes, macrophages, and activated eosinophils in the bronchial mucosa in intrinsic asthma. Relationship to symptoms and bronchial responsiveness. Am Rev Respir Dis 1992; 146:500-6.

(7) Bentley AM, Maestrelli P, Saetta M, Fabbri LM, Robinson DS, Bradley BL, et al. Activated T-lymphocytes and eosinophils in the bronchial mucosa in isocyanate-induced asthma. J Allergy Clin Immunol 1992; 89:821-9.

(8) Sjaheim T, Halstensen TS, Lund MB, Bjortuft O, Drablos PA, Malterud D, et al. Airway inflammation in aluminium potroom asthma. Occup Environ Med 2004; 61:779-85.

(9) Saetta M, Turato G, Maestrelli P, Mapp CE, Fabbri LM. Cellular and structural bases of chronic obstructive pulmonary disease. Am J Respir Crit Care Med 2001; 163:1304-9.

(10) Kay AB. The role of T Lymphocytes in Asthma. Chem Immunol Allergy 2007; 91:59-75.

(11) O'Sullivan S, Cormican L, Faul JL, Ichinohe S, Johnston SL, Burke CM, et al. Activated, cytotoxic CD8(+) T lymphocytes contribute to the pathology of asthma death. Am J Respir Critical Care Med 2001; 164:560-4.

(12) Yagi H, Nomura T, Nakamura K, Yamazaki S, Kitawaki T, Hori S, et al. Crucial role of FOXP3 in the development and function of human CD25+CD4+ regulatory T cells. Int Immunol 2004; 16:1643-56.

(13) Chatila TA. Role of regulatory T cells in human diseases. J Allergy Clin Immunol 2005; 116:949-59.

(14) Umetsu DT, DeKruyff RH. The regulation of allergy and asthma. Immunological Reviews 2006; 212:238-55.

(15) Larche M. Regulatory T Cells in Allergy and Asthma. Chest 2007; 132:1007-14.

(16) Hartl D, Koller B, Mehlhorn AT, Reinhardt D, Nicolai T, Schendel DJ, et al. Quantitative and functional impairment of pulmonary CD4+CD25hi regulatory T cells in pediatric asthma. J Allergy Clin Immunol 2007; 119:1258-66.

(17) Lee JH, Yu HH, Wang LC, Yang YH, Lin YT, Chiang BL. The levels of CD4+CD25+ regulatory T cells in paediatric patients with allergic rhinitis and bronchial asthma. Clin Exp Immunol 2007; 148:53-63.

(18) Shi HZ, Li S, Xie ZF, Qin XJ, Qin X, Zhong XN. Regulatory CD4+CD25+ T lymphocytes in peripheral blood from patients with atopic asthma. Clin Immunol 2004; 113:172-8.

(19) Sullivan P, Stephens D, Ansari T, Costello J, Jeffery P. Variation in the measurements of basement membrane thickness and inflammatory cell number in bronchial biopsies. Eur Respir J 1998; 12:811-5.

(20) Verhagen J, Akdis M, Traidl-Hoffmann C, Schmid-Grendelmeier P, Hijnen D, Knol EF, et al. Absence of T-regulatory cell expression and function in atopic dermatitis skin. J Allergy Clin Immunol 2006; 117:176-83.

(21) Wan YY, Flavell RA. Regulatory T-cell functions are subverted and converted owing to attenuated Foxp3 expression. Nature 2007; 445:766-70.

(22) Suto A, Nakajima H, Kagami SI, Suzuki K, Saito Y, Iwamoto I. Role of CD4+ CD25+ Regulatory T Cells in T Helper 2 Cell-mediated Allergic Inflammation in the Airways. Am J Respir Crit Care Med 2001; 164:680-7.

(23) Tran DQ, Ramsey H, Shevach EM. Induction of FOXP3 expression in naive human CD4+FOXP3 T cells by T-cell receptor stimulation is transforming growth factor-{beta} dependent but does not confer a regulatory phenotype. Blood 2007; 15;110:2983-90.

(24) Allan SE, Crome SQ, Crellin NK, Passerini L, Steiner TS, Bacchetta R, et al. Activation-induced FOXP3 in human T effector cells does not suppress proliferation or cytokine production. Int Immunol 2007; 19:345-54.

(25) Fisson S, Darrasse-Jeze G, Litvinova E, Septier F, Klatzmann D, Liblau R, et al. Continuous Activation of Autoreactive CD4+ CD25+ Regulatory T Cells in the Steady State. J Exp Med 2003; 198:737-46.

(26) Walker LSK, Chodos A, Eggena M, Dooms H, Abbas AK. Antigen-dependent Proliferation of CD4+ CD25+ Regulatory T Cells In Vivo. J Exp Med 2003; 198:249-58.

(27) Vukmanovic-Stejic M, Zhang Y, Cook JE, Fletcher JM, McQuaid A, Masters JE, et al. Human CD4+ CD25hi Foxp3+ regulatory T cells are derived by rapid turnover of memory populations in vivo. J Clin Invest 2006; 16:2423-33.

(28) Diedrichs M, Schendel DJ. Differential surface expression of class II isotypes on activated CD4 and CD8 cells correlates with levels of locus-specific mRNA. J Immunol 1989; 142:3275-80.

(29) Kamoun M, Zerva L, Sloan S, Zmijewski C, Monos D, Trinchieri G. Induction of HLA class II molecules on human T cells: relationship to immunoregulation and the pathogenesis of AIDS. DNA Cell Biol 1992; 11:265-8.