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Induction of a non-allergic inflammation in the human respiratory tract by organic dust

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To my family

Original Articles

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I. Larsson B-M, Palmberg L, Malmberg P O, Larsson K. Effect of exposure to swine dust on levels of IL-8 in airway lavage fluid. *Thorax* 1997; 52:638-642.

II. O´Sullivan S, Dahlén S-E, Larsson K, Larsson B-M, Malmberg P, Kumlin M, Palmberg L. Exposure of healthy volunteers to swine house dust increases formation of leukotrienes, prostaglandin D_2, and bronchial responsiveness to methacholine. *Thorax* 1998; 53:1041-1046.

III. Larsson B-M, Sundblad B-M, Larsson K, Dahlén S-E, Kumlin M, Palmberg L. Effects of the 5-lipoxygenase inhibitor zileuton on airway responses to inhaled organic dust in healthy subjects. Submitted

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V. Palmberg L, Larsson B-M, Malmberg P, Larsson K. Induction of IL-8 production in human alveolar macrophages and human bronchial epithelial cells *in vitro* by swine dust. *Thorax* 1998; 53:260-264.

VI. Larsson B-M, Larsson K, Malmberg P, Palmberg L. Gram positive bacteria induce IL-6 and IL-8 production in human alveolar macrophages and epithelial cells. *Inflammation* 1999; 23:217-230.

Abbreviations

AA	Arachidonic acid, eicosa-5,8,11,14-tetraenoic acid
BAL	Bronchoalveolar lavage
BHR	Bronchial hyperresponsiveness
COPD	Chronic obstructive pulmonary disease
COX	Cyclooxygenase
Cys-LT	Cysteinyl-leukotrienes (LTC_4, LTD_4, LTE_4)
ELISA	Enzyme-linked immunosorbent assay
EIA	Enzyme immunoassay
FEV_1	Forced expiratory volume in one second
FVC	Forced vital capacity
IgE	Immunoglobulin E
IL	Interleukin
LPS	Lipopolysaccharide
NO	Nitric oxide
NSAIDs	Non-steroidal anti-inflammatory drugs
ODTS	Organic dust toxic syndrome
$PD_{20}FEV_1$	Cumulative provocation dose of methacholine causing a 20% decrease in FEV_1
PEF	Peak expiratory flow
PG	Prostaglandin
PGHS	Prostaglandin endoperoxide H synthase
SEM	Standard error of the mean
TNF	Tumour necrosis factor
VC	Vital capacity
5-LO	5-lipoxygenase
5-HPETE	5-hydroperoxy-eicosatetraenoic acid

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1. Introduction

1.1 Background

Farmers have an increased prevalence of respiratory symptoms and lung diseases, which has been known for centuries. One of the first scientific reports concerning occupational lung disease within farming, was written in the beginning of the 18th century by Bernardino Ramazzini (Sakula, 1983). In this publication Ramazzini proposed that farming was connected with respiratory problems among the workers. The farming environment is not only noxious to humans. Thus pulmonary lesions have *e.g.* frequently been found in pigs at slaughter (Bahnson et al., 1992). Consequently, there is a need for studies regarding health problems in farming environment. This thesis is, however, focused on the human aspect.

1.2 Exposure levels in swine confinement buildings

The swine confinement building is a hazardous working environment. The farmers are exposed to an aerosol containing constituents such as feed particles, micro-organisms originating from faecal material, swine dander, moulds, pollen, insect parts etc (Donham et al., 1986). Gas exposure is also a concern and ammonia, carbon dioxide, methane and hydrogen sulphide are detected in the air of swine confinement buildings, although often below hygienic threshold levels (Von Essen et al., 1999). Dust levels ranging from 1.7 to 21 mg/m^3 have been reported in swine farms with corresponding endotoxin concentrations ranging from less than 0.1 and up to 1.9 μg/m^3 (Attwood et al., 1987; Crook et al., 1991; Donham et al., 1986; Duchaine et al., 2000; Haglind et al., 1987; Heederik et al., 1991; Holness et al., 1987). Endotoxin, often referred to as lipopolysaccharide (LPS), is a cell wall constituent of Gram-negative bacteria (Rietschel et al., 1992). Intensive working operations like weighing of pigs prior to slaughter are associated with high exposure levels to inhalable dust, often exceeding 20 mg/m^3, and endotoxin concentrations from 0.6 to 1.2 μg/m^3 (Larsson et al., 1994; Larsson et al., 2001; Wang et al., 1997; Wang et al., 1996). Of the microorganisms found in the confinement buildings, bacteria are most frequent and often found in concentrations between 10^5 and 10^6 cfu/m^3 while fungi generally are quite sparse (Attwood et al., 1987; Cormier et al., 1990; Crook et al., 1991; Donham et al., 1986; Duchaine et al., 2000; Haglind & Rylander, 1987). Gram-positive species are the dominating type of bacteria.

Ammonia is one of the most frequently measured gases in this context. Levels of ammonia ranging from less than 1 ppm to > 30 ppm have been reported (Attwood et al., 1987; Crook et al., 1991; Donham et al., 1985; Duchaine et al., 2000; Haglind & Rylander, 1987). The higher levels have probably been measured during the winter season when the ventilation is lowest (Duchaine et al., 2000).

1.3 Occupational health problems among swine farmers

Swine farmers are thus exposed to several potentially harmful agents during work. Studies of swine farmers health and respiratory problems have indeed confirmed that work in a swine confinement building is hazardous to the health of the farmers. Swine farmers report higher prevalence of respiratory symptoms than other farmers and non-farming subjects (table 1) (Brouwer et al., 1986; Donham et al., 1989; Dosman et al., 1987; Heederik et al., 1991; Iversen et al., 1988; Zejda et al., 1993). Work-related respiratory symptoms were reported by 52 % of the swine farmers in a study by Heederik et al (Heederik et al., 1991). Increased prevalence of symptoms during or shortly after work has been described in several health surveys. Cough has been reported by 17-64%, shortness of breath in 3-12 %, phlegm production in 16-46 % and clogged nose in 8-23 % of swine farmers (Brouwer et al., 1986; Choudat et al., 1994; Heederik et al., 1991; Zejda et al., 1993).

A number of studies have reported normal lung function (FEV_1; forced expiratory volume in one second; FVC; forced vital capacity) (Choudat et al., 1994; Cormier et al., 1991; Larsson et al., 1992; Pedersen et al., 1996; Rylander et al., 1990; Schwartz et al., 1992; Zejda et al., 1993; Zhou et al., 1991), although some studies have indicated airflow obstruction in swine farmers (Cormier et al., 1991; Zejda et al., 1993) in comparison with non-farming control subjects.

Iversen et al showed that symptom-free farmers (69 % swine farmers) had normal lung function, whereas farmers experiencing respiratory symptoms, had significantly lower FEV_1 and VC (vital capacity) than predicted (Iversen et al., 1989). There was also a tendency towards increased bronchial responsiveness to histamine in farmers with symptoms. Schwartz et al, did not find a relationship between impaired lung function and the presence of work-related or chronic symptoms in swine farmers (Schwartz et al., 1992). However, in that study, symptomatic swine farmers also had increased bronchial responsiveness compared to farmers with minor respiratory symptoms. The implication of these results seems to be that an impaired lung function already is established when respiratory symptoms appear. This is also supported by two other studies in which FEV_1/FVC was slightly reduced in symptomatic swine farmers compared to asymptomatic farmers (Pedersen et al., 1996; Vogelzang et al., 2000).

Small decreases in FEV_1 (0.4-3%) over a work-shift period have also been observed in swine farmers indicating acute effect of exposure (Donham et al., 1989; Haglind & Rylander, 1987; Reynolds et al., 1996; Rylander et al., 1990).

Table 1. Prevalence (%) of respiratory symptoms in swine farmers.

	Cough	Phlegm	Wheezing	Shortness of breath
Brouwer et al 1986[a)]	9.9	5.3	12.9	9.8
Dosman et al 1987[b)]	—	14.5**	27.4***	33.3***
Iversen et al 1988[c)]	37.2**	—	26.2**	23.1**
Donham et al 1989[c)]	23**	—	12	—
Heederik et al 1991[a)]	15.8	12.0	21.9	9.3
Zejda et al 1993[b,c)]	20.9*	28.5*	25.3*	—

* $p<0.05$, ** $p<0.01$, *** $p<0.001$
a) Comparison with control group not made
b) Comparison made with non-farmers
c) Comparison made with other farmers

In a longitudinal four year study in swine farmers, Senthilselvan et al found a FEV_1 decline of approximately 62 ml/year compared to ~ 50 ml/year in a non-farming control group. Forced vital capacity fell ~ 34 ml/year in swine farmers but only 10 ml/year in the control group (Senthilselvan et al., 1997). The additional decline in lung function among swine farmers compared to healthy controls was 36 ml/year for FEV_1 and 34 ml/year for FVC, after correcting for age, height, smoking and baseline lung function. In non-smoking swine farmers, a FEV_1 decline of 53 ml/year was found. The corresponding decrease in dairy farmers was 36 ml/year. This corresponds to an additional decline in FEV_1 of approximately 0.5 L during 30 years of work in a swine farm (Iversen et al., 2000). Decline in FVC, on the other hand was normal. These studies strongly indicate that swine farmers are at risk of developing chronic airflow obstruction.

Increased bronchial responsiveness in swine farmers has been demonstrated in several studies and there are data supporting an association between the presence of respiratory symptoms and increased bronchial responsiveness. Zhou and colleagues, reported increased bronchial responsiveness in swine farmers compared to non-farmers, and 90% of the farmers reported acute respiratory symptoms during work (Zhou et al., 1991). Bronchial responsiveness was also increased in swine farmers with higher prevalence of respiratory symptoms compared to swine farmers with minor symptoms (Schwartz et al., 1992). Choudat et al showed a high prevalence of respiratory symptoms and lower $PD_{20}FEV_1$ to methacholine in swine farmers compared to a reference group of non-farmers (Choudat et al., 1994). Larsson et al reported that healthy symptom-free farmers had normal bronchial responsiveness (Larsson et al., 1992). Pedersen et al could not detect a difference in farmers with and without respiratory symptoms regarding reactivity to inhaled histamine (Pedersen et al., 1996). In that study bronchial responsiveness in the farmers did not differ significantly from non-farming controls. In a longitudinal study by Vogelzang et al, the bronchial responsiveness expressed as PC_{20} increased by 1.36 dose doubling steps over a three years period (Vogelzang et al., 2000). The significance of this finding is, however, difficult to assess since no control group was included.

The prevalence of asthma in swine farmers is not higher than in non-farming subjects (Senthilselvan et al., 1997; Vogelzang et al., 1999b). This could be related to vocational selection. For example, swine farmers reported less symptoms of atopy during childhood than did control subjects. In a study by Zuskin et al, there was no difference in total IgE or positive skin prick tests for allergen extracts of floor material from the swine confinement building, swine feed, corn flour, swine hair, moulds and house-dust mites between swine farmers and a control group of industrial workers (Zuskin et al., 1991). Brouwer at al failed to demonstrate the presence of IgE antibodies against pig-derived antigens such as hair and urine in a population of 57 swine farmers, although IgG antibodies against both feed and pig-derived antigens were generally found (Brouwer et al., 1986). In a study of farmers, van Hage-Hamsten et al demonstrated low prevalences of positive skin prick tests for animal dander, moulds and pollen (0.7-2.7%) but higher for house dust mite (Der p, 6%) (van Hage-Hamsten et al., 1987).

Chronic bronchitis is defined as chronic productive cough persistent for more than 3 months/year for at least two consecutive years. A common feature of chronic bronchitis is a significant influx of neutrophils into the airway lumen (Hoidal, 1994). The prevalence of chronic bronchitis is higher in swine farmers than in the normal population and other farmers (table 2) (Cormier et al., 1991; Donham et al., 1989; Dosman et al., 1987; Iversen et al., 1988; Vogelzang et al., 1999b; Zejda et al., 1993). The increased prevalence among farmers was not due to smoking habits.

Table 2. Prevalence (%) of chronic bronchitis in swine farmers. For statistical analysis smoking habits are taken into consideration.

	Swine farmers	Farmers-no pigs	Non-farmers
Dosman et al 1987	11.1*	—	7.7
Iversen et al 1988	32.0**	17.5	—
Donham et al 1989	38*-61***[a)]	13	—
Cormier et al 1991	17.5*	—	11.6
Zejda et al 1993	15.3*	7.2	5.7
Vogelzang et al 1999	20.2***	—	7.7

* $p<0.05$, ** $p<0.01$, *** $p<0.001$
a) Figures given for farmers with 1-13 respectively 14-30 years of work in swine farming.

Episodes of influenza-like symptoms are observed among farmers in connection with work and heavy exposure to organic dust with high microbial content. The condition is commonly referred to as organic dust toxic syndrome (ODTS) and is characterised by fever, chills, malaise, dry cough, headache, mild dyspnea and muscle pain (for review see (Von Essen et al., 1990)). Influx of neutrophils has also been demonstrated by bronchoalveolar lavage. The onset of symptoms is commonly observed within 4-12 hours after exposure and has normally disappeared 24 hours after exposure without any need for medical treatment and without sequel. Chest X-ray is usually normal. The incidence of ODTS among

Swedish farmers has been reported to 1 per 100 farmers every year (Malmberg et al., 1988). Vogelzang and colleagues reported an ODTS prevalence of 15.4 % among farmers with less than 5 years of swine farming, whereas, in farmers with longer experience the ODTS prevalence was only 5.9% (Vogelzang et al., 1999a). This difference was, however, not statistically significant. Nevertheless these findings indicate that the swine farmers, by increased working experience, have learnt to avoid heavy exposure situations or that adaptive mechanisms to repeated exposures occurs. It may also reflect a "healthy worker effect", *i.e*. farmers with health problems leave pig farming.

Organic dust toxic syndrome shares many clinical features with allergic alveolitis, sometimes called hypersensitivity pneumonitis or farmer's lung, which is a respiratory disorder associated with exposure to organic dust. The yearly incidence of allergic alveolitis is only 2-3/10000 farmers, and the symptoms are induced by repeated exposure to organic dust. There are several distinctions between ODTS and allergic alveolitis. In allergic alveolitis chest X-ray often shows signs of pulmonary infiltrates and precipitating antibodies against antigens present in the dust are detected in serum. The recovery from an attack of allergic alveolitis often takes several months and medical treatment with corticosteroids may be needed. Elevated numbers of lymphocytes are observed in BAL fluid, but in the acute phase, increased levels of neutrophils can also be detected. In addition, there is a risk for development of chronic lung function impairment in allergic alveolitis.

In 1990 Pedersen et al observed macroscopic signs of inflammation the bronchial mucosa in 17 out of 26 non-smoking swine farmers with normal lung function (Pedersen et al., 1990). Moreover, farmers with inflamed mucosa demonstrated increased bronchial responsiveness to histamine (PC_{20}=14 mg/ml) compared to in those with macroscopically normal mucosa (PC_{20}=30 mg/ml). Evidence for an ongoing inflammation in the lower airways of swine farmers has been supplied by a number of studies using the bronchoalveolar lavage (BAL) technique. A study by Larsson et al, compared 20 healthy, non-smoking swine farmers with a control group comprised of 20 non-smoking office workers. The swine farmers had increased concentrations of neutrophils in BAL fluid, while the levels of alveolar macrophages, lymphocytes and eosinophils were similar in the two groups (Larsson et al., 1992). There were no differences between the groups regarding lung function or bronchial responsiveness to methacholine. Skin-prick tests with a panel of common aeroallergen and allergen extracts from the swine confinement environment were negative in all but one subject. In a subsequent, study Pedersen et al compared 27 non-smoking swine farmers of whom 8 had mild chronic bronchitis, with 53 healthy, non-smoking non-farmers. FEV_1 was similar in both groups and bronchial reactivity to histamine tended to be somewhat, although not statistically, higher among farmers (Pedersen et al., 1996). The lower airway mucosa presented more signs of inflammation such as oedema, erythema and secretions in swine farmers compared to controls ($p<0.01$), and this was related to the proportion of neutrophils in BAL fluid ($r=0.47$,

p=0.01). Interestingly, there was no correlation between macroscopic inflammatory signs of the mucosa and respiratory symptoms. The percentage of neutrophils and lymphocytes was significantly higher in swine farmers. The alveolar macrophages recovered from BAL fluid displayed enhanced spontaneous migration as well as chemotaxis in swine farmers compared to the control group. It is possible that the oedema formation and the cellular influx lead to increased narrowing of the airway lumen and thereby enhanced effect of bronchoconstricting agents such as histamine and methacholine.

The two above mentioned studies using the BAL technique (Larsson et al., 1992; Pedersen et al., 1996) are, however, in conflict with a study by Schwartz et al, in which no increase in BAL fluid neutrophils or other cell types could be detected in swine farmers (Schwartz et al., 1992). The probable explanation of this discrepancy is that cellular distribution in the study by Schwartz et al was presented as percentage values, with no consideration taken to the total cell concentration. Hence, the conclusion that the cellular population was as similar in swine farmers and the controls could very likely be erroneous. This particular study also indicated an increased thickness of the basement membrane of the bronchial wall in symptomatic swine farmers.

When comparing induced sputum from 24 swine farmers and 14 urban citizens, with no history of allergy or asthma, the concentration of macrophages was significantly higher in swine farmers, whereas the number of neutrophils were similar between the groups (Von Essen et al., 1998). The differences between BAL fluid and induced sputum could be related to sampling of different compartments of the lung, the more distal airways with BAL and the proximal airways with sputum technique. Eosinophils could not be detected at all in the sputum samples. Exhaled nitric oxide was slightly, but significantly increased in these farmers, also indicative of an inflammatory process.

In summary, swine farmers report increased prevalences of chronic respiratory symptoms as well as chronic bronchitis. The absence of IgE antibodies to environmental antigens and no increased concentrations of eosinophils, together with obstructive lung function impairment in some studies, indicate that work in swine confinement buildings may not be associated with an increased risk for development of asthma, but rather a condition more similar to chronic obstructive pulmonary disease (COPD).

1.4 The inflammatory process

1.4.1 Cytokines

Inflammation is the host response to physical injury, antigenic stimuli or invading microorganisms. Main features of the reaction are elevated blood flow, increased vascular permeability leading to oedema formation and recruitment of inflammatory cells such as neutrophils to the site of inflammation. This is a complex process highly regulated by different molecules. Cytokines consist of a group of pleiotropic signalling peptides that can exert their effects in an autocrine (on the cell source), paracrine (on neighbouring cells) or an endocrine (distributed to

target cell by circulation) fashion. Interleukin-1 (IL-1) and tumour necrosis factor (TNF) are cytokines involved in pro-inflammatory events, possessing a wide range of biological effects connected to the early inflammatory host response. Their effects are often coinciding and sometimes also synergistic although they have well-defined individual receptors (Oppenheim et al., 1990). TNF exists in two forms, TNF-α and TNF-β, also called lymphotoxin (Porter, 1990). They share 30% of the amino acid sequence and bind to the same receptor and they also elicit similar biological effects (Ulich, 1993). Henceforth we will focus on the effects of TNF-α, which is produced by a number of cells of which monocytes and macrophages are major sources. Also natural killer cells, neutrophils, lymphocytes, keratinocytes, astrocytes, tumour cells, endothelial cells, epithelial cells, mast cells and smooth muscle cells are TNF-α producers (Barbara et al., 1996; Brouckaert et al., 1996; Khair et al., 1996), and these cells are also capable of secreting IL-1 (Aksamit et al., 1993). Stimuli like LPS, IL-1 and TNF-α induce production of IL-1 and TNF-α. Injection of LPS in humans induces a sequential order of cytokine release, where TNF-α increases in serum, with a maximal level after 90 minutes (Hesse et al., 1988). This is followed by a peak in IL-1 concentration within short. Another study, where primates were given a lethal dose of live *E. coli* reported a similar successive cytokine release prompted by TNF-α secretion, followed by increasing levels of IL-1 and IL-6 (Fong et al., 1989). When the monkeys were pre-treated with a monoclonal antibody directed against TNF-α, the subsequent cytokine release were attenuated, suggesting that TNF-α is necessary for both IL-1 and IL-6 production. In healthy, human subjects submitted to a exposure during three hours of weighing pigs, TNF-α in serum increased from a median value of 2.5 ng/L to10 ng/L, with peak values at 3-5 hours after the start of the exposure (Wang et al., 1996). IL-6 in serum increased significantly from 1.5 ng/L to 21.4 ng/L, peak levels were reached approximately 4-11 hours after the start of exposure. In general, a maximal IL-6 response was obtained 1-5 hours after the maximal TNF-α increase. IL-6 is a cytokine active in the inflammatory response albeit more belonging to the anti-inflammatory side since it has a negative feedback on IL-1 and TNF-α synthesis (Schindler et al., 1990). Essentially the same cell types serve as sources for IL-6 as for synthesis of IL-1 and TNF-α (Zitnik et al., 1993). One of the more prominent pro-inflammatory effects of IL-1 and TNF-α are their ability to stimulate neutrophil migration into inflamed tissue. This is achieved by complex mechanisms including expression of P-selectins important for leukocyte rolling on endothelial cell walls. The migration process also require increased expression of adhesion molecules like ICAM-1 (intracellular adhesion molecule –1) on endothelial cells and their counterpart receptors on neutrophils, LFA-1 (lymphocyte associated antigen-1, CD11/CD18, α_L/β_2) and Mac-1 (macrophage associated antigen-1, CD11/CD18, α_M/β_2) (for review see (Meager, 1999; Wagner et al., 2000)).

Interleukin-1 and TNF-α are together with IL-6 involved in the induction of fever, thereby acting as pyrogens, although the exact course of events leading to increase in body temperature is not yet fully elucidated (Luheshi et al., 1996). The

mechanism is considered to involve induction of prostaglandin E_2 (PGE_2) synthesis by these cytokines, although there are some controversies and new theories are emerging (Blatteis et al., 1998). The actions of IL-6 also includes stimulation of IgG synthesis by B-lymphocytes and increased production of acute-phase proteins like CRP (C-reactive protein) and fibrinogen by hepatocytes (Zitnik & Elias, 1993). The pro-inflammatory cytokines IL-1 and TNF-α are involved in the induction of IL-8 synthesis from epithelial, endothelial and smooth muscle cells (Bédard et al., 1993; Lukacs et al., 1995; Standiford et al., 1990). Neutrophils, fibroblasts, monocytes and macrophages also contribute to IL-8 synthesis (Strieter et al., 1992; Strieter et al., 1993). Burns et al demonstrated that endothelial cells stimulated with *Borrelia burgdorferi* produced IL-8 regardless of the presence of IL-1 receptor antagonist and a neutralising antibody directed against TNF-α (Burns et al., 1997), suggesting an alternative pathway for IL-8 synthesis. IL-8 is a potent chemotactic factor for neutrophils and a member of the chemokine family (Leonard et al., 1990). Chemokines are divided in 4 subfamilies and IL-8 belongs to the CXC family, in which two cysteins are separated by a single amino acid (Kunkel, 1999). Other CXC neutrophil chemoattractants includes ENA-78 (epithelial neutrophil activating protein), NAP-2 (neutrophil activating protein-2) and GRO-α,β,γ (growth-regulated oncogene protein-α,β,γ). Increased IL-8 levels have been discerned in a number of inflammatory disorders like COPD, fibrotic lung diseases, rheumatoid arthritis and inflammatory bowel disease (Jatakanon et al., 1999; Mitsuyama et al., 1994; Szekanecz et al., 1998; Vaillant et al., 1996; Williams et al., 2001). Interleukin-8 also induces activation of neutrophils resulting in degranulation and induction of the respiratory burst system, increased expression of CD11/CD18 leukocyte integrins, stimulation of LTB_4 production and is also chemotactic for T-lymphocytes and basophils.

1.4.2 Arachidonic acid metabolites

Many metabolites originating from arachidonic acid (AA) are mediators of significance for the inflammatory response. The essential polyunsaturated fatty acid, arachidonic acid, is stored in phospholipids in different cell membranes. Biosynthesis of various arachidonic acid products is initiated when AA is liberated from the phospholipid stores by the action of different phospholipases, for instance cytosolic phosphoplipase A_2 ($cPLA_2$) (Glaser et al., 1993). A number of pro-inflammatory stimuli such as IL-1 or TNF-α can trigger this process and one key event involves increased intracellular Ca^{2+}, which in turn promotes the translocation of $cPLA_2$ from the cytosol to cellular membranes (Murakami et al., 1997)). The freed AA can then be further metabolised by different enzyme systems into leukotrienes, prostaglandins, lipoxins, thromboxanes and hydroxy-eicosatetraenoic acids. Leukotrienes are synthesised by the 5-lipoxygenase (5-LO) pathway (Samuelsson, 1983) (figure 1).

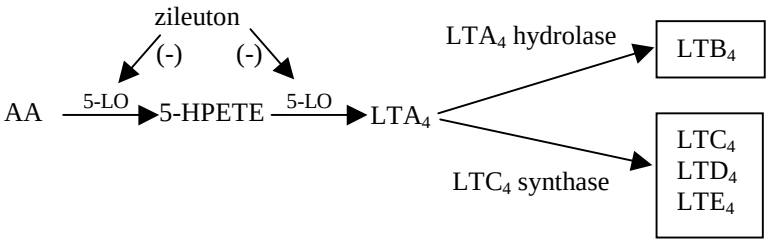


Figure 1. Biosynthesis of leukotrienes via 5-lipoxygenase pathway.

The first step is the formation of 5-HPETE (5-hydroperoxy-eicosatetraenoic acid) catalysed by 5-LO, which also catalyses the subsequent formation of the unstable intermediate LTA_4. The forthcoming fate of LTA_4 is either to be hydrolysed by LTA_4 hydrolase into LTB_4, or to be conjugated with glutathione, a reaction controlled by LTC_4 synthase, leading to formation of LTC_4. This compound can then be further metabolised into LTD_4 by γ-glutamyltranspeptidase, which removes glutamic acid, and into LTE_4 by dipeptidase that removes glycine from LTD_4. Leukotrienes C_4,D_4 and E_4 are collectively called the cysteinyl-leukotrienes (cys-LTs) due to their cysteine content and common biological properties. The 5-LO enzyme is present in neutrophils, eosinophils, basophils, monocytes, macrophages, mast cells and B-lymphocytes. Leukotriene A_4 hydrolase is present in a wide range of cells within the body (Haeggström, 2000), whereas LTC_4 synthase is restricted to a more selective group of cells such as eosinophils, basophils, mast cells, platelets, macrophages and monocytes (Lam et al., 2000). Cells lacking certain critical enzymes can be overcome this deficiency by transcellular biosynthesis. This is achieved by export of an intermediate product, such as LTA_4, from one cell type to surrounding cells in possession of additional enzymes, such as LTA_4 hydrolase and LTC_4 synthase, leading to further conversion of LTA_4 (Lindgren et al., 1993). In general, cells are committed to release either LTB_4 or cys-LTs. Thus, neutrophils and human alveolar macrophages mainly produce LTB_4, whereas mast cells and eosinophils release LTC_4 and monocytes/macrophages make both types (Henderson, 1994).

Leukotriene B_4 is as IL-8 a potent chemoattractant for neutrophils (Borgeat et al., 1990). LTB_4 and IL-8 share other biological effects such as activation and degranulation of neutrophils and increased integrin (CD11/CD18) expression leading to enhanced adhesion of neutrophils to the endothelium (Crooks et al., 1998). Leukotriene B_4 also induces production of IL-1, IL-6, IL-8 and TNF-α in monocytes and macrophages.

The biological actions of cys-LTs possess several phenomena that are typical for the inflammation observed in asthma. They have ability to recruit eosinophils, induce bronchoconstriction, increase vascular permeability leading to oedema formation, elevate mucus production and impair mucociliary clearance (Dahlén, 2000). The bronchoconstrictive effect of cys-LTs on human airways has been confirmed in a number of studies using selective 5-LO inhibitors or cys-LT receptor antagonists. The degree of cys-LT involvement seems to depend on type of stimulus (for review see (Drazen, 1998)). The airway response after exercise/

cold air challenge is only to some extent mediated by leukotrienes, but the aspirin induced airway obstruction seems to be totally mediated by leukotrienes. The 5-lipoxygenase inhibitor zileuton has demonstrated beneficial effects in asthmatic subjects. A long-term medication with zileuton (400-600 mg four times a day for 6 months) of subjects with mild to moderate asthma, resulted in lung function improvement measured as a 16% improvement of baseline FEV_1, which was significant compared to a placebo group (Liu et al., 1996). In a study by Meltzer et al, a two days treatment with zileuton (600 mg four times a day) attenuated the exercise-induced bronchoconstriction by 40% in patients with exercise-induced asthma (Meltzer et al., 1996). There are also studies supporting the hypothesis that leukotrienes are involved in the development of bronchial hyperresponsiveness in asthma. Asthmatic subjects treated with zileuton for 13 weeks (400-600 mg four times a day) demonstrated attenuated airway responsiveness to cold air (Fischer et al., 1995). Dahlén et al also displayed an additive effect of zileuton to glucocorti-costeroid treatment. In aspirin-intolerant asthmatic patients under continuously treatment with steroids, the bronchial responsiveness to histamine was reduced by 1.5 doubling doses after 6 weeks of zileuton treatment (600 mg q i d) (Dahlén et al., 1998). However, as zileuton in steroid treated asthmatics, have also been reported to have an acute effect on bronchial hyperresponsiveness to histamine and ultrasonic water that was dissociated from changes in baseline airway calibre (Dekhuijzen et al., 1997), it may have multiple actions that require further studies

Prostaglandins (PG) and thromboxanes (TX) are lipid mediators synthesised by the cyclooxygenase (COX) route. The cyclooxygenase, more correctly named prostaglandin endoperoxide H synthase, PGHS, has been identified in two isoforms, COX-1 and COX-2 (Smith et al., 1996). Most cells constitutively express COX-1, which in general catalyses the formation of PGs involved in physiological functions such as controlling the arterial blood pressure and the integrity of intestinal mucosa. Cyclooxygenase-2 on the other hand, is little expressed under normal conditions, but is induced in different cell types such as neutrophils, monocytes, macrophages and mast cells. This is often a consequence of activation by inflammatory stimuli, *e.g.* IL-1, TNF and LPS (Ley, 2001).

Both cyclooxygenases catalyse the reaction where AA is transformed into prostaglandin G_2 (PGG_2), which then serves as a substrate for the formation of PGH_2 (figure 2) (Smith et al., 1991).

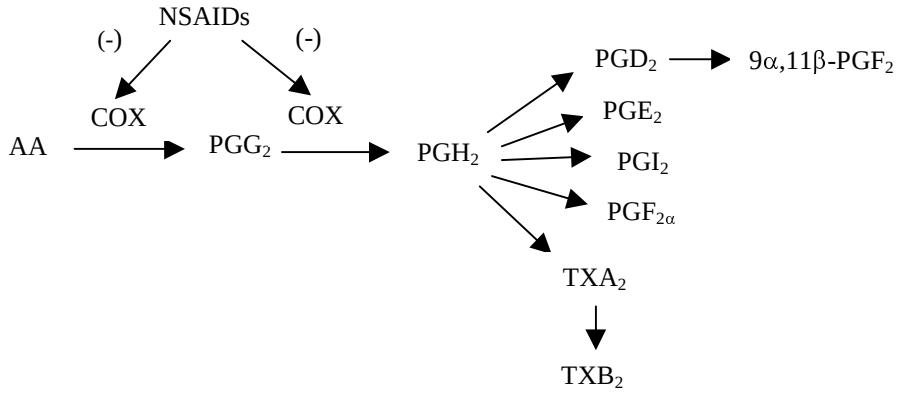


Figure 2. Biosynthesis of prostaglandins (PG) and thromboxanes (TX) via the cyclooxygenase pathway (COX).

The metabolism of PGH_2 can then continue in different directions. Non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin inhibit both cyclooxygenases, although to different extent.

Prostacyclin (PGI_2) is formed by PGI synthase and PGD_2/ E_2/$F_{2\alpha}$ are the product of the conversion of PGH_2 by PGD-, PGE-, and PGF-synthase, respectively. Thromboxane A_2 is also derived from the intermediate product PGH_2, a reaction driven by the enzyme thromboxane synthase (Smith et al., 1991). Thromboxane A_2 is a biologically active, highly unstable product and is non-enzymatically hydrolysed to TXB_2. The actions of TXA_2 include induction of vasoconstriction and platelet aggregation.

Prostaglandin D_2 is a mast cell mediator and a potent bronchoconstrictor (O´Sullivan, 1999). Prostaglandin D_2 may be used as a marker for mast cell activation especially *in vitro*, but as it is rapidly degraded into 9α,11β-PGF_2, this compound has been found a more useful marker of mast cell activation *in vivo* (O´Sullivan, 1999). Incidentally, 9α,11β-PGF_2 retains the bronchoconstrictive potency of PGD_2. The bronchoconstrictive effect of PGD_2 is counteracted by PGE_2 (Wenzel, 1997), which is a bronchodilator and a potent inhibitor of mast cell mediator release (Raud et al., 1988).

1.4.3 Nitric oxide

Nitric oxide (NO) is formed by the action of three isoforms of nitric oxide synthase (NOS), two constitutive forms, cNOS (endothelial NOS and neuronal NOS) and one inducible form, iNOS. Nitric oxide formed by the constitutive forms of NOS is involved in physiological functions. Nitric oxide has a vasodilatory effect on arterioles, thereby regulating blood pressure and may be an inhibitory neurotransmitter in non-adrenergic, non-cholinergic nerves (for review see (Barnes, 1995; Singh et al., 1997)). Inducible NOS is formed upon stimulation with LPS, ozone or pro-inflammatory cytokines like TNF-α, IL-1 or IFN-γ. The induction leads to production of NO levels that are almost 1000 times higher than by cNOS. The cytotoxic effect of NO is important in the host-defence reaction against invading microorganisms. Elevated levels of NO have been reported in

several inflammatory disorders. In ulcerative colitis increased colonic NO concentrations have been detected (Middleton et al., 1993) and in asthma exhaled NO is increased (Alving et al., 1993). In a recent report, it was claimed that exhaled NO levels are increased in patients with chronic bronchitis (Delen et al., 2000). However, in that study, the definition of the patients was controversial. The role of NO is dual. On one hand, it plays a role in regulatory functions in the normal situation and has beneficial effects in inflammation by *e.g.* bactericidal properties. On the other hand, excess production could induce a prolonged vasodilatation leading to extreme hypotension during septic reactions (Moncada et al., 1993). Nitric oxide could also have cytotoxic effects on cells leading to tissue destruction. In addition, NO seems to have a stimulatory effect on COX activity demonstrated by *in vitro* studies. Nitric oxide and PGs were released in a macrophage cell line stimulated with LPS. After treatment with a nonselective NOS inhibitor both NO and prostaglandin responses were attenuated (Salvemini, 1997).

1.4.4 Bronchial responsiveness

Bronchial hyperresponsiveness (BHR) is a hallmark of asthma and is believed to be causally related to airway inflammation. It is described as an enhanced bronchial response to constrictive stimuli (Sterk et al., 1993)). The degree of responsiveness is assessed by inhalation of increasing concentrations of broncho-constricting agents such as methacholine or histamine. The result is expressed as the cumulative dose or the concentration causing a 20% decrease of FEV_1 ($PD_{20}FEV_1$ or $PC_{20}FEV_1$). An alternative way to express the degree of bronchial reactivity is to calculate the slope of the dose-response curve obtained from the inhalation challenge. In normal subjects a plateau of the dose-response curve is reached, while in asthmatics a plateau level is not always observed (Woolcock et al., 1984).

Methacholine and histamine are directly acting bronchoconstrictors, *e.g.* they activate airway smooth muscle and possibly other elements (blood vessels) that cause bronchoconstriction. Another method to estimate the degree of bronchial responsiveness is by provocation with indirect stimuli supposed to cause broncho-constriction indirectly by induction of mediator release. Provocation with hyper-ventilation of dry air or exercise is two examples of indirect stimuli. A positive test is defined as 10% decrease in FEV_1 during a standardised challenge.

The pathophysiological basis of BHR is not yet concluded, but it is often assumed to be related to airways inflammation. It is obvious that several factors may cause this phenomenon. Airway inflammation with recruitment of eosino-phils, mast cells and neutrophils from the blood stream to the airways could be one important factor. The accumulated cells could subsequently release mediators that either could influence smooth muscle cells (PGD_2, cys-LTs, histamine) or confer to epithelial damage (proteinases, O_2-radicals etc) (O´Byrne et al., 2000; Pauwels et al., 1990). Many inflammatory mediators also increase the permea-bility of the epithelial layer, thereby making target receptors more accessible for

triggering factors (like methacholine). In addition, during an inflammatory situation where the airway mucosa is swollen due to oedema formation, the same degree of smooth muscle contraction will lead to a much more severe airway narrowing than in a normal, non-oedematous mucosa (Moreno et al., 1986)).

The thickening of the subepithelial membrane, lamina reticularis, and the shedding of epithelial cells observed in asthmatic airway inflammation, could be one explanation of the bronchial hyperresponsiveness in asthmatics. Hypertrophy of smooth muscle in asthma, and perhaps extended propensity to contract could also lead to bronchial hyperresponsiveness. The neural control system in the airways comprised of the adrenergic, cholinergic, and the non-adrenergic/non-cholinergic system, is involved in the regulation of contraction/relaxation of smooth muscle and an imbalance in stimulatory/inhibitory pathways may also influence the degree of bronchial responsiveness.

2. Aims of the Thesis

The general aim of the thesis was:

- To investigate the inflammatory process induced by exposure to swine confinement environment.

Specific aims were:

- To assess the role and effects of some chemotactic factors suggested to be involved in the inflammatory reaction.
- To find out whether a respiratory protection device influenced the outcome measures.
- To study if leukotrienes and mast cells contributed to organic dust induced airway inflammation.
- To elucidate the inflammatory responses found *in vivo* by the use of *in vitro* models for screening of possible pro-inflammatory agents in the swine confinement environment.

3. Subjects, Materials and Methods

3.1 Human studies *in vivo*

3.1.1 Subjects exposed in swine confinement buildings

The number and age of the participating subjects in each study is reported in table 3. They were all previously unexposed or only occasionally exposed to farming environment and residents in suburbs of Stockholm. All participants filled in a questionnaire in which they denied past or present symptoms of allergy and airways diseases. All had normal lung function.

Table 3. Age and gender distribution of subjects in human *in vivo* studies.

	Study I	Study II	Study III	Study IV
No. of subjects (men)	31 (16)	10 (2)	23 (12)	16 (14)
mean age (years)	31	39	27	24
(range)	(18-50)	(26-60)	(20-47)	(20-32)

3.1.2 Designs and main questions in exposure studies

I. Study I was performed in order to investigate airway release of the chemokine IL-8, following exposure while weighing pigs for three hours in a swine confinement building with 600-900 pigs. A possible relationship between increase of IL-8 levels and neutrophil concentration was also studied. Nasal and bronchoalveolar lavages were performed before and 7 respectively 24 hours after the start of the exposure. Personal samplers were used to assess exposure levels.

II. In study II the purpose was to evaluate if leukotrienes were released and mast cells activated during the airway inflammation induced by organic dust. Exposure was made in the same manner and took place in the same facility as in study I. In addition to the measurements mentioned in study I (except BAL), lung function and bronchial responsiveness to methacholine were measured prior and 7 hours after the start of the exposure. Urine samples were also collected at hourly intervals for approximately 12 hours during one day at three different occasions, five days before exposure, during the exposure day and the day after exposure.

III. An intervention study using the 5-lipoxygenase inhibitor zileuton was undertaken to evaluate a possible role of leukotrienes in the development of bronchial responsiveness in healthy subjects exposed to swine farming environment. The same measurements were performed as in study II. Exposure took place in a swine confinement facility, housing approximately 300 pigs.

IV. The impact of a respiratory protection device (Sundström® half mask) on health effects induced by exposure in a swine confinement building was determined. Sixteen subjects, of whom 7 were provided with a half mask, were exposed for three hours. The mask was supplied with a particle filter of P3 class, which ensures protection of all particles ranging from dust, smoke, fog, spray, asbest, bacteria and viruses. Effectiveness of the filters is tested according to the European Standard EN 143:1990. No gas filter was added to the mask. Exposure took place during a cleaning procedure using a high-pressure cleaner (water), after a completed breeding period when all the pigs were evacuated from the stable. During the exposure, a swine farmer was cleaning the stable with a high-pressure cleaner. The subjects stayed within a radius of 2 - 5 meters from the swine farmer. The same study protocol mentioned above was used (BAL and urine sampling were not included).

3.2 Cellular studies *in vitro*

3.2.1 Cells

For *in vitro* studies the human pulmonary epithelial carcinoma cell line A549 (American Type Culture Collection, Rockville, Maryland, USA), and normal human bronchial epithelial cells (NHBE) in primary culture (Clonetics Corporation, San Diego, California, USA) were used. Alveolar macrophages were obtained by bronchoalveolar lavage of healthy subjects.

3.2.2 Designs and main questions in cell studies

V. The capacity of IL-8 release after stimulation with crude swine house dust and different constituents (LPS, glucan, grain dust) was evaluated in A549 cells, normal human bronchial epithelial cells (NHBE) and alveolar macrophages.

VI. Gram-positive bacteria dominate the bacterial flora in swine house confinement buildings. Four Gram-positive bacteria (*Bacillus subtilis*, *Staphylococcus lentus*, *Staphylococcus hominis* and *Micrococcus luteus*) and one Gram-negative (*Escherichia coli*), were evaluated regarding their ability to induce cytokine release from A549 cells and alveolar macrophages. A bacteria-free supernatant was prepared from each bacterial strain and thereafter used for stimulation in cell culture.

3.3 Methods

Methods are briefly summarised below, for details see individual manuscripts.

3.3.1 Symptoms (study I, III and IV)

In study I and IV, symptoms like headache, chills, mental fatigue, muscle pain and malaise were recorded using a questionnaire with grades ranging from 1 to 5 (1 = no symptoms, 5 = severe symptoms). Only rates of 4 and 5 were classified as

significant. In study III the symptoms were recorded using a visual analogue scale (VAS) ranging from 0-100 mm.

3.3.2 Exposure (study I-IV)
IOM samplers with filter cassettes (25 mm) (SKC LTD, Dorset, England) were used to measure inhalable dust and sampling was performed at an airflow of 2 L/min. This sampler simulates the dust entering the nose and mouth, and approves with the sampling criteria for inhalable dust according to ACGIH (American Conference of Governmental Industrial Hygienists). Plastic cyclones (25 mm) (Casella LTD, London, England) were used to monitor respiratory dust levels. The cut-off size of dust particles sampled with a cyclone is approximately 5 μm. The filters were weighed and thereafter extracted and analysed regarding endotoxin concentration using the *Limulus amebocyte* lysate assay (QCL-1000, Endotoxin, BioWhittaker, Walkersville, USA,

Muramic acid, an amino sugar present only in eubacteria (Black et al., 1994), was measured with a GC-MS (gas chromatography - mass spectrometry) method (Mielniczuk et al., 1995) in study I. This amino sugar is a constituent of peptido-glycan, a cell wall component of both Gram negative and Gram positive bacteria.

3.3.3 Nasal lavage (study I-IV)
Nasal lavage was performed using a procedure described by Bascom and Pipkorn (Bascom et al., 1988; Pipkorn et al., 1988) with minor modifications (Larsson et al., 1997).

3.3.4 Bronchoalveolar lavage (study I)
Bronchoscopy was performed through the mouth or the nose with a flexible fibreoptic bronchoscope under local anaesthesia. A total of 250 ml lavage fluid was used.

3.3.5 Peripheral blood (study III and IV)
A total and differential white blood cell count of peripheral blood was determined by flow cytometry using fluorescent cell surface markers.

3.3.6 Cytokines (study I-VI)
Interleukin-6 (IL-6) in peripheral blood and nasal lavage fluid and IL-8 in nasal lavage fluid were determined using a specific ELISA validated in our laboratory (Larsson et al., 1998) using commercially available antibody pairs (R&D systems, Europe, Abingdon, UK). The lower detection limit was 3 ng/L for IL-6 and 50 ng/L for IL-8. Regarding studies I, II, V and VI, IL-8 was measured using a commercial ELISA kit with a lower detection limit of 31 ng/L(Quantikine, R&D systems).

3.3.7 Measurements of LTB_4, LTE_4 and 9α,11β-PGF_2 in nasal lavage and urine (study II and III)

Analysis of 9α, 11β-PGF_2 and LTE_4 in urine, as well as LTE_4 and LTB_4 in nasal lavage fluid were performed with enzyme immunoassays (Cayman Chemical, Ann Arbor, MI, USA) using rabbit polyclonal antisera and acetylcholinesterase linked tracers essentially as described previously (Kumlin et al., 1995; O´Sullivan et al., 1996). Creatinine was determined in all urine samples using a commercial available alkaline picrate colorimetric assay (Sigma Chemical Company, St Louis, MO, USA).

3.3.8 Lung function and bronchial responsiveness (study II-IV)

Lung function was measured with a wedge-spirometer (Vitalograph®, Buckingham, UK) according to the American Thoracic Society criteria (ATS, 1995). Local reference values were used (Hedenström et al., 1985; Hedenström et al., 1986). Peak expiratory flow (PEF) was measured using a peak flow meter (Mini-Wright, Clement Clarke International Ltd, London, UK).

Bronchial responsiveness was assessed by a methacholine challenge (Malmberg et al., 1991). The result was expressed as the cumulative dose causing a 20% decrease in FEV_1 ($PD_{20}FEV_1$). The method has been designed to achieve a maximal deposition of the inhaled methacholine in the lower airways, using a drying device for the methacholine solution. Due to this modification, it is possible to obtain a PD_{20} value for nearly 80% of healthy subjects, thus, giving us a possibility to measure changes in airway reactivity in healthy subjects (Sundblad et al., 2000).

3.3.9 Exhaled nitric oxide (study III)

Exhaled nitric oxide (NO) was determined using single-breath exhalations according to accepted standards (ATS, 1999; Kharitonov et al., 1997; Lundberg et al., 1996).

3.3.10 Preparation of cell supernatants (study V-VI)

After incubation with different stimuli for 8 or 24 hours, cell supernatants were collected and frozen at –70°C until cytokine analysis

3.4 Statistics

Regarding human studies results are presented as median value (25th to 75th percentiles) except for lung function data and oral temperature which are presented as mean value (95% confidence interval). Wilcoxon's signed rank test was used for paired comparisons (pre- and post-exposure) and differences between groups were assessed by Mann-Whitney U-test. Student's t-test was used for analysis of lung function data, oral temperature and symptom scores. Correlations were estimated by Spearman Rank correlation test. Differences in baseline concentration of mediators measured in urine were assessed by one way ANOVA. Results from *in vitro* studies are presented as mean values (SEM). Comparisons

were performed by ANOVA with post hoc Fisher's PLSD when appropriate for each tested agent separately. A p-value <0.05 was considered significant.

4. Results and Discussion

4.1 Human *in vivo* exposure studies

4.1.1 Exposure levels in swine confinement buildings

Results from exposure measurements assessed by personal samplers are presented in table 4. Studies I and II were performed in the same swine confinement building housing approximately 900 pigs and study III and IV were performed in two different swine farms with stable units containing approximately 300 pigs. This is the most likely explanation of the lower exposure levels in studies III and IV compared to study I and II. In study IV, the observations were made when another working-operation, cleaning of an empty swine stable with a high-pressure cleaner, was performed, whereas in the other studies the exposure involved weighing of pigs (study I-III). Thus, it seems that different work tasks and different confinement units led to different exposure.

Table 4. Exposure levels.

	Study I	Study II	Study III	Study IV
	----------Weighing of pigs----------			Cleaning
Inhalable dust (mg/m^3)	23.3 (20.9-29.3)	28.5 (26.5-29.5)	10.4 (9.7-16.2)	0.94 (0.74-1.55)
Respirable dust (mg/m^3)	NM	NM	0.74 (0.54-0.83)	0.56 (0.51-0.63)
Endotoxin (inhalable) ($\mu g/m^3$)	1.3 (1.1-1.4)	NM	0.58 (0.36-0.83)	0.083 (0.051-0.063)
Endotoxin (respirable) ($\mu g/m^3$)	NM	NM	0.039 (0.034-0.040)	0.023 (0.0047-0.024)
Peptidoglycan ($\mu g/m^3$)	6.6 (5.2-14)	NM	NM	NM

Median (25th – 75th percentiles)
NM not measured

The inhalable dust level in study IV was only 3-9% of the levels observed in study I-III and in two studies by Wang et al using the same exposure model (Wang et al., 1996; Wang et al., 1998), while the respirable dust fraction in study IV was similar to the other studies (0.56 mg/m^3 compared to 0.7-1.0 mg/m^3) (Wang et al., 1996; Wang et al., 1998). The higher proportion of respirable dust in study IV could originate from pulverising of larger dust particles by the high-pressure cleaner used in this particular study. The absence of pigs in the stable could have influenced the inhalable dust levels. Also the use of a splashguard in front of the IOM sampler could have influenced the airflow and hence the sampling efficiency (see figure 1 in manuscript IV). This device was needed in order to protect the filter orifice from being soaked with water due to splashing

from the high pressure cleaner. Nonetheless, preliminary data from exposure measurement with or without a splashguard did not give any signs of decreased sampling efficiency (measurements during weighing of pigs, 2.46 mg/m^3 with splashguard versus 2.66 mg/m^3 without).

Markers of exposure to microorganisms were measured. Thus, the airborne levels of LPS, cell wall constituent of Gram-negative bacteria, and peptidoglycan, present in cell walls of both Gram-negative and Gram-positive bacteria were measured (table 4). Gram-positive bacteria have a thick multi-layer of peptidoglycan in their cell wall compared to a single layer in Gram-negative bacteria (Glauser, 1996). Gram-positive bacteria have also been shown to dominate the bacterial flora in swine confinement buildings (Attwood et al., 1987; Crook et al., 1991; Donham et al., 1986; Martin et al., 1996).

A significant correlation between clinical parameters and exposure markers, could only be detected in study I where a correlation between the increase of neutrophils in BAL fluid and the concentration of peptidoglycan was demonstrated (ρ=0.66, p<0.02) (I). This indicates that exposure to Gram-positive bacteria have an influence on the airway inflammation following exposure in a swine farm. When compiling the data from study I-IV, significant correlations between exposure and clinical parameters measured in blood and nasal lavage fluid were found (table 5). All measured parameters in nasal lavage fluid (cytokines, leukotrienes, and neutrophils) displayed significant correlations with inhalable dust levels. Only changes in IL-6 concentration in serum and nasal lavage fluid correlated with endotoxin. This may suggest a greater importance for dust constituents other than endotoxin, for the inflammatory response in this exposure model.

Table 5. Spearman correlations between exposure and changes in clinical markers in nasal lavage fluid and peripheral blood. Data compiled from studies I-IV.

	Inhalable dust	Inhalable endotoxin
Blood Neutrophils[a)]	ns	ns
Serum IL-6[a)]	ns	ρ=0.56, p<0.05
Nasal lavage		
Neutrophils[b)]	ρ=0.52, p<0.01	ns
IL-6[a)]	ρ=0.74, p<0.01	ρ=0.78, p<0.01
IL-8[b)]	ρ=0.77, p<0.01	ns
LTB_4[c)]	ρ=0.67, p<0.05	ns
LTE_4[c)]	ρ=0.60, p<0.05	ns

a) Data from study III-IV, b) Data from study I-IV, c) Data from study II-III
ns=not significant
Due to multiple comparisons (n=14), the significance levels are adjusted according to Bonferroni. A p-value < 0.0036 is required for a significance at the 5% level and a p-value <0.0007 for a significance at 1% level.

4.1.2 Inflammatory responses assessed by lavage fluid from upper and lower airways

Exposing healthy subjects, naive to farming environment for three hours during weighing of pigs (I-III) or during a cleaning procedure in a swine confinement building (IV), resulted without any exception in an inflammatory airways response. The main feature of the reaction was a massive accumulation of neutrophilic granulocytes in the airways. The neutrophil concentration increased between 10 to 66 times in the upper airways assessed by nasal lavage (I-IV) and approximately 70-fold in the lower airways assessed by bronchoalveolar lavage (I) (table 6).

Table 6. Concentration of neutrophils in airway lavage fluids.

		Nasal lavage (cells $x10^6$/L)		BAL (cells $x10^6$/L)	
		Before	After	Before	After
Study I		3.4 (0.33-11)	66 (31-171)***	1.6 (0.7-2.5)	114 (64-226)***
Study II		3.3 (1.0-14)	133 (63-222)**		
Study III	placebo	0.83 (0.05-6.8)	55 (10-78)**		
	zileuton	0.28 (0.15-2.0)	38 (21-88)**		
Study IV	no mask	1.4 (0.58-4.2)	14 (11-18)*		
	mask	0.87 (0.13-3.8)	1.7 (0.074-5.2)		

Median (25th – 75th percentiles)
* $p<0.05$, ** $p<0.01$, *** $p<0.001$ (pre- and post-exposure comparison)

This strong neutrophilic migration confirms the results from an earlier study using this exposure model (Larsson et al., 1994), where a 75-fold post-exposure increase of neutrophilic granulocytes was detected in BAL fluid from healthy subjects. Similar findings in naive subjects following swine house exposure have been reported by Cormier et al (Cormier et al., 1997). The total cell concentration in BAL fluid was nearly 3 times higher in the naive subjects after three hours of exposure than what have been found in swine farmers with normal lung function and normal bronchial responsiveness (Larsson et al., 1992). A mean BAL neutrophil concentration of $83x10^6$/L, has been found in patients with chronic bronchitis during exacerbation (Balbi et al., 1997). This is in the same order of magnitude as we have demonstrated in BAL fluid of healthy subjects following exposure in a pig house.

Chemotactic factors such as IL-8, C5a, PAF, NAP-2, GRO-α,β,γ, ENA-78 and LTB_4 may be involved in the migration of neutrophils into the airways. These factors are produced by cells present in the airways, *i.e.* macrophages/monocytes, epithelial cells and neutrophils (Wagner & Roth, 2000). Up to approximately a 10-fold increase in IL-8 was detected in nasal lavage fluid from healthy subjects following exposure to swine house dust (I-IV), from 100 ng/L prior to exposure to approximately 1000 ng/L post-exposure (table 7).

Table 7. Chemotactic factors in airway lavage fluid in nasal lavage (I-IV) and BAL (I).

		IL-8 (ng/L)		LTB_4 (ng/L)	
		Before	After	Before	After
Study I	NAL	144 (97-227)	1064 (864-1437)***		
	BAL	<31 (<31-<31)	63 (41-109)***		
Study II		132 (66-161)	1561 (1255-2104)**	26 (15-35)	138 (106-251)***
Study III	placebo	68 (<50-119)	391 (303-513)**	44 (37-71)	124 (59-153)*
	zileuton	68 (<50-113)	289 (218-604)**	28 (24-33)	31 (29-49)
Study IV	no mask	<50 (<50-71)	102 (69-163)*		
	mask	<50 (<50-78)	<50 (<50-<50)		

Median (25th – 75th percentiles).
* $p<0.05$, ** $p<0.01$, *** $p<0.001$ (pre- and post-exposure comparison)

These levels appear to be higher than the IL-8 levels in nasal lavage fluid of 367±85 (mean±SEM) ng/L, found during pollen season in subjects with a seasonal allergic rhinitis to birch pollen (Linden et al., 1999). The time points for post-exposure nasal and bronchoalveolar lavage were different. Nasal lavage was performed 7 hours and BAL 24 hours after the start of the exposure. In a study by Deetz et al, where repeated BAL was performed (on different bronchial segments) 4, 24, 48, 96 and 168 hours after a grain-dust inhalation, maximal levels of neutrophils and cytokines (TNF-α, IL-6 and IL-8) were found at the first BAL at 4 hours (Deetz et al., 1997). The peak levels of IL-8 in BAL fluid were 800-900 ng/L, which is in the same range as what we have detected in post-exposure nasal lavage fluid (I-IV). Concentrations thereafter decreased, but the neutrophils and IL-8 levels remained significantly elevated after 48 hours and IL-6 prevailed significantly increased for 96 hours whereas TNF-α was increased only for 12 hours. The IL-8 concentration in BAL fluid had decreased at 24 hour to approximately 1/3 of the value obtained 4 hours after exposure and the corresponding figure for neutrophils was a reduction by half. These results suggest that, we might have been able to demonstrate a stronger IL-8 response if we had performed BAL earlier.

In COPD patients a median IL-8 concentration of 40 ng/L (range 0 to 2600) was reported in BAL fluid by Rutgers et al (Rutgers et al., 2000). This is similar to our observations after organic dust exposure. However, Soler et al reported higher IL-8 levels of 255±83.7 (mean±SD) ng/L in BAL fluid from patients suffering from mild COPD (Soler et al., 1999).

In addition, increased levels of LTB_4 were found in nasal lavage following exposure (II-III, see table 7). In study II, IL-8 increased approximately 12 times while LTB_4 increased 5.5 times, while in study III the corresponding figures were

about 6 times for IL-8 and 3-fold increase for LTB_4. Furthermore, no correlation was observed between the increase in neutrophil concentration and change in LTB_4 levels in nasal lavage fluid in study II and III. A significant correlation was observed between increased levels of IL-8 and changes in neutrophil concentration in nasal lavage fluid ($p<0.02$, $\rho=0.54$) in study I, but a corresponding correlation was not observed for changes in BAL fluid. This discrepancy in correlation might be caused by the study design as mentioned above. It is possible that other chemoattractants such as LTB_4, ENA-78 and GRO-α,β,γ are more important than IL-8 for recruiting neutrophils in the lung than in the nasal region. In BAL fluid obtained from HIV- infected patients with a bacterial pneumonia, IL-8 levels, but not LTB_4, were significantly elevated compared to healthy controls, and furthermore, the IL-8 concentrations correlated significantly with neutrophil levels (Krarup et al., 1997). This study strengthens the hypothesis that IL-8 is an important contributing factor for neutrophil recruitment in the airways. On the other hand, LTB_4 levels might have reached peak levels at an earlier stage than IL-8, and hence the lower increase of LTB_4 could erroneously be taken as a sign of lower contribution to migration. An *in vitro* study using LPS stimulated alveolar macrophages from healthy donors, showed a sequential release of LTB_4 and IL-8. The LTB_4 release started at one hour after stimulation, reaching peak levels at 3 hours, while IL-8 production was detected after 3-5 hours with maximum levels at 24 hours after stimulation (Rankin et al., 1990).

The fact that we have demonstrated higher levels of IL-8 compared to LTB_4 is by no means an evidence for a more prominent role of IL-8, although there are data to support such an assumption. IL-8 was proved to be far more potent than both LTB_4 and PAF in a canine study investigating chemotaxis (Thomsen et al., 1991). Tan et al showed that using neutrophils isolated from human cord blood, IL-8 was 5 respectively 50 times more potent in inducing chemotaxis of neutrophils than LTB_4 and PAF at the same concentration (Tan et al., 1995). Since elevated IL-8 levels have been reported in a number of respiratory disorders such as COPD, adult respiratory distress syndrome and cystic fibrosis (Kunkel et al., 1995), there is strong indication that IL-8 may have a distinct role in the neutrophil recruitment observed in the swine house dust induced inflammation.

When pooled data from study I-IV were analysed, increased concentrations of neutrophils correlated with changes in IL-8 levels in nasal lavage fluid (study I-IV; figure 3, $\rho=0.58$, $p<0.01$), but not with LTB_4 (study II-III; $\rho=0.29$, $p=0.12$). This finding adds further evidence for a prominent role of IL-8 in the inflammatory reaction following exposure in a swine farm.

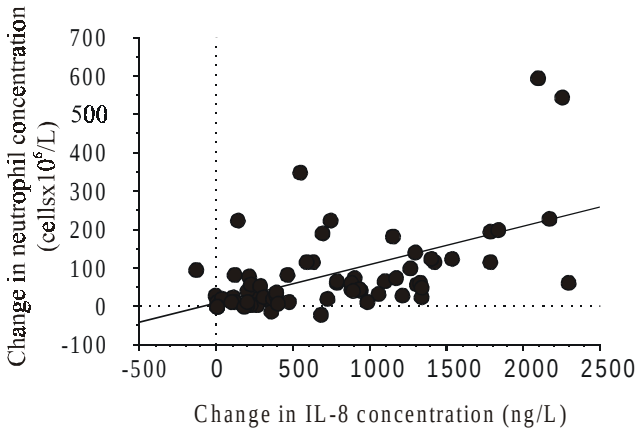


Figure 3. Correlation between changes in concentration of neutrophils and IL-8 levels in nasal lavage fluid following exposure to swine house dust. Data compiled from study I-IV. ρ=0.58, p<0.01.

IL-6 levels were increased in nasal lavage fluid following exposure while weighing pigs (III) and during the cleaning procedure (IV). Pre-exposure levels were all below detection limit (3ng/L).). In study III post-exposure median values were 33 ng/L in the placebo group (p<0.01) and 67 ng/L in the zileuton group (p<0.01). Median post-exposure values of 4.5 ng/L (p<0.05) were observed in subjects exposed without a mask during a cleaning procedure, while IL-6 levels in nasal lavage fluid from subjects equipped with a mask during exposure were still below detection limit (IV). For comparison, a study by Linden et al, comprising subjects with seasonal allergic rhinitis, mean levels of 3.2±2.5 ng/L (mean±SEM) were detected in nasal lavage fluid during pollen season (Linden et al., 1999), which is lower than what we have reported in healthy subjects following organic dust exposure. This indicates that the swine farm environment encompass a strong inflammatory stimulus.

4.1.3 Findings in peripheral blood

The cellular reaction in peripheral blood displayed an increase in total leukocyte concentration, mainly comprising elevated levels of neutrophilic granulocytes, which increased approximately 2-fold (table 8). In study III, an interesting observation was that the cellular response was greater in the zileuton group than in the placebo group.

The concentration of IL-6 also increased in peripheral blood after exposure while weighing pigs, from pre-exposure levels below the detection limit (<3ng/L) to post-exposure concentrations of 17.5 (12.6-26.7) ng/L in the zileuton group (p<0.01). In the placebo group the increase was only minor and reached a post-exposure level of <3(<3 – 7.94) ng/L (p<0.05). Exposure during the cleaning procedure of the swine stable did only result in increased serum levels of IL-6 in subjects exposed without respiratory protection, from below the detection limit up to 4.5 (3.2-19) ng/L post-exposure (p<0.05). In comparison, serum levels of 21.6

(<0.5-141) ng/L (mean (range)), have been demonstrated in asthmatic subjects (Yokoyama et al., 1995).

Table 8. Neutrophilic granulocytes in peripheral blood (study III and IV).

		Neutrophils ($x10^9$/L)	
		Before	After
Study III	placebo	3.6 (3.0-5.3)	7.7 (6.8-11)***
	zileuton	4.9 (3.6-5.4)	12 (9.8-15)***
Study IV	no mask	3.2 (2.9-4.3)	8.7 (5.6-9.9)**
	mask	3.9 (3.2-4.6)	4.9 (4.3-6.5)*

Median (25th – 75th percentiles).
* $p<0.05$, ** $p<0.01$, *** $p<0.001$ (pre- and post-exposure comparison)

There was a tendency towards an association between increased symptom scores for chills and high serum levels of IL-6 in study III. Symptom scores for chills increased significantly only in subjects treated with zileuton, and there was also a tendency for higher IL-6 levels in serum in those individuals reporting chills. As a whole, IL-6 increased more in zileuton treated subjects compared to the placebo group ($p<0.001$), and the increase in oral temperature tended to be somewhat higher in the former group (0.73°C compared to 0.41°C in placebo group). This was, however, not statistically significant. The increased oral temperature and symptom scores for chills could very likely reflect an effect of IL-6. However, when data from study III and IV were pooled, no correlation was observed between changes in serum IL-6 and oral temperature. Since we used different symptom questionnaires in study III and IV, no comparison could be made regarding serum IL-6 and symptoms of chills.

The greater response in the zileuton group regarding IL-6 and neutrophils in peripheral blood could be due to an effect of the 5-lipoxygenase inhibition of the formation of lipoxins. Lipoxin A_4 and B_4 inhibit neutrophil migration (Lee et al., 1989), possibly by affecting P-selectin and beta-2 integrin (CD18) mediated cell adhesion and transmigration (Papayianni et al., 1996). Lipoxin A_4 also inhibits IL-1β induced IL-6 and IL-8 release from human synovial fibroblasts (Sodin-Semrl et al., 2000). Lipoxins are formed by the action of 5- and 15-lipoxygenase activity (for review (Serhan, 1994)), and a reduction of lipoxin levels by zileuton, might account for the increased blood concentrations of IL-6 and neutrophils in the zileuton group compared to placebo. Another explanation to the higher concentrations of neutrophils in the zileuton group could be that attenuated LTB_4 levels in nasal lavage might have lead to a retention of neutrophils in the blood-stream.

4.1.4 Lung function data and bronchial responsiveness

Lung function measured as vital capacity (VC) and forced expiratory volume in one second (FEV_1), was reported in study II (only FEV_1), III and IV. There were

merely minor changes, that reached statistical significance only in study III (placebo group), with a 7.2 % decrease in FEV_1 and 2.8% in VC. In another study using the same exposure model similar decrease in lung function has been recorded for healthy subjects, a 5% decrease for FEV_1 and a 2 % reduction in VC (Wang et al., 1997). The changes in FEV_1 are modest in comparison to the 10-20% decrease that is required to regard a bronchial provocation as positive.

Table 9. Bronchial responsiveness before and after exposure in swine confinement building.

	N	Group	$PD_{20}FEV_1$ (mg) Before	After	Change (doubling)
Study II	10		0.82 (0.33 - >15)	0.40 (0.15 - 0.83)	1.4 (1.1-3.6)**
Study III	12	placebo	1.76 (0.98 - 10.3)	0.38 (0.13 - 0.58)	3.4 (1.7-4.3)**
	11	zileuton	0.90 (0.48 - 2.48)	0.21 (0.12 - 0.28)	2.3 (1.2-2.8)**
Study IV	9	no mask	1.42 (0.60 - 2.00)	0.16 (0.12 - 0.24)	3.2 (1.6- 3.9)**
	7	mask	0.92 (0.51 – 2.67)	0.70 (0.25 – 0.76)	1.1 (0.74-1.7)*

[o]

Median (25th – 75th percentiles)
* $p<0.05$, ** $p<0.01$ (pre- and post-exposure comparison)

The results from the bronchial provocation tests with methacholine demonstrated an indisputable pattern with increased bronchial responsiveness in all but one subject submitted to organic dust exposure for three hours (table 9). The lack of differences in bronchial responsiveness regardless the wide range of inhalable dust exposure levels (0.94-28 mg/m^3) between the studies suggests that a maximal response in bronchial responsiveness may be achieved already at low exposure levels and that additional exposure does not further affect the response to methacholine. Increased responsiveness to histamine has been reported by Kölbeck et al using the same exposure model in healthy subjects (Kölbeck et al., 2000). The responsiveness to histamine did not seem to be as pronounced as that to methacholine. In asthmatic subjects there is a correlation between histamine and methacholine responsiveness (Hargreave et al., 1981). This relationship may not be as strong in healthy subjects following organic dust inhalation. Bronchial responsiveness to eucapnic hyperventilation of dry air was not altered by swine house dust inhalation (Sundblad et al., 2001). In asthmatic subjects increased responsiveness to hyperventilation of dry air is recorded (Hurwitz et al., 1995). Methacholine and histamine are direct bronchoconstrictive stimuli (acting on airway smooth muscle), while hyperventilation of dry air is claimed to act indirectly through the release of mediators (Sterk et al., 1993). The different "profiles" in reactivity to indirect and direct stimuli, in healthy subjects exposed to organic dust and asthmatic subjects, imply that the mechanism behind increased bronchial responsiveness differs between the two groups. Analysing the compiled data from study II-IV did not result in any correlations between change in

bronchial responsiveness and any of the measured inflammatory parameters (cells and mediators). These findings strengthen the theory that the organic dust induced increase in bronchial responsiveness observed in healthy subjects is not due to a direct effect of mediator release from inflammatory cells. Instead, the inflammatory reaction might cause a swelling of the airway mucosa leading to a decrease in airway diameter. This in turn, implies that addition of bronchoconstrictive stimulus leads to a more pronounced bronchial obstruction. The subjects in study II had lower pre-exposure $PD_{20}FEV_1$ compared to the other studies. Eight out of ten subject in study II were women, and since women have smaller airways, a geometrical factor could explain the increased baseline bronchial responsiveness in study II (Sundblad, unpublished data).

4.1.5 Measurements of cys-LTs in nasal lavage fluid and urine

In study II, the aim was to evaluate the role of cys-LTs in the organic dust induced inflammation and in study III the focus was on the potential effect of leukotrienes on the increased bronchial responsiveness. Leukotriene E_4 in nasal lavage fluid from healthy subjects increased significantly in study II, though in the intervention study with zileuton, no change could be detected in either group (table 10). Lower exposure levels compared to the previous study may explain the lack of increase in LTE_4 levels in the latter study (III). The changes in LTE_4 levels of approximately 28 ng/L in study II are less than the maximal mean increase of 152 ng/L found in nasal lavage fluid in subjects with aspirin-intolerant asthma after ingestion of aspirin (Fischer et al., 1994).

Table 10. Measurements of cysteinyl-leukotrienes and LTB_4 in nasal lavage fluid and urine.

		LTE_4 (ng/L)		LTB_4 (ng/L)	
		Before	After	Before	After
Study II (10)		32 (28-34)	60 (46-83)**	26 (15-35)	138 (106-251)***
Study III	placebo (12)	34 (31-50)	41 (34-50)	44 (37-71)	124 (59-153)*
	zileuton (11)	31 (23-34)	30 (23-33)	28 (24-33)	31 (29-49)

Median (25th – 75th percentiles)
* $p<0.05$, ** $p<0.01$, *** $p<0.001$ (pre- and post-exposure comparison)

Whole body formation of cys-LTs, was assessed by analysis of urinary LTE_4. Urinary excretion of LTE_4 induced by this exposure increased significantly in both study II and III and the increase was in the same order as in asthmatics after an allergen challenge (Roquet et al., 1997) (se figure 2, II; figure 3, III). In study III, only subjects in the placebo group demonstrated significantly increased urinary LTE_4 levels. Although there was no significant difference between zileuton and placebo treated groups, the lack of post-challenge increase in the zileuton group could signify a minor inhibitory effect on cys-LT biosynthesis by zileuton.

The pre-treatment with zileuton did not affect the increased bronchial responsiveness in healthy volunteers after three hours of exposure during weighing of pigs. The bronchial responsiveness was in the same magnitude as what have been demonstrated in several earlier studies using the same exposure model. The pre-treatment period (five days) might have been too short or the dose (600 mg q i d) too low, to influence the intense inflammatory reaction evoked by the exposure. This is, however, a dose that have been proven to have beneficial effects in asthma. Nonetheless, this exposure model provides a very strong inflammatory response in healthy, non-allergic subjects and we cannot exclude that the effect of the inhibitor is overcome by upregulation of enzymes involved in leukotriene biosynthesis that may occur during the inflammatory reaction. This leads to the assumption that a more aggressive pre-treatment with zileuton might be required. However, the post-exposure increase of LTB_4 in nasal lavage fluid was completely extinguished in zileuton treated subjects (table 10), implying that zileuton was able to inhibit leukotriene formation. Leukotriene B_4 measurements in nasal lavage fluid might reflect a local reaction in the nasal mucosa, whereas measurements of urinary levels of LTE_4 monitors whole body formation of cys-LTs, indicating that the treatment with zileuton causing a threshold inhibition of 5-lipoxygenase, preferentially acts on a local level.

4.1.6 Urinary excretion of 9α,11β-PGF_2

Evidence of mast cell activation was assessed by analysis of urinary levels of the PGD_2 metabolite 9α,11β-PGF_2. Increased levels of 9α,11β-PGF_2 were reported in study II and III (see figure 2 (II), figure 3 (III)). The change was similar to what has been measured in aspirin-intolerant asthmatics subjected to an aspirin challenge (O´Sullivan et al., 1996). In study III, the excretion of the PGD_2 metabolite was abolished in the zileuton group but increased in placebo treated subjects ($p<0.01$), displaying a significant difference between the groups ($p<0.05$). Since zileuton is only known to inhibit 5-lipoxygenase activity and not cyclo-oxygenase, this is probably due to an indirect effect. Mast cell activation induced by exposure to swine house dust has been supported by another study where pre-medication with sodium cromoglycate diminished the post-exposure increase of neutrophilic granulocytes, IL-6 and TNF-α in BAL fluid, yet with no effect on bronchial responsiveness (Larsson et al., 2001). TNF-α is a representative cytokine product for mast cells (Gordon et al., 1990) and sodium cromoglycate has a stabilising effect on mast cells (Orr et al., 1969), which taken together support that mast cells contribute to the inflammatory response evoked by swine house dust exposure. There are no data on the effect of cromoglycate on macro-phages, which are also potent TNF-α producers.

PGD_2 is a potent bronchoconstrictor in man (Hardy et al., 1984) and exerts this particular effect through the thromboxane receptor (TP-receptor) (Beasley et al., 1989). It could therefore be assumed that PGD_2 is involved in the development of bronchial responsiveness after exposure to farming environment. Mast cells possess the capability to release an array of different mediators like proteases,

eicosanoids and cytokines, pre-formed or rapidly produced upon activation, that are known to influence the airway inflammation in asthma. Some of these mediators PGD_2, LTC_4 and histamine, influence bronchiolar smooth muscle by inducing contraction (Drazen, 1998; Hardy et al., 1984; White, 1990). Mast cells were previously thought to mainly play an important role in the IgE-mediated allergic response and in the defence system aimed at parasitic infections, but there are studies indicating a role for mast cells in the defence against bacterial infections (for review (Mekori et al., 2000)). Upon activation, which is usually conceived through interaction of the high affinity FcεRI receptor on the cell surface and the IgE molecule followed by a cross-linking of IgE by an allergen, mast cells can release a number of cytokines like TNF-α, IL-1, IL-6 and IL-8 (Metcalfe et al., 1997) which could influence a non-allergic inflammation. They are also phagocytes, and in mice deficient of mast cells, an impaired defence against bacterial infection was demonstrated as well as attenuated neutrophil recruitment and TNF-α levels (Malaviya et al., 1996)

4.1.7 Exhaled nitric oxide

A doubling of exhaled nitric oxide (NO) levels was demonstrated in healthy subjects following a three hours exposure in the swine confinement building ($p<0.01$). There was no effect of pre-treatment with the 5-LO inhibitor zileuton, indicative of absence of influence of cys-LTs on NO production. This is supported by a study by Deykin et al in which inhalation of LTE_4 did not change NO levels in asthmatic subjects (Deykin et al., 2000).

Swine house dust exposure includes exposure to LPS and the inflammatory response following this exposure comprehends increased concentrations of TNF-α in serum and in BAL fluid (Wang et al., 1997; Wang et al., 1996), and both LPS and TNF-α could induce iNOS expression. The increased levels of NO could reflect a normal host-defence response to bacteria in the inhaled organic dust.

4.1.8 Intervention study with respiratory protection device

The exposure during a cleaning procedure in a swine stable using a high-pressure cleaner induced an inflammatory reaction similar to what has been described after exposure during weighing of pigs (IV) (Larsson et al., 1994; Wang et al., 1997; Wang et al., 1996). The bronchial responsiveness increased significantly in both groups, by 3.2 doubling steps in the group exposed without a mask and 1.1 doubling dose steps in subjects exposed with a respiratory protection ($p<0.05$ between groups). The increase in bronchial responsiveness was of the same order of magnitude as observed during exposure while weighing of pigs (II-III) (table 9), despite much lower exposure levels in study IV (table 4). The inhalable dust level was 20 times lower during the cleaning procedure compared to the operation of weighing pigs. The respirable fraction of the dust aerosol measured in the stable was, however, similar during the different working situations.

Respirable dust levels were similar in the different studies and could possibly be responsible for the increased bronchial responsiveness. The fact that the increase in bronchial responsiveness was still detected in subjects exposed with a mask

supplied with a P3 particle filter (removes all particle exposure down to viruses according to the manufacturer), suggests that other components than particles, possibly gases, may contribute to the reaction. Gases like ammonia, carbon dioxide, methane and hydrogen sulphide are present in the swine farm indoor environment, although the concentrations seldom exceed hygienic threshold values (Von Essen & Donham, 1999). No gases were measured in study IV, but in studies performed in similar farms under similar conditions, levels of ammonia below the Swedish hygienic threshold limit 25 ppm (The Occupational Safety and Health Administration in Sweden) have been recorded. Still, this does not exclude the possibility that gas exposure is of importance in this context.

Lung function (VC and FEV_1) remained unaffected by the exposure, but PEF (peak expiratory flow) decreased significantly in subjects exposed without a mask from 503±89 to 471±70 L/min ($p<0.05$), but remained unaffected in subjects equipped with a mask ($p<0.01$ between groups). Peak expiratory flow was measured right before entering the swine stable and immediately following accomplished exposure, indicating that this was an acute bronchoconstriction.

In nasal lavage fluid a cellular increase, predominantly neutrophils, was observed only in subjects exposed without a mask ($p<0.05$), significantly different to mask equipped subjects ($p<0.01$). Significant increase of IL-6 and IL-8 in nasal lavage fluid (see paragraph 4.1.2), was only observed in subjects without respiratory protection, although no significant difference between the groups was found.

In peripheral blood the neutrophil concentration increased significantly in both groups, with a significant higher increase in subjects exposed without mask. IL-6 in serum increased in the group without respiratory protection and not in the mask group, the difference between the groups was, however, not significant.

The protective effect of the respiratory device was more clear-cut regarding reactions in the upper airways, whereas less pronounced effects were observed as far as systemic reactions and changes in bronchial responsiveness were concerned. From this it could be hypothesised that particle exposure is more important for the nasal reaction and gases have a greater influence on the bronchial and systemic response. In addition, all measured parameters in the upper airways showed significant correlations with inhalable dust levels, when data from all studies (I-IV) were analysed together (table 5).

A swine dust exposure study with the focus on effects of a half mask (3M™ 9322) on personal exposure levels and health effects during weighing of pigs was recently accomplished by Palmberg et al (unpublished data). This mask was of a disposable type with a class P2 protective effect. The mask used in study IV was of a reusable type (Sundström® half mask), with class P3 filter, which was changed before every exposure. The fitting was also more tight with the Sundström half mask, than with the 3M filter. For individual exposure assessment, personal intranasal samplers were used in the study by Palmberg et al, which facilitated exposure measurements behind the half mask during the exposure while weighing pigs for three hours. The intranasal endotoxin concentration was approximately 350 ng/30 min (10 min measurements/hr) in subjects exposed

without a mask, and the use of a mask during exposure reduced the endotoxin levels by 90%. Bronchial responsiveness to methacholine increased significantly in both groups, yet the increase was significantly higher in subjects exposed without a respiratory protection device. The increase of total cells in nasal lavage fluid was higher in the group exposed without a mask and furthermore, the use of a mask abolished the cytokine release in nasal lavage fluid and the cell increase in peripheral blood. These results, in addition to study IV, may indicate a prominent role for gas exposure in the inflammatory reaction, although the particle fraction of the swine house aerosol seems to have a great impact on the nasal response.

Further support for this suggestion is provided in a study by Cormier et al where they explored the possibility that nasal filtration of large particles and soluble gases would reduce the inflammatory reaction in healthy subjects after exposure in a swine farm (Cormier et al., 1998). The subjects were exposed either during normal breathing or with the nose or mouth occluded. No difference in the inflammatory response depending on the route of breathing could be detected in the lung assessed by BAL and in peripheral blood suggesting that respirable particles and/or gases are responsible for the reactions.

4.2 *In vitro* studies with alveolar macrophages and airway epithelial cells

4.2.1 Induction of IL-8 release by swine house dust constituents

In epithelial cells, crude swine house dust extract (100 μg/ml) was outstandingly the most potent stimuli for IL-8 release, and induced a 28- and 20-fold increase of IL-8 release in A549 cells and NHBE cells, respectively (figure 3 and 4). LPS (100 μg/ml) induced 1/5 of the IL-8 produced in A549 cells stimulated with swine dust and in NHBE the difference was even greater. In A549 cells glucan induced a small but significant IL-8 release, but grain dust failed to stimulate cytokine release. In NHBE cells, it was the other way around. The IL-8 production capacity per cell was higher in NHBE than A549 cells, both at baseline ($p<0.001$) and after stimulation with the swine dust (100 μg/ml, $p<0.001$), most likely reflecting differences between cells in primary culture (NHBE) and a carcinoma cell line (A549).

In alveolar macrophages the scenario was somewhat different (figure 5). Swine dust (100 μg/ml) and LPS (100 μg/ml) were equally potent in inducing IL-8 release. Similar to the findings in epithelial cells, glucan and grain dust were much weaker stimuli.

Stimulation with swine dust and LPS also induced release of cytokines such as IL-1β, IL-6 and TNF-α in alveolar macrophages (Wang et al., 1999). In A549 cells, swine dust stimulation only enhanced IL-6 release and not IL-1β or TNF-α, whereas LPS did not induce release of any of these cytokines. Both swine dust and LPS stimulation of NHBE cells, induced IL-6 but not TNF-α release. Since TNF-α could not be detected in supernatant from neither A549 cells nor NHBE cells, this suggests that these cells may not be totally representative to epithelial cells *in vivo*, which are known to be able to produce TNF-α.

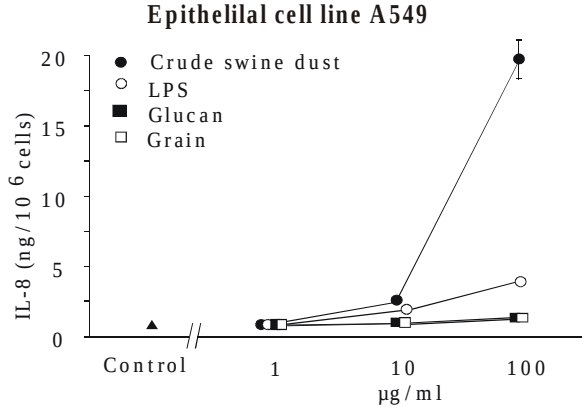


Figure 3. Mean (SEM) IL-8 production (ng) per million epithelial cells (A549). Swine dust ($p<0.001$), LPS ($p<0.001$) and glucan ($p<0.01$) induced a dose-dependent increase in IL-8 release from A549 cells. Grain dust did not influence IL-8 release.

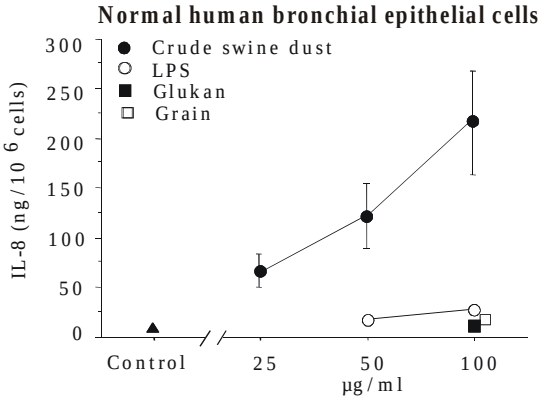


Figure 4. Mean (SEM) IL-8 production (ng) per million normal human bronchial epithelial cells. Swine dust ($p<0.001$) and LPS ($p<0.01$) induced a dose-dependent IL-8 release from NHBE cells. Grain dust also induced significant IL-8 release ($p<0.05$), while glucan did not influence IL-8 release.

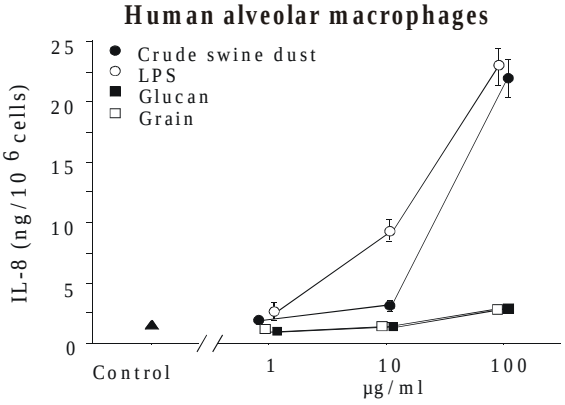


Figure 5. Mean (SEM) IL-8 production (ng) per million alveolar macrophages. Swine dust ($p<0.001$), LPS ($p<0.001$), grain dust ($p<0.01$) and glucan ($p<0.001$) stimulated macrophages to produce IL-8 in a dose-dependent manner.

The greater cytokine response to LPS in macrophages than in epithelial cells is most likely due to stimulation of additional LPS receptors like CD11/CD18 and the scavenger receptor (Wright, 1991) present on macrophages. There are, however, several other LPS binding receptors such as CD14, which was first characterised on phagocytes. CD14 exists in both a membrane bound form (mCD14) present on macrophages, monocytes, neutrophils and epithelial cells, and a soluble form (sCD14) detected in human serum (for review see (Diamond et al., 2000; Su et al., 1995)). LPS can form a complex with the acute phase protein LBP (lipopolysaccharide binding protein) which then binds to the membrane bound CD14 receptor (Tobias et al., 1993). Another activation route available for cells lacking mCD14, also involves formation of a LPS/LBP complex followed by a relocation of LPS to sCD14, and this complex can subsequently react with a hitherto unknown receptor on epithelial or endothelial cells (Pugin et al., 1993). This is, however, somewhat in contradiction to the results from study II, where LPS induced approximately the same IL-8 levels in A549 epithelial cells stimulated with or without serum. Further evidence for a CD14-independent LPS binding to epithelial cells is provided by a study by Striz et al (Striz et al., 1998), in which anti-CD14 monoclonal antibodies did not affect the binding of LPS to human bronchial epithelial cells. Recently, a new family of receptors called Toll-like receptors (TLR) has been demonstrated on phagocytes and epithelial cells (Diamond et al., 2000). These are involved in innate immune response to microbial antigens like lipopolysaccharide, but also components from Gram-positive bacteria bind to TLRs. Five human Toll-like receptors have been identified (Rock et al., 1998). The presence of TLRs on epithelial cells may explain why incubation of A549 cells stimulated with swine dust or LPS under serum-free conditions did not display any major decrease in cytokine release compared to stimulation in the presence of serum (study V).

The endotoxin concentration in the highest swine dust dose used for stimulation *in vitro* was only 2.2 ng/100 μg dust (0.0022 ‰), which is lower than the lowest endotoxin concentration (1 μg/ml) tested *in vitro*. This *in vitro* study thus indicates that other constituents of the organic dust than LPS must be involved in the inflammatory response following exposure in a swine confinement building.

4.2.2 Cytokine release induced by bacteria

All tested bacteria strains, known to be present in the swine confinement environment, induced a dose dependent release of both IL-6 and IL-8 from A549 cells ($p<0.001$)(IL-6 figure 1 in IV, IL-8 figure 6). *Escherichia coli* was the most potent, followed by *S. hominis*, *B. Subtilis*, *S. lentus* and *M. luteus* regarding both IL-6 and IL-8 release. Additionally, stimulation with bacteria-free supernatants from all bacteria (except *M. luteus* regarding IL-6 release) resulted in significant cytokine release. Swine dust and LPS at 100 μg/ml were used as positive controls. *Escherichia coli* at the highest concentration induced 11 times higher cytokine levels than LPS.

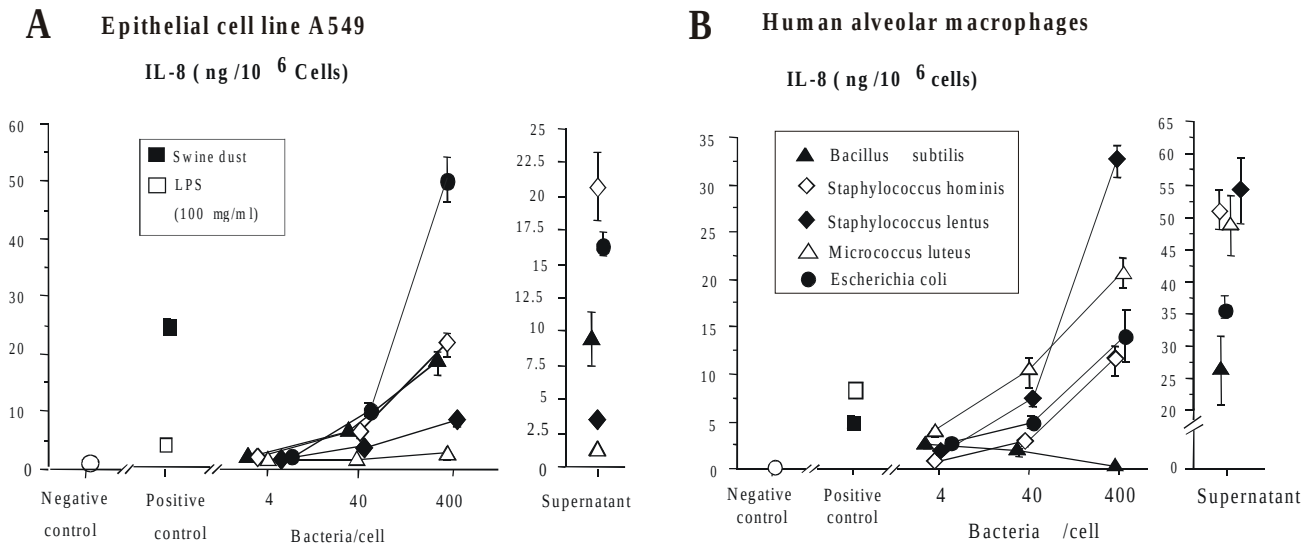


Figure 6. Mean (SEM) IL-8 production (ng) A) per million epithelial cells (A549) . Swine dust and LPS induced a significant IL-8 release ($p<0.001$). All bacteria induced a dose-dependent IL-8 release ($p<0.001$). Supernatants from all bacteria induced a significant IL-8 release (*B. subtilis* $p<0.001$; *S. hominis* $p<0.001$; *S. lentus* $p<0.001$; *M. luteus* $p<0.01$; *E. coli* $p<0.001$). B) per million alveolar macrophages. Swine dust and LPS induced a significant IL-8 release ($p<0.001$). All bacteria except *B. subtilis*, induced a dose-dependent IL-8 release ($p<0.001$). Supernatants from all bacteria induced a significant IL-8 release ($p<0.001$).

Experiments with alveolar macrophages demonstrated some differences regarding IL-6 and IL-8 release (IL-6; figure 3 IV; IL-8 figure 6). All bacteria except *B. subtilis* and *M. luteus* induced a dose-dependent IL-6 release ($p<0.001$). In macrophage cultures *S. lentus* was the most potent stimulus at the highest concentration, followed by *S. hominis*, *E. coli*, *M. luteus* and *B. subtilis*. All bacteria free supernatants induced significant IL-6 release. Stimulation with all bacteria except *B. subtilis* resulted in elevated IL-8 concentrations in the cell supernatants ($p<0.001$), with *S. lentus* being the most potent at the highest concentration, followed by *M. luteus*, *E. coli* and *S. hominis*, *B. subtilis*. All bacteria-free supernatants stimulated IL-8 production ($p<0.001$).

Bacteria-free supernatants were stronger stimuli than whole bacteria in alveolar macrophages, while in A549 cells whole bacteria induced the strongest cytokine response. Perhaps this reflects differences in bacterial defence mechanisms. Macrophages possess a high phagocytic capacity (Lohmann-Matthes et al., 1994) and probably start ingesting whole bacteria at once, and therefore do not produce cytokines to the same extent as when stimulated with soluble components from bacteria. Epithelial cells have no phagocytic activity, instead they produce chemotactic cytokines such as IL-8 to attract phagocytic cells including neutrophilic granulocytes.

Our results are confirmed by several other studies. IL-8 release has been demonstrated in A549 epithelial cells (Gudmundsson et al., 1999) and in human

colonic epithelial cells (Eckmann et al., 1993) after stimulation with whole bacteria. Cell wall components such as lipopeptides, lipopolysaccharide, peptidoglycan and lipoteichoic acid, have also been shown to have stimulatory effects on cytokine release from epithelial cells and monocytes/macrophages (Hauschildt et al., 1995; Heumann et al., 1994; Khair et al., 1996; Kreutz et al., 1997; Timmerman et al., 1993).

In addition to endotoxin, CD14 also binds to the Gram positive bacterial component peptidoglycan (Gupta et al., 1996; Weidemann et al., 1997). The Toll-like receptors are also putative receptors for components from Gram-positive bacteria (Yoshimura et al., 1999). LPS is nonetheless a more potent stimulus for cytokine production than peptidoglycan with up to 1000-fold difference (Heumann et al., 1994; Timmerman et al., 1993). A peptidoglycan-polysaccharide complex did not induce IL-6 or IL-8 release from A549 cells (Saraf et al., 1999). In contrast, house dust samples with a muramic acid (a constituent of peptido-glycan) content in the same range as the tested peptidoglycan complex, induced a dose-dependent IL-6 and IL-8 release from A549 cells.

Gram-positive bacteria as well as bacteria-free supernatants from the different bacteria have been demonstrated to be potent inducers of cytokine release from alveolar macrophages and airway epithelial cells. The results from this study together with the fact that Gram-positive bacteria dominate the microbial flora in swine confinement buildings, suggest that bacteria and their soluble components and/or products, may participate in the development of inflammation following inhalation of swine house dust by inducing release of cytokines.

5. General Discussion

5.1 Effects of exposure

Endotoxin exposure in swine confinement buildings has been correlated with changes in lung function in a number of studies, both acute changes during exposure or chronic impairment following long-term occupational exposure. In 1989 Donham et al reported significant association between endotoxin concentrations in total dust and work-shift decrease in FEV_1 (Donham et al., 1989). In a study comprising 160 Dutch farmers no correlation between FEV_1 and endotoxin exposure was observed, but when analysing a sub-group consisting of 62 farmers with expanded exposure measurements a statistically significant correlation was found (Heederik et al., 1991). Reynolds et al presented a weak but significant correlation between work-shift decreases in FEV_1 and endotoxin levels in total dust. This correlation was stronger in farmers with less than seven years of exposure (Reynolds et al., 1996). In a cohort study with 171 pig farmers spanning over three years, a significant association between yearly decline in FEV_1 and endotoxin concentrations in inhalable dust was found (Vogelzang et al., 1998). The positive correlations with endotoxin levels do not, however, provide binding evidence that endotoxin is the target, since no other microbial markers have been analysed in this context. Other dust constituents of microbial origin displaying parallel exposure levels to endotoxin could be associated with changes in lung function in swine farmers. In the present studies (I-IV), no relationship with endotoxin exposure and clinical effects in healthy subjects could be observed when each study was analysed separately. When the material from study I-IV was compiled into one database significant correlations with endotoxin exposure and some of the measured parameters were found (table 5). Yet almost all measured responses regarding cells and cytokines in nasal lavage fluid and peripheral blood following exposure were better correlated with airborne inhalable dust levels.

The role of endotoxin in exposure induced inflammation has been thoroughly studied. Michel et al reported blood neutrophilia in normal subjects exposed to LPS inhalation (20 μg), with approximately a doubling of neutrophil concentration in blood (Michel et al., 1995). The lung function measured as FEV_1 was, however, unaffected by the exposure. Inhalation of 100 μg LPS in healthy subjects, where the effective dose to the lung was estimated to be approximately 25 μg, resulted in a neutrophilia in BAL fluid with a 145-fold increase three hours after the exposure (Sandström et al., 1992). A dose-response inhalation study with LPS ranging from 0.5 μg to 50 μg, demonstrated significantly increased levels of neutrophils in peripheral blood and induced sputum performed 6 hours after exposure to 5 respectively 50 μg LPS (Michel et al., 1997). A minor, but not significant, decrease in FEV_1, and no alterations of the bronchial responsiveness to histamine was observed at the highest LPS concentration. Other studies have also evaluated the effect of LPS inhalation on lung function and bronchial

responsiveness. Inhalation of 22 μg LPS did not induce any significant change in FEV_1 or bronchial responsiveness to histamine (Michel et al., 1989). In a study by Rylander and colleagues inhalation of 200-300 μg LPS (with an estimated lung deposition of 50 %) resulted in a significant decrease in FEV_1 by 5-8 % in normal subjects ($p<0.001$) (Rylander et al., 1989), and a slight but significant increase in bronchial responsiveness (PC_{15} before 11.5±5.4 mg and 10±7.3 mg 4 hours after exposure, $p=0.04$). Eighty % of the subjects experienced fever, airway irritation and chest tightness were reported by 52 % and 44 % of the subjects, respectively. Nasal provocation with swine dust in healthy subjects, induced increased numbers of neutrophils 180 minutes following exposure, but not at 60 minutes (Nowak et al., 1994). The response was not related to the endotoxin concentration of the dust and no spirometric changes were observed. The above-mentioned results concerning cellular reactions and minor changes in FEV_1 are in agreement to what we have demonstrated in healthy subjects, after an acute exposure in a swine farm. Assuming a breathing frequency corresponding to a light workload with an approximate minute ventilation of 16 L (~$1m^3$/hour) and exposure levels to endotoxin about 1 $\mu g/m^3$, the total endotoxin inhalation would be no more than 3 μg in subjects exposed for three hours during weighing of pigs. Regarding the strong inflammatory response and the striking increase in bronchial responsiveness we have observed after the swine house dust exposure, endotoxin is unlikely to be the only toxic agent underlying the inflammatory response in normal subjects. This is also supported by the lack correlation between airborne endotoxin levels and the majority of measured inflammatory parameters (table 5).

This view is also supported by the *in vitro* experiments where swine dust was a far more potent cell stimulus than was LPS , despite the fact that swine dust only contained 0.0022 ‰ of endotoxin. Wang et al reported significant correlations between endotoxin in inhalable dust and changes in serum IL-6, $PD_{20}FEV_1$ and VC after exposure during weighing of pigs (Zhiping et al., 1996). In a consecutive study IL-6 in BAL fluid correlated with endotoxin exposure while the majority of measured parameters did not display any association with endotoxin (Wang et al., 1997). Results from a study using intranasal exposure samplers (Palmberg et al, unpublished data) also add evidence to the hypothesis that endotoxin is not the sole pro-inflammatory agent in swine dust. Albeit more than 90 % reduction of the endotoxin exposure by the mask, bronchial responsiveness increased by 1.6 doubling dose step in subjects exposed with a mask.

Swine house dust comprises a lot of feed dust, grain dust, which could contribute to the inflammatory response, though the findings from *in vitro* study V do not support such a hypothesis. Studies regarding grain dust exposure have presented a similar inflammatory profile with increase of inflammatory cytokines and neutrophils in BAL fluid (Clapp et al., 1994; Deetz et al., 1997; Jagielo et al., 1996) and endotoxin has been postulated as the triggering factor (Von Essen, 1997).

β-(1-3)-D-glucan is a glucose polymer present in plants (barley) and microorganisms, especially in fungi. There are limited results from occupational

exposure to glucan, but one study has reported a concentration of 4.34 μg/m^3 in swine farms with a mean dust concentration of 2.1 mg/m^3 (Douwes et al., 1996). In our *in vitro* studies using the β-(1-3)-D-glucan curdlan as stimulus, we failed to elicit any secretory cytokine response. We cannot, however, exclude glucan from the list of possible pro-inflammatory agents since the structure (branching) of the glucan may influence the cytokine response (Adachi et al., 1994). Fogelmark et al exposed guinea pig to aerosols of endotoxin and glucan (water insoluble β-(1-3)-D-glucan) which resulted in neutrophilia in BAL fluid in animals exposed to endotoxin, but not in those exposed to glucan. When glucan was transformed to a soluble form, the reaction was similar to the one after endotoxin exposure (Fogelmark et al., 1992). A five weeks exposure (4 hr/day, 5 days/week) of guinea-pigs to endotoxin, curdlan or the two in combination, revealed an enhancing effect of curdlan on the endotoxin evoked inflammatory response regarding neutrophils, lymphocytes and macrophages in lung lavage fluid (Fogelmark et al., 1994). Curdlan alone did not influence the cell population in the lung. This is, however, in contradiction to results from the earlier study by Fogelmark et al, where glucan from baker's yeast significantly decreased the neutrophil response after endotoxin stimulation (Fogelmark et al., 1992). Differences in the types of glucan used or the exposure design possibly explain the discrepancy. Non-atopic subjects exposed to β-(1-3)-D-glucan at a concentration of 210 ng/m^3 for 4 hours, did not develop increased bronchial responsiveness measured by methacholine challenge test (Rylander, 1996). This is although not comparable to the levels of β-(1-3)-D-glucan documented in swine farms. Since we have not measured β-(1-3)-D-glucan levels during our exposure studies in swine confinement buildings, we cannot speculate about correlations with clinical parameters. Hence the influence of β-(1-3)-D-glucan on the inflammatory response after exposure to organic dust needs further studies.

Peptidoglycan, the marker for Gram positive bacteria, correlated significantly with the increase of neutrophils in BAL fluid (study I). In a previous swine house exposure study, significant correlations between peptidoglycan and IL-6 increase in serum and changes in granulocyte concentrations in peripheral blood were demonstrated (Zhiping et al., 1996). In study VI, we have demonstrated that Gram-positive bacteria, as well as cell wall components and/or products released from these bacteria are potent stimuli for cytokine release from alveolar macrophages and epithelial cells. Gram positive bacteria and their cell wall components seem to stimulate cellular response in a similar way to LPS, using CD14 (Gupta et al., 1996) and Toll-like receptor 2 (Yoshimura et al., 1999).

The findings from study IV that explored the effect of a respiratory protection device during exposure, together with the study by Palmberg et al (Palmberg et al, unpublished data) raised the new possibility that gas exposure also could contribute to the reaction. The mask with a class P3 filter did not provide a full protective effect although the inflammatory response was attenuated. Apart from a particle exposure, gases seem to have a significant contributing effect on *e.g.* the development of bronchial responsiveness. Health surveys among swine farmers

display somewhat disparate results regarding ammonia exposure and effects on lung function. Vogelzang et al presented a study comprising 196 swine farmers, 96 with chronic respiratory symptoms and 100 symptom-free. No relationship between exposure to ammonia and bronchial responsiveness was established among the farmers (Vogelzang et al., 1997). However, when re-examination of 171 out of the 196 farmers was performed three years later, increased bronchial responsiveness, was significantly associated with ammonia exposure (Vogelzang et al., 2000). A significant correlation with baseline lung function (PEF and FEV_1) and ammonia exposure has been demonstrated by Preller et al (Preller et al., 1995). Cross-shift change in FEV_1 and ammonia exposure was weakly associated in a study by Reynolds et al (Reynolds et al., 1996). Donham et al showed that ammonia exposures ≥ 7.5 ppm predicted a work-shift change of ≥ 3% in FEV_1 (Donham et al., 1995). A longitudinal study stretching over 5 years could not relate annual decline in FEV_1 and FVC with ammonia levels (Kirychuk et al., 1998). Furthermore, Heederik et al did not find a relationship between ammonia exposure and lung function in swine farmers (Heederik et al., 1991). Still, there is no study that has focused on the relationship between gas exposure and inflammatory reactions in the airways of swine farmers. Ammonia exposure is present in other occupational setting than the farming environment. Workers in a soda ash plant were exposed to a time-weighted average ammonia concentration of 9.2±1.4 (mean±SEM) ppm while a control group was exposed to 0.3±0.1 ppm. No difference between the two group regarding base line lung function or work-shift changes was reported, and accordingly, no association to ammonia exposure could be found (Holness et al., 1989). There are a limited number of human experimental inhalation studies regarding exposure to gases like ammonia and hydrogen sulphide in concentrations that are likely to be found in swine houses and what implications exposure have on lung function and bronchial responsiveness.

The occupational exposure limit for hydrogen sulphide is 10 ppm, and in swine confinement buildings the concentration under normal conditions seldom exceeds 5 ppm (Von Essen & Donham, 1999). In the study by Palmberg et al, the exposure levels of hydrogen sulphide were below the detection limit of 0.5 ppm.

Exposing healthy subjects to 10 ppm of hydrogen sulphide during 15 minutes of cycling (50 % of their maximal aerobic power) did not lead to changes in lung function compared to control situation (Bhambhani et al., 1996). A 30 minutes long exposure to 2 ppm of hydrogen sulphide did not significantly influence lung function in asthmatic subjects (Jäppinen et al., 1990).

5.2 Possible mechanisms behind increased bronchial responsiveness

According to our initial hypothesis, cys-LTs might contribute to the increase in bronchial responsiveness following exposure to swine house dust. The participation of leukotrienes in the inflammatory reaction induced by organic dust inhalation was supported by study II, where increased excretion of urinary LTE_4 was detected together with increased levels of LTB_4 and LTE_4 in nasal lavage fluid following exposure. In a subsequent study (III), the effect of cys-LTs on the

bronchial responsiveness was evaluated using pharmacological intervention with the 5-lipoxygenase inhibitor zileuton. The lack of a significant inhibition on whole body formation of cys-LTs, measured as urinary LTE_4 excretion, leaves the question whether cys-LTs are involved in the organic dust induced increase of bronchial responsiveness in healthy non-allergic subjects unanswered. The dose of the 5-LO inhibitor zileuton and the treatment period are the same as have been clinically used with positive effects (for review see (Devillier et al., 1999)). Still, this exposure model comprises a very strong stimulus that induces an intense inflammatory reaction in healthy subjects, and perhaps therefore the pre-treatment dose may have been too low.

Another alternative is to use selective cys-LT_1 receptor antagonists to elucidate the role of cys-LTs. There is, of course, the possibility that the cys-LTs are not pivotal in this non-allergic inflammatory reaction in healthy subjects leading to increased bronchial responsiveness. Increased urinary levels of a PGD_2 metabolite have been demonstrated following inhalation of organic dust and this mediator possesses the ability to induce bronchoconstriction (Hardy et al., 1984) as well as cys-LTs. A study using knock-out mice deficient of the PGD receptor and sensitised to ovalbumin, revealed that these mice failed to develop increased airway hyperreactivity after ovalbumin challenge (Matsuoka et al., 2000).

In our studies, no correlation after swine dust exposure, was observed between bronchial reactivity and inflammatory response measured as changes in concentration of both inflammatory cells and mediators (cytokines, leukotrienes and PGD_2) in healthy, previously unexposed subjects. Hence these results do not support the hypothesis that the increased bronchial responsiveness following organic dust exposure is directly caused by mediator release. The increased swelling and secretions of the airway mucosa induced by an inflammatory reaction will lead to a reduction of the diameter of the airway lumen. The airway resistance (R) is proportional to the radius of the airway lumen (r) demonstrated by the formula $R=1/r^4$ (Nadel et al., 1987). Therefore the same bronchoconstrictive stimulus will lead to a more pronounced bronchial obstruction in airways with reduced lumen prior to the challenge. Thus, a healthy normal subject would display a higher tolerance to airway narrowing prior exposure in a swine farm, than what is demonstrated in the same person following exposure.

Increased bronchial responsiveness despite the use of a respiratory protection device during the exposure in a swine confinement building, suggested a contributing role of gas exposure in the development of augmented bronchial reactivity. Inhalation of irritant gases such as ammonia could induce acute health effects including irritation and inflammation of the airways and oedema formation (Schwartz, 1987). Although the levels of ammonia and other gases usually are below hygienic threshold limits, gases could present a potential health hazard, and possibly promote the development of an increased bronchial responsiveness following exposure in a swine farm.

5.3 Swine farmers versus non-farmers

The airway response following swine house dust exposure during weighing of pigs was compared in swine farmers and healthy, naive subjects (Palmberg et al., 1999). The swine farmers did not show a significant increased bronchial responsiveness after exposure in contradiction to the non-farmers. Furthermore, the cellular increase was of a higher magnitude in naive subjects compared to the swine farmers. This could be a manifestation of an adaptive mechanism in the farmers leading to a reduced inflammatory response. Endotoxin tolerance, or adaptation has been reported after repeated endotoxin exposure with decreased cytokine release as a consequence (Cavaillon, 1995). A study evaluating airway responsiveness to LPS inhalation discovered characteristic differences in response between individuals. They could be divided into a "hyperresponsive" group in which subjects reacted with a decrease $\geq 20\%$ after inhalation of 6.5μg LPS or less, and a "hyporesponsive" group, where FEV_1 still was $\geq 90\%$ after inhaling 41.4 μg LPS. Moreover, ex vivo release of IL-6 and IL-8 from peripheral blood monocytes stimulated with LPS was higher in the group with LPS sensitive subjects (Kline et al., 1999). Interestingly, women were significantly more often found in the sensitive group. This discrepancy has been further studied by the same group and found to be related to mutations of the endotoxin receptor Toll-like receptor 4 (TLR4) (Arbour et al., 2000). The attenuated inflammatory response in swine farmers together with the discovery of differences in LPS tolerance among healthy normal subjects, could implicate a "healthy worker" effect, where subjects tolerant to the environmental exposure in swine confinement building continue their profession.

5.4 Acute inflammation versus chronic bronchitis

Chronic bronchitis is characterised by chronic cough and increased mucus production in the lower airways, and is a common airway disorder among farmers. Elevated number of neutrophils in BAL is observed in patients with chronic bronchitis (Lusuardi et al., 1994). An accumulation of neutrophils in the airways assessed by BAL has been demonstrated in healthy swine farmers, with no symptoms of chronic bronchitis (Larsson et al., 1992). A single, 3 hours long exposure in a swine confinement building, leads to an acute inflammatory airway response with a prominent increase of neutrophils in healthy, previously unexposed subjects. The question rises how this acute response could transform into a chronic inflammatory state in subjects submitted to exposure during daily work in the swine farm. The inflammatory profile in both chronic bronchitis as well as COPD shows similarities to the acute reaction seen in healthy subjects following dust exposure with increased concentration of both neutrophils and IL-8. In a study comparing the inflammatory response in swine farmers with healthy subjects exposed to swine house dust during 3 hours while weighing pigs, swine farmers reacted with a similar increase of IL-8 in nasal lavage fluid as healthy subjects (Palmberg et al, unpublished data), but with a weaker cellular increase

and a smaller increase in bronchial responsiveness. Nevertheless, these findings demonstrate that the swine-farmers still respond to inflammatory stimuli during work in the confinement building, yet with signs of an adaptation. Activated neutrophils can release proteolytic enzymes that could contribute to the pathogenesis of chronic bronchitis. We have confirmed neutrophil activation by measuring MPO (myeloperoxidase) in both BAL fluid (Larsson et al., 2001) and in peripheral blood (Palmberg, unpublished data) in healthy subjects following exposure in a swine farm. Neutrophil elastase and cathepsin G are proteases released from neutrophils, and together with mast-cell derived chymase, they are highly potent secretagogues for airway gland serous cells (Sommerhoff et al., 1989; Sommerhoff et al., 1990). Elastase has also been shown to reduce ciliary-beat frequency and it also causes epithelial injury (Amitani et al., 1991). Another factor contributing to the neutrophil accumulation could be a pro-longed life span of the neutrophil. A study by Rubel et al demonstrated a delayed apoptosis of neutrophils in the presence of the plasma protein fibrinogen (Rubel et al., 2001). Elevated fibrinogen levels in peripheral blood have been detected in healthy subjects after exposure to swine house dust inhalation (Sjögren et al., 1999).

To elucidate which steps in the inflammatory response that are detrimental in the process of transforming a normal host response into a chronic disease, one would need to perform a longitudinal study starting with presumptive swine farmers. The selected cohort would then be followed during an extensive period of time and submitted to regular examinations regarding both lung function and bronchial reactivity. Perhaps even more important, changes in cellular populations and mediator release in the airways and in peripheral blood should be monitored. Of special interest could be the mechanisms that regulate the synthesis (transcription factors) and release of inflammatory mediators. This would, of course, be a very costly and labour-intensive study. An alternative is to use an animal model. The only disadvantage would then be the uncertainty in to what degree the findings are applicable to human.

6. Conclusions

- Swine dust exposure caused an inflammatory reaction characterised by a massive influx of neutrophils and release of inflammatory mediators in the upper (IL-6, IL-8, LTB_4, LTE_4) and in the lower (IL-8) airways. Elevated levels of urinary LTE_4 were observed, indicative of increased whole-body formation of cysteinyl-leukotrienes. Evidence of mast cell activation was provided by increased urinary levels of 9α,11β-PGF_2, a metabolite of the mast cell product PGD_2.

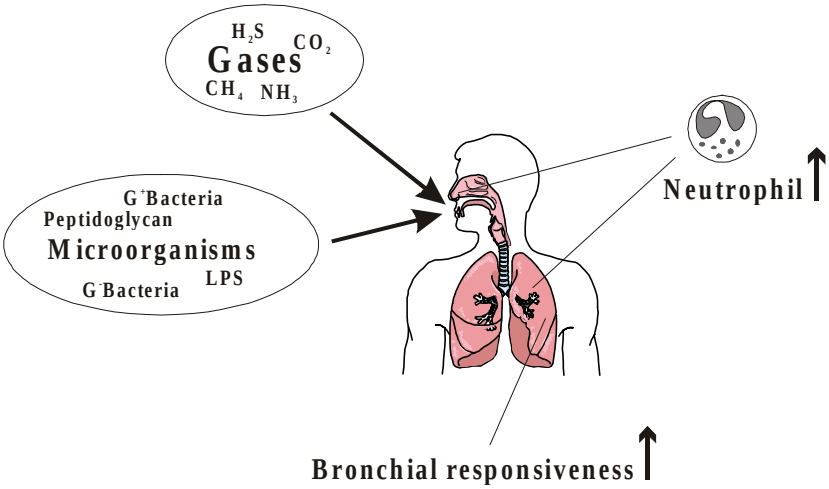
- A significant correlation was observed in the upper airways between changes in concentration of neutrophils and elevated IL-8 levels, but not with changes in LTB_4, suggesting a prominent role for IL-8 in the recruitment of neutrophils into the airways.

- Crude swine dust was more potent stimulus for *in vitro* cytokine production than LPS at the same concentration. β-glucan and grain dust were poor stimuli *in vitro*. Gram positive bacteria and products/cell wall components originating from these bacteria were also potent *in vitro* stimuli. The finding that LPS was a much weaker stimuli *in vitro* together with the lack of significant correlations between clinical parameters in the inflammatory reaction and airborne levels of LPS, conclude that LPS cannot be the sole contributing factor to the inflammation induced by organic dust inhalation in healthy subjects.

- The use of a respiratory protection device significantly attenuated but did not completely abolish the inflammatory response although all particle exposure was eliminated. This indicates that health effects also may be caused by exposure to gases present in the farm environment, though levels of e.g. ammonia and hydrogen sulphide, seldom exceed hygienic threshold limits. In case no improvement of the indoor air quality of swine confinement buildings are made, a respiratory protection device equipped with particle- as well as gas-filter may be needed during work in a swine confinement building, in order to avoid exposure that could initiate an inflammatory response. Repeated exposure during daily work resulting in a prolonged inflammatory reaction could subsequently lead to the development of chronic respiratory disorders, like chronic bronchitis.

- The role of cysteinyl-leukotrienes in the development of increased bronchial responsiveness remains to be concluded. No inflammatory mediators could be related to the increase in bronchial responsiveness. Increased swelling of the airway lumen due to the inflammation may be responsible for the increased responsiveness to methacholine in healthy subjects following exposure.

- This exposure model induces an inflammatory reaction in healthy subjects that displays several similarities to pathological conditions observed in chronic bronchitis and COPD. Considering the increased prevalence of chronic bronchitis among swine farmers, the present exposure model may be suitable to study underlying mechanisms in the development of chronic bronchitis and COPD in swine confinement workers.



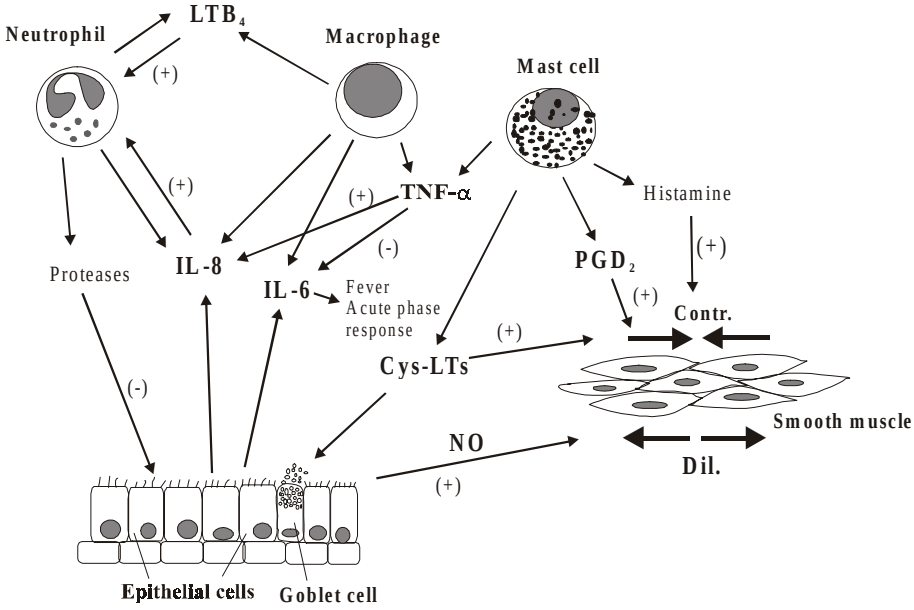


Figure 4. Main events in the non-allergic inflammation induced by exposure to swine house environment.

7. Summary

Larsson, B-M.(2001) *Induction of a non-allergic inflammation in the human respiratory tract by organic dust*. Arbete och Hälsa 2001:8

Swine farming is an occupation associated with high exposure levels to organic dust, but gases such as ammonia and hydrogen sulphide are also contributing to the potentially hazardous exposure. The prevalences for respiratory symptoms, chronic or work-related, are increased among swine farmers, compared to both the normal population as well as other farming categories.

Healthy subjects, previously unexposed to farming environment, developed an intense airway inflammation following three hours of exposure during weighing pigs or during cleaning procedure of an evacuated stable using a high-pressure cleaner. The inflammation was characterised by an increased bronchial responsiveness to methacholine between approximately 1 to 3 dose-doubling steps as well as an extensive migration of inflammatory cells, predominantly neutrophils, into the upper and lower airways. Elevated levels of the chemotactic factor IL-8 found in lavage fluid from the airways most likely achieve this accumulation of neutrophils. The significant correlation between changes in IL-8 levels and increased concentrations of neutrophils found in nasal lavage fluid supports such a theory. Other chemoattractants, could however, also contribute to the recruitment of neutrophils, since increased levels of LTB_4 were demonstrated in the upper airways. Other signs of an induced inflammatory reaction were increased post-exposure levels of the inflammatory cytokine IL-6 in nasal lavage fluid and peripheral blood.

Involvement of cysteinyl-leukotrienes (cys-LTs) was also established. A local production of LTE_4 was detected in the upper airways following exposure as well as an increased whole body formation of cys-LTs measured as increased levels of urinary LTE_4. The cys-LTs were hypothesised to be contributing factors to the increased bronchial responsiveness. The intervention study with an inhibitor of leukotriene biosynthesis could not clarify this assumption due to an insufficient inhibitory effect on urinary LTE_4 excretion.

Signs of mast cell activation were also demonstrated by increased levels of PGD_2, a mast cell mediator, measured as increased urinary levels of the PGD_2 metabolite 9α, 11β-PGF_2.

No relationship between measured inflammatory mediators and increased bronchial responsiveness could be detected, indicating that the airway response was not caused directly by mediator release. Instead, swelling of the airway mucosa and increased secretions due to the general inflammatory reaction could lead to an airway narrowing that would enhance the post-exposure response to methacholine.

Endotoxin has been postulated as the main factor behind respiratory illness in the farming environment. This hypothesis is not supported by our studies. Other agents, originating from Gram-positive bacteria are probably important contributors to the induction of the inflammatory reaction after swine house exposure. The perhaps most intriguing finding regarding exposure in this thesis, was the discovery that even if the healthy volunteers were equipped with a half mask with a particle filter during the exposure, they still responded with an increased bronchial responsiveness, although somewhat attenuated. However, the respiratory device showed better effect regarding reactions in the upper airways. This implicates a role for gases (e.g. ammonia) in the development of increased bronchial responsiveness in healthy, previously unexposed, subjects following an acute exposure in the swine farm environment. On the other hand, the particles seem to have a more prominent role for the reactions in the upper airways.

Keywords: swine farmers, airway inflammation, bronchial responsiveness, organic dust, interleukins, lekotrienes

8. Sammanfattning

Larsson, B-M. (2001) *En icke-allergisk inflammation i humana luftvägar inducerad av organiskt damm.* Arbete och Hälsa 2001:8

Svinuppfödning är ett yrke associerat med höga exponeringsnivåer för organiskt damm, men även gaser som t ex ammoniak och svavelväte kan påvisas i svinstallsmiljön och bidra till den potentiellt skadliga exponeringen. Prevalensen av kroniska eller arbetsrelaterade luftvägssymtom, är högre hos svinbönder än hos normalpopulationen, och andra typer av lantbrukare.

Friska, tidigare oexponerade försökspersoner utvecklade en kraftig luftvägsinflammation efter tre timmars exponering vid vägning av grisar och vid rengöring av svinstall med högtryckstvätt. Inflammationen karakteriserades av en invandring av inflammatoriska celler, huvudsakligen neutrofiler, till såväl de övre som de nedre luftvägarna, och ökad bronkiell reaktivitet mot metakolin med ungefär 1 till 3 dos-dubbleringssteg. Ackumuleringen av neutrofiler i luftvägarna orsakades troligen av förhöjda nivåer av den kemotaktiska faktorn IL-8, som påvisades i sköljvätska från luftvägarna. En signifikant korrelation mellan ökningen av IL-8 och förändringen av antalet neutrofiler i nässköljvätska styrkte denna teori. Andra kemoattraktanter kan bidra till rekryteringen av neutrofiler, och vi fann förhöjda halter av den kemotaktiska faktorn LTB_4 i nässköljvätska efter exponering. Ökad koncentration av den inflammatoriska cytokinen IL-6 påvisades i perifert blod, liksom i sköljvätska från de övre luftvägarna.

Cysteinyl-leukotriener är involverade i den inflammatoriska reaktion efter exponering i svinhus. En ökad lokal produktion av LTE_4 påvisades i de övre luftvägarna efter exponering, liksom en ökad produktion av cysteinyl-leukotriener uppmätt som en ökad utsöndringen av LTE_4 i urin. Hypotesen var att cysteinyl-leukotriener var bidragande faktorer till den ökade bronkiella reaktiviteten efter exponering i svinhus. I en interventionsstudie med en leukotrien biosyntes hämmare (zileuton) fann vi emellertid ingen effekt på utsöndringen av LTE_4 i urin efter exponering.

Förhöjda halter av PGD_2, en mast cells produkt, uppmätt som en ökad utsöndringen av PGD_2 metaboliten 9α, 11β-PGF_2 i urin, talade för en aktivering av mast celler i denna inflammatoriska reaktion.

Avsaknaden av samband mellan uppmätta inflammatoriska mediatorer och ökad bronkiell reaktivitet, tydde på att den ökade luftvägsreaktiviteten inte direkt orsakades av en frisättning av inflammatoriska mediatorer. En ökad svullnad och sekretion i luftvägarnas slemhinna orsakad av den inflammatoriska reaktionen leder sannolikt till en luftvägsförträngning vilket i sin tur resulterar i en kraftigare luftvägsobstruktion vid inhalation av samma dos metakolin efter exponeringen.

Exponering för endotoxin har ansetts vara den huvudsakliga orsaken till luftvägsproblem i lantbruksmiljöer. Denna avhandling har dock inte kunnat styrka

ett sådant antagande. Komponenter från Gram positiva bakterier är förmodligen också betydelsefulla för uppkomsten av den inflammatoriska reaktionen vid exponering i svinstall. En betydelsefull upptäckt i denna studie var att friska försökspersoner som exponerades under tre timmar i svinstallsmiljö med andningsskydd utrustat med partikelfilter, fortfarande uppvisade en ökad bronkiell reaktivitet efter exponeringen. Ökningen var dock mindre än den som observerades efter exponering utan filter. Andningsskyddet uppvisade en tydligare effekt i de övre luftvägarna. Detta kan tyda på att exponering för gaser som t ex ammoniak, i svinstall spelar en roll för uppkomsten av den ökade bronkiella reaktiviteten hos friska, tidigare oexponerade försökspersoner. Exponeringen för partiklar tycks spela en större roll för uppkomsten av inflammation i de övre luftvägarna.

Nyckelord: Svinbönder, luftvägsinflammation, bronkiell reaktivitet, organiskt damm, interleukiner, leukotriener

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