MINNA PUROKIVI

Assessment of airway inflammation in association with exposure to microbes present in moisture-damaged buildings

Doctoral dissertation

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Department of Respiratory Medicine
University of Kuopio
Distributor: Kuopio University Library
P.O.Box 1627
FIN-70211 Kuopio
FINLAND
Tel. +358 17 163 430
Fax +358 17 163 410

Serial editors: Professor Esko Alhava, M.D., Ph.D.
Department of Surgery
Professor Martti Hakumäki, M.D., Ph.D.
Department of Physiology

Authors Address: Department of Respiratory Medicine
Kuopio University Hospital
P.O. Box 1777
FIN-70211 Kuopio
FINLAND

Supervisors: Professor Hannu Tukiainen, M.D.
Department of Respiratory Medicine
University of Kuopio
Docent Maija-Riitta Hirvonen, Ph.D.
National Public Health Institute
Kuopio

Reviewers: Docent Kari Reijula, M.D.
Finnish Institute if Occupational Health
Helsinki
Docent Lauri Tammilehto, M.D.
Finnish Institute if Occupational Health
Helsinki

Opponent: Docent Hannu Puolijoki, M.D.
Seinäjoki Central Hospital

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ABSTRACT

Several epidemiological studies have reported that there is an association between adverse health effects and exposure to microbial growth in moisture-damaged buildings. However, there has been a serious lack of objective biochemical data linking together qualitative characteristics of the microbial exposure, the large variety of symptoms among the occupants and the inflammatory changes occurring in the respiratory tract mucosa of the exposed subjects. The aim of this study was to determine the feasibility of non-invasive sampling methods induced sputum (IS) and exhaled nitric oxide (NO) measurement and to identify markers that could be used in evaluating possible inflammatory changes in lower airways after exposure to microbes encountered in moisture-damaged buildings.

In the validation study the measurement of exhaled NO and analyses of pro-inflammatory cytokines (tumour necrosis factor (TNF)α, interleukin (IL)-4 and IL-6) and eosinophil cationic protein (ECP) measured from IS supernatant of healthy volunteers were shown reproducible when sampled 48 h apart. In addition, differential cell counts, despite of lymphocytes, were found highly reproducible. Inducible nitric oxide synthase (iNOS) was not detectable in sputum cells from the healthy volunteers supporting the general assumption that expression of iNOS is related to inflammatory diseases and activation of host defence.

To evaluate the value of IS and exhaled NO measurement in assessing the inflammatory status of lower airways after exposure to microbes present in moisture-damaged buildings, subjects with occupational exposure in such building and their controls without exposure were studied. Microbial analyses performed from indoor air, surface and material samples confirmed the classification of the studied school buildings into the moisture-damaged test-school and control school. Inflammatory mediators were determined from IS and nasal lavage (NAL) samples, and exhaled NO was measured from all subjects during both working and vacation periods. During working period statistically significant elevations of IL-1, TNFα, and IL-6 in NAL and IL-6 in IS of exposed subjects were found. The exposed subjects reported sore throat, phlegm, eye irritation, rhinitis, nasal obstruction and cough in parallel with these findings. Nitrite levels in NAL and IS as well as level of exhaled NO did not show any significant differences between the two groups. iNOS expression was not detected from NAL and IS cells during exposure or vacation periods. Although the levels of NO and IL-4 in NAL did predict their level in IS in a statistically significant manner, their predictive values were low.

In conclusion, the high reproducibility of studied measurements provide a foundation for their use in investigating inflammatory changes in association with exposure to indoor air bioaerosols. The present data point to an association between microbial exposure, reported symptoms, and the pro-inflammatory mediators assayed from IS and NAL samples in subjects exposed to indoor air bioaerosols in moisture-damaged buildings. These results also show that inflammatory mediators determined from NAL are not alone reliable in evaluating the inflammatory status of the lower airways. Moreover, the measurement of exhaled NO alone it is not sufficient in assessing airway inflammation in this context.

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Minna Purokivi
ABBREVIATIONS

BAL Bronchoalveolar lavage
CD4/CD8 Helper/suppressor lymphocyte
cNOS Constitutive nitric oxide synthase enzyme
COPD Chronic obstructive pulmonary disease
CRP C-reactive protein
D Dalton
DTT Dithiotreitol
ECP Eosinophil cationic protein
EIA Enzyme immunoassay
ELISA Enzyme-linked immunosorbent assay
eNOS Endothelial nitric oxide synthase enzyme
EPO Eosinophil peroxidase
EPX Eosinophil protein X
FEV1 Forced expiratory volume in one second
GM-CSF Granulocyte-macrophage colony-stimulating factor
GMP Guanylate monophosphate
HIV Human immunodeficiency virus
HNl Human neutrophil lipokalin
IFN-γ Interferon gamma
Ig Immunoglobulin
IL Interleukin
iNOS Inducible nitric oxide synthase enzyme
IS Induced sputum
LPS Lipopolysaccharide
MBP Major basic protein
MGG May Grünwald Giemsa
MPO Myeloperoxidase
MVC Microbial derived volatile organic compounds
NADPH Nicotinamide adenine dinucleotide phosphate, reduced form
NANC Non-adrenergic, non-cholinergic
NAL Nasal lavage
nNOS Neuronal nitric oxide synthase enzyme
NO2 Nitric dioxide
NO Nitric oxide
O3 Ozone
ODTS Organic dust toxic syndrome
PEFR Peak expiratory flow rate
RANTES Regulated on activation, normal T cell-expressed, and secreted protein
RIA Radioimmunoassay
SPT Skin prick test
TCC Total cell count
Th T helper lymphocyte
TNFα Tumour necrosis factor alpha
VOC Volatile organic compound
LIST OF ORIGINAL PUBLICATIONS

This thesis is based on original publications, which will be referred to by their Roman numerals.


IV Purokivi M., Hirvonen M-R., Randell J., Roponen M., Tukiainen H. Nitric oxide alone is an insufficient biomarker of exposure to microbes in a moisture-damaged building. Inhalation Toxicology 2002; in press.
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APPENDIX: ORIGINAL ARTICLES
1 INTRODUCTION

Several cross-sectional epidemiological studies in different countries have emphasized to the strong association between microbial growth in building structures and adverse health effects in the occupants of the buildings. (Dales et al. 1991b, Brunekreef 1992, Jaakkola et al. 1993, Spengler et al. 1994, Pirhonen et al. 1996, Bornehag et al. 2001) This is of special interest, since in studies based on questionnaire data the prevalence of moisture damages in UK is 30% (Platt et al. 1989), in the Netherlands 20-25% (Brunekreef 1992), in Canada 14-32% (Dales et al. 1991) and in Finnish houses it varies between 3-15% (Pirhonen et al. 1996, Kilpeläinen et al. 2001). In the report of Nevalainen et al. (1998), in which 450 houses where thoroughly investigated by trained civil engineers, as many as 55% of houses in Finland were in need of repair due to moisture defects. In a Finnish questionnaire study concerning moisture problems in schools, twenty percent of 1164 evaluated schools suffered from serious moisture damage, 60% less severe damage, and in 26% of schools visible mould growth as well as mould odour was found (Kurnitski et al. 1996). Thus, moisture damage appears to be a common phenomenon in the modern building stock. As a consequence of moisture damage, microbial growth is also common in buildings causing exposure to biological particles among the occupants. During the year 2000 from the Finnish Institute of Occupational Health, altogether 42 new cases of asthma, 24 of allergic rhinitis, 6 of allergic alveolitis (in addition to 36 Farmer’s lung cases) and 8 cases of ODTs (out of 15) in relation to exposure to microbes in moisture-damaged buildings were sent to the Finnish Registry of Occupational Diseases (Karjalainen et al. 2001).

In previous epidemiological studies, the assessment of exposure in moisture-damaged buildings was mainly based on visual observations of moisture and mould growth, or smell of mould odour reported by the occupants. Among objective methods to evaluate the microbial contamination of an indoor environment, measurements of airborne viable fungi (Hyvärinen et al. 1993, Nevalainen et al. 1998) or determinations of microbial components such as endotoxins (Park et al. 2001), 1,3-β-D-glucan (Rylander et al. 1997), ergosterol (Dharmage et al. 1999) or mycotoxin (Tuomi et al. 2001) from indoor air or house dust have been used. However, the results of the studies concerning exposure assessment in mouldy buildings are contradictory. The concentrations and content of microbial flora is affected by a number of factors including climate, season, ventilation and other building characteristics, the activities of the occupants as well as microbial sources such as microbial growth (Nevalainen et al. 1991, Samson et al. 1994). Microbial growth in water damaged building
materials often includes microbial genera or groups not belonging to the normal mycoflora of the indoor environments (Hyvärinen et al. 2002). These "indicator" microbes are considered to indicate building-related moisture (Samson et al. 1994). Emissions into indoor air may include microbial spores, cells and products, such as volatile end products of metabolism or other secondary metabolites, such as microbial toxins (Sorensen et al. 1987, Andersson et al. 1997). Especially the production of secondary metabolites is dependent on the strains (Betina 1989, Ruotsalainen et al. 1998, Huttunen et al. 2000) and on building material on which the growth takes place (Murtoniemi et al. 2001, Roponen et al. 2001b) as well as on growing conditions (Hirvonen et al. 2001).

Little is known about the biochemical link between exposure to microbes in a moisture-damaged building and the symptoms experienced by the occupants. There is evidence that indicator microbes of moisture-damaged buildings cause specific inflammatory responses i.e. elevation in pro-inflammatory cytokines and nitric oxide (NO), in both rodent and human cells in vitro (Hirvonen et al. 1997a, Huttunen et al. 2000, Jussila et al. 1999) and cause inflammation and tissue damage in mouse lungs in vivo (Nikulin et al. 1996, Nikulin et al. 1997, Jussila et al. 2001).

Previously bronchoalveolar lavage (BAL) and bronchial biopsies have been used in occupational environmental studies evaluating the effects of air pollutants and bioaerosols on the lower airways (Graham and Koren 1990, Larsson et al. 1997, Wang et al. 1997). These methods are invasive, expensive and time consuming, and not suitable for repeated sampling. Sputum induction by hypertonic saline does not suffer from these disadvantages, and seems suitable method for studying lower respiratory tract inflammation (Fahy et al. 1995a, von Essen et al. 1998, Nigthingale et al. 2000). Similarly, measurement of exhaled NO by chemiluminescence method has become a valuable tool in assessing lower airway inflammation e.g. in asthma (Kharitonov et al. 1994), but it has not been used in studying airway inflammation related to exposure to abnormal microbial flora typically present in the indoor air of moisture-damaged buildings. In this context, inflammation in nasal mucosa has been evaluated with nasal lavage (NAL), showing an elevation of pro-inflammatory cytokines, NO, ECP, and MPO during occupational exposure (Hirvonen et al. 1999, Roponen et al. 2001a, Wålinder et al. 2001). Since the nasal mucosa is the first barrier to inhaled air and there are many similarities in its histology and the histology of bronchial mucosa, it has been proposed that NAL alone could also be used to evaluate lower respiratory tract inflammation (Graham and Koren 1990, Noah et al. 1995). The aim of the present study was to evaluate the usefulness of the non-invasive sampling methods, induced
sputum (IS) and measurement of exhaled NO, to identify inflammatory markers which could be used in determining inflammatory changes in lower airways in association with exposure in moisture-damaged buildings, and to evaluate if NAL samples could be used alone in assessing inflammation in the lower airways.
2 REVIEW OF THE LITERATURE

2.1 Health effects and exposure to microbes in moisture-damaged building

Several epidemiological studies evaluating health effects related to exposure to moisture-damaged building microbes based on interview (Platt et al. 1989) or questionnaire data (Dales et al. 1991b, Brunekreef 1992, Spengler et al. 1994, Pirhonen et al. 1996, Timonen et al. 1995) have revealed the association between lower respiratory symptoms, such as cough, phlegm, wheezing, dyspnea, and damp housing and the presence of mould in building materials. This association was seen even though subjects with known allergies and asthma were excluded from the analyses (Dales et al. 1991b). In addition to respiratory symptoms of upper and lower airways, several authors have reported also non specific symptoms, such as fatigue and difficulties in concentration, in both adults and children associated with moisture-damaged indoor environments (Pirhonen et al. 1996, Koskinen et al. 1999a, Koskinen et al. 1999b).

The connection between allergy and asthma and exposure to a high total level of indoor air bacteria has been suggested to be a risk factor for asthma related symptoms and wheezing, since both ECP and IgE in serum have been associated with the high number of viable bacteria, and IgE also to the total number of fungi (Björnsson et al. 1995). However, no correlation has been found between pulmonary function tests and the number of airborne bacteria, fungi or house dust mites (Björnsson et al. 1995). Approximately up to 6 % of individuals in the general population have respiratory allergy to fungi (Kurup et al. 2000). It has been shown that allergy to moulds was also rare in children in a moisture-damaged school (Taskinen et al. 1997). Moreover, recent data demonstrate that although there was a correlation between reported lower respiratory symptoms and exposure, there was no statistically significant association between PEFR, FEV1, SPT and IgE to fungal antigens in serum, and microbial exposure among school children (Taskinen et al. 1997, 1999, 2001). In a three-year follow-up study with the same population, positive findings of specific IgE to fungal antigens were still rare, and were mostly found from children with multiple atopy without any association with exposure in a moisture-damaged school (Immonen et al. 2000). Furthermore, no evidence of progression of atopy during this time was found (Immonen et al. 2001).

There is, however, evidence of an increased risk of asthma in children related to dampness and indoor mould (Andrea et al. 1988, Dekker et al. 1991, Jaakkola et al. 1993,
Lindfors et al. 1995, Maier et al. 1997). This has also been shown among adults (Williamson et al. 1997). The increased risk for asthma or asthmatic symptoms in water-damaged buildings observed in various studies has been OR 1.5-3.5 (Peat et al. 2001). Home dampness also maintains currently symptomatic asthma, allergic rhinitis and atopic dermatitis, and also increases the susceptibility to common colds (Kilpeläinen et al. 2001).

Assessment of the microbial status of the moisture-damaged buildings can provide further information about the exposure in the indoor environment (Hyvärinen et al. 1993, Nevalainen et al. 1998). There is a considerable variation in the indoor air microbial flora in climate with a distinct seasonal range. However, the composition of the microbial genera in the problem buildings differs from the normal flora which consists of outdoor air microbes entering the buildings via the ventilation systems and of emissions from normal sources such as handling vegetables or firewood (Lehtonen et al. 1993). The concentrations of airborne bacteria and fungal spores in moisture-damaged buildings are higher than in reference buildings (Hyvärinen et al. 1993, Meklin et al. 2002), but in addition to microbes and their structural components also metabolic products released by the microbes are among the possible exposing agents of mouldy indoor environments. Recently it was reported that even among the same bacteria the cytotoxicity can vary between different strains (Ruotsalainen et al. 1998, Huttunen et al. 2000), and the growing conditions have an important influence on the spores’ ability to induce inflammatory responses in vitro (Hirvonen et al. 2001, Murtoniemi et al. 2001, Roponen et al. 2001b).

It has been shown in vitro that epithelial cells may contribute to the pathogenesis of airway diseases by their interaction with inhaled biologic substances. These extracts, e.g. allergens, may enhance airway inflammation by means other than IgE-binding activity (Tomee et al. 1998). In studies concerning occupational exposure, structural components of microbial cells such as gram negative bacterial lipopolysaccharides (LPS) (Rylander 1995, Michel et al. 1997) and 1,3-β-D-glucan of the cell wall of actinomycetes and fungi (Fogelmark et al. 1992), have been suggested to be responsible for the inflammatory changes occurring in the airways. Bacterial endotoxins are known to cause inflammation by activating macrophages and bronchial epithelial cells, and increase B-cell mediated antibody formation (Rylander 1998). Various microbial components together can exert synergistic effects on pulmonary inflammation as shown in laboratory animals (Fogelmark et al. 1994). Andersson et al. (1999) have reported significantly lower endotoxin and 1,3-β-D-glucan concentrations in the indoor air of a day care centre (0.2-0.3 ng/mg dust and 0.5-1.4 ng/mg dust, respectively) than in animal sheds (8-35 ng/mg of dust and 0.3-2 up to 5-41 ng/mg
dust, respectively). Although massive exposure to biological dust may explain the excess of respiratory diseases occurring in farmers (Tammiilehto et al. 1994), it could be argued that microbes may not be the principal exposing component in the indoor air of moisture and mould contaminated schools and day care centres.

Mycotoxins, secondary metabolites of many moisture indicative moulds, are usually produced under conditions not optimal for growth. Key factors that are known to influence fungal growth are temperature, relative humidity, moisture and growth substrate. (Hendry and Cole 1993, Andersson et al. 1997, Nielsen et al. 1999, Tuomi et al. 2000) Consumption of mouldy food contaminated with mycotoxins can cause adverse side effects or even be fatal. Severe health hazards, even cancer, have been described in case reports after exposure to inhaled mycotoxins in occupations related to grain processing (Olsen et al. 1988), laboratory work (Dvorackova 1990) and in association with mouldy housing (Croft et al. 1986, Johanning et al. 1996). In vitro studies have given evidence that *Stachybotrys atra* spores contaminated by satratoxins have higher pulmonary toxicity than mycotoxin free spores of the same microbe (Nikulin et al. 1996, Nikulin et al. 1997). Exposure via inhalation in indoor environments has rarely been shown. However, in most of the studies the lack of exact information about inhaled particles and their chemical structures in indoor environment, make the estimation of health risk of inhaled mycotoxins difficult. (Nielsen et al. 1999, Robbins et al. 2000)

Microbiologically produced volatile organic compounds, MVOCs, include various chemical structures i.e. alcohols, ketones and organic acids. Mixtures of these compounds account for mouldy odours, and they are considered to provoke respiratory symptoms among inhabitants of mouldy buildings (Jaakkola et al. 1993, Becher et al. 1996, Norbäck et al. 1999). In a placebo controlled, double blinded study Pappas et al. (2000) reported dose-related increases in respiratory symptoms in upper and lower airways after inhalation challenge to a VOC mixture, but were not able to detect any changes in inflammatory markers (i.e. cell counts, IL-8) in NAL and IS or in pulmonary function tests of the subjects. Furthermore, a study with an animal model suggests that MVOSs may have synergistic effects for the sensory irritation response but their contribution to the symptoms may be less crucial (Korpi et al. 1999a). Korpi et al. (1998, 1999b) have recently reported that moisture-damaged building materials may emit similar VOCs whether they are sterile or contaminated by microbes suggesting that VOCs are not a specific indicators of microbial contamination. Their effects on respiratory symptoms of occupants and the possible inflammatory pattern behind the symptoms need further evaluation.
The association between exposure to airborne fungi and the development of IgG antibodies to microbial antigens has been shown in adults in occupational environments (Kurup et al. 1987, Terho et al. 1987, Erkinjuntti-Pekkanen et al. 1999). However, no clear association with IgG antibodies and exposure was found in children who were exposed to moisture-damaged building microbes at school (Taskinen et al. 2002).

2.2 Evidence of biochemical linkage

Recent in vivo and in vitro studies have provided further evidence of the biochemical link between exposure to microbes in moisture-damaged buildings and the health effects of the occupants. Actinomycetes, which are known to be indicator microbes of moisture damage in building structures (Nevalainen et al. 1991), cause expression of iNOS, and subsequent production of NO and cytokines as well as cytotoxicity in rodent macrophages, thus latter effect not being dependent on bacterial viability (Hirvonen et al. 1997b). Moisture indicating fungi, i.e. Stachybotrys, Cladosporium, Aspergillus and Penicillium, have been found to elevate cytokine production in rat alveolar macrophages (Shahan et al. 1998) though not to the same extent as actinomycetes (Hirvonen et al. 1997a, 1997b). Also mycobacterium species which had originated from moisture infested buildings evoked a time and dose dependent production of pro-inflammatory cytokines IL-6 and TNFα, expression of iNOS and production of NO in rodent macrophages (Huttunen et al. 2000). Jussila et. al. (2001) have shown that similar inflammatory responses can be seen in BAL fluid of mice exposed via tracheal instillation to spores of Streptomyces californicus. In parallel to this finding, LPS challenge caused a rapid but not as high elevation in inflammatory markers.

In line with the in vitro and in vivo findings, it has been shown that the concentrations of the inflammatory markers in NAL of occupants of a moisture-damaged building are elevated during the exposure to microbes in indoor environment. These findings run parallel to questionnaire data concerning the respiratory symptoms of the same occupants. (Hirvonen et al. 1999, Roponen et al. 2001a, Wålinder et al. 2001)

2.3 Inflammatory markers in airways

In host defence mechanisms the inflammatory mediators are often the same though the trigger for the inflammation varies. In the network of immune defence, leukocytes, epithelial
and parenchymal cells communicate through complex signalling systems, such as cytokines and NO.

2.3.1 Nitric oxide

NO is synthesised from the amino acid L-arginine by a family of enzymes, the nitric oxide synthases (NOS). (Figure 1.) These enzymes are either calcium dependent and constitutive (cNOS) or calcium independent and inducible (iNOS). The cNOS enzymes, expressed in unique subsets of epithelial cells in various organs, i.e. human bronchial epithelium (Shaul et al. 1994), produce NO in picomolar concentrations for intracellular signalling, maintaining blood vessel tone, neurotransmission, host defence and immunity. (Clancy and Abramson 1995) Inducible NOS is produced in nanomicromolar concentrations (Clancy and Abramson 1995) by many cell types, i.e. epithelium in paranasal sinuses (Lundberg et al. 1996b), human alveolar (Asano et al. 1994) and bronchial (Hamid et al. 1993) epithelium, murine macrophages (Hibbs et al. 1988) and neutrophils (Moncada et al. 1993). iNOS has an important role in host defence and immunological reactions, and it is thought to be a mediator in vasodilatation, edema, cytotoxicity and other cytokine dependent processes.

![Figure 1. - Biosynthesis of nitric oxide from L-arginine to NO and L-citrulline is catalysed by nitric oxide synthase (NOS) enzyme in the presence of oxygen and NADPH.](image-url)
Endogenous NO is measurable from exhaled air (Gustafsson et al. 1991), and it has a regulatory role in both physiological and pathophysiological functions in the upper and lower airways. (Figure 2.) The production of NO has been found to be disturbed in several inflammatory diseases. (Table 1.) Elevated levels of NO have also been measured from NAL after occupational exposure to indoor air microbes (Hirvonen et al. 1999, Douwes et al. 2000, Roponen et al. 2001a).

Figure 2. – Effects of nitric oxide (NO) in the airways. Different inhaled irritants may stimulate NOS (iNOS, eNOS, nNOS) expression in various cell types leading to activation of inflammatory processes in the airway mucosa.
Table 1. Exhaled nitric oxide (NO) from upper and lower airways in some inflammatory diseases measured by chemiluminescence analyser.

<table>
<thead>
<tr>
<th>Disease or exposure</th>
<th>Oral NO</th>
<th>Nasal NO</th>
<th>Author (year)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asthma</td>
<td>↑</td>
<td>NM</td>
<td>(Alving et al. 1993)</td>
</tr>
<tr>
<td></td>
<td>↑</td>
<td></td>
<td>(Kharitonov et al. 1994)</td>
</tr>
<tr>
<td>Lower respiratory tract infection</td>
<td>↑</td>
<td>NM</td>
<td>(Alving et al. 1993)</td>
</tr>
<tr>
<td>Upper respiratory tract infection</td>
<td>↑</td>
<td>NM</td>
<td>(Kharitonov et al. 1995c)</td>
</tr>
<tr>
<td>Bronchiectasis</td>
<td>↑</td>
<td>NM</td>
<td>(Kharitonov et al. 1995b)</td>
</tr>
<tr>
<td>Kartagener’s syndrome</td>
<td>NM</td>
<td>↓</td>
<td>(Lundberg et al. 1994)</td>
</tr>
<tr>
<td>Cystic fibrosis</td>
<td>NM</td>
<td>↓</td>
<td>(Lundberg et al. 1996b)</td>
</tr>
<tr>
<td>Seasonal rhinitis</td>
<td>↑</td>
<td>↑</td>
<td>(Martin et al. 1996)</td>
</tr>
<tr>
<td>Laboratory animal allergy</td>
<td>↑</td>
<td>↑</td>
<td>(Adisesh et al. 1998)</td>
</tr>
<tr>
<td>COPD</td>
<td>↑</td>
<td>NM</td>
<td>(Maziak et al. 1998)</td>
</tr>
<tr>
<td>Pulmonary sarcoidosis</td>
<td>↑</td>
<td>NM</td>
<td>(Moodley et al. 1999)</td>
</tr>
<tr>
<td>Smoking</td>
<td>↓</td>
<td>↓</td>
<td>(Persson et al. 1994),</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(Kharitonov et al. 1995a)</td>
</tr>
<tr>
<td>Smoking cessation</td>
<td>↑</td>
<td>NM</td>
<td>(Robbins et al. 1997)</td>
</tr>
<tr>
<td>Exposure in swine confinement building</td>
<td>NM</td>
<td>↑</td>
<td>(von Essen et al. 1998)</td>
</tr>
</tbody>
</table>

NM = not measured; ↑ = elevated NO; ↓ = decreased NO.

2.3.2 Cytokines

Cytokines are small, non-structural proteins with molecular weights ranging from 8 to 40 000 D. Most of the cytokines are involved in host responses to disease or infection. (Dinarello 2000) They are multifunctional depending on the activating factor, and they seldom are expressed alone in inflammation, instead they operate in networks. Some of the cytokines that commonly take part into airway inflammation are presented in more detail in table 2.
Table 2. Origin and function of pro-inflammatory cytokines (interleukin (IL)-1, IL-6, TNFα), and IL-4, IL-5, IL-8 and IFNγ. (Barnes et al. 1998)

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Producing cells</th>
<th>Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1</td>
<td>lymphocytes, eosinophils, macrophages, monocytes, fibroblasts, epithelial cells</td>
<td>Induction of endothelial adhesion molecules, growth factor for T and B cells, release of neutrophils, induces IL-6 production and fever</td>
</tr>
<tr>
<td>TNFα</td>
<td>lymphocytes, eosinophils, macrophages, monocytes, epithelial cells</td>
<td>Apoptosis, NO-production and cytotoxicity, chemotaxis of eosinophils, expression of IL-6 and 8</td>
</tr>
<tr>
<td>IL-4</td>
<td>T-lymphocytes, eosinophils</td>
<td>B-cell activation and production of IgE, promotes Th0 → Th2, inhibits TNFα, IL-1, IL-8 and INFγ and iNOS production in macrophages, promotes growth of eosinophils and basophils, induces fibroblasts chemotaxis and activity</td>
</tr>
<tr>
<td>IL-5</td>
<td>T-lymphocytes</td>
<td>Production, growth, maturation, activity and survival of eosinophils</td>
</tr>
<tr>
<td>IL-6</td>
<td>lymphocytes, monocytes, macrophages, epithelial cells, fibroblasts, smooth muscle cells</td>
<td>Induces production of immunoglobulins, growth regulatory effects on lymphocytes, inhibits IL-1 and TNFα in macrophages</td>
</tr>
<tr>
<td>IL-8</td>
<td>lymphocytes, neutrophils, macrophages, epithelial cells</td>
<td>Neutrophil recruitment and activation</td>
</tr>
<tr>
<td>IFNγ</td>
<td>T-lymphocytes</td>
<td>Inhibits cell proliferation, enhances cytotoxicity of TNFα, inhibitor of IL-4</td>
</tr>
</tbody>
</table>

In different pathological conditions, the cytokine pattern may show significant similarities. The pro-inflammatory cytokines TNFα, IL-1, IL-6 are activated not only in asthma (Hamid et al. 1993, Gelder et al. 1995), allergic rhinitis (Bachert et al. 1995) and prolonged cough (Jatakaran et al. 1999) but also in viral infections or bacterial pneumonia (Baumann and Gauldie 1994). Similarly their elevation in airway inflammation has been related to exposure to different inhaled irritants (Dahlgvist et al. 1996, Douwes et al. 2000). Th2-type cytokines, such as IL-4 and IL-5, are mainly connected to atopy, and symptoms and diseases related to atopy (Shi et al. 1998, Olivenstein et al. 1999). After occupational exposure to microbes present in moisture-damaged building the IL-4 levels have been elevated though atopy among the exposed subjects did not correlate with the cytokine result (Roponen et al. 2001a). The main functions of IL-8 are the recruitment and activation of neutrophils, and it is present in acute bacterial infections but also in chronic inflammatory conditions in the airways such as COPD (Keatings et al. 1996), cystic fibrosis and bronchiectasis (Richman-Eisenstat et al. 1993), and exposure to occupational (Douwes et al. 2000) or environmental irritants (Fahy et al. 1995b).
2.3.3 Inflammatory cells

The distribution of inflammatory cells in upper and lower airways has been examined in numerous studies. In addition, the total cell count in nasal and bronchial samples as differential cell counts have given information about the inflammatory pathways in the mucosa.

Monocytes, cells which are formed in the bone marrow maturate within the tissues into macrophages. These are phagocytic cells which are activated by pro-inflammatory cytokines as well as bacterial toxins. After activation and actual phagocytosis, they augment the inflammation response by excreting cytokines such as TNFα, IL-1, IL-6, prostaglandins, leukotrienes and expression of iNOS. They also process and present antigens to lymphocytes. In induced sputum samples of healthy volunteers, most of the leukocytes are macrophages (Belda et al. 2000).

Neutrophils are phagocytes whose main task in immune defence is to kill bacteria. They have intracellular granules containing various proteins i.e. myeloperoxidase (MPO), lysozyme and human neutrophil lipokalin (HNL). Neutrophils appear in larger airways of both healthy and diseased subjects. In addition to microbes and their products, such as endotoxin (Nightingale et al. 1998), neutrophils also are activated by non-specific irritants e.g. inhaled diesel exhaust particles (Nightingale et al. 2000) and ozone (Graham and Koren 1990, Liu et al. 1999).

Eosinophils are important mediators in allergic inflammation (Kirby et al. 1987). Elevated levels of eosinophils are seen in parasite infections, as well as in atopic diseases, such as asthma or allergic rhinitis, but in smaller amounts also in other inflammatory diseases such as COPD (Keatings and Barnes 1997). Eosinophil granules contain several inflammatory mediators, which can be secreted into the extracellular space after stimulation. These mediators include basic proteins (i.e. ECP, EPO, EPX), cytokines (GM-CSF, IL-5, IL-6, TNFα), lipid mediators (cysteiny1 leukotrienes) and oxygen radicals.

Basophils and mast cells, which are tissue basophils, play an important role in anaphylaxis and in allergic reactions. They release inflammation promoting substances, for example histamine and leukotrienes. Basophils and mast cells are found in the bronchial walls and also the nasal mucosa of both healthy and asthmatic subjects. (Dolovich et al. 1989)

Lymphocytes can be subdivided into T- and B-lymphocytes. When T-lymphocytes are activated by antigen presenting macrophages, they induce antigen specific immunoglobulin
production in B-lymphocytes. T-lymphocytes are grouped into CD4 and CD8 cells, and CD4 cells further to Th1 and Th2 cells depending on the cytokine profile they produce. Th1 cells can secrete INFγ, an agent which inhibits IgE production in B-lymphocytes. Th2 cells secrete IL-3, IL-4, IL-5 and IL-10, which are crucially involved in atopy and allergic diseases and are also measurable from airway excretions of diseased subjects (Walker et al. 1991, Gelder et al. 1995, Benson et al. 1997).

2.4 Non-invasive methods to study airway inflammation

The assessment of airway inflammation is important if one wishes to investigate the underlying mechanisms of airway diseases. The measurements of lung function or airway hyperresponsiveness may not always reveal diseased airways. Recently, non-invasive sampling methods, e.g. measurement of exhaled NO, IS and NAL, have become valuable tools in investigating the effects of various pollutants on health (Hirvonen et al. 1999, Nightingale et al. 2000) as well as in assessing inflammatory changes and the effects of therapy in common airway diseases, such as asthma (Kharitonov et al. 1994, Rytilä et al. 2000) or COPD (Maziak et al. 1998, Peleman et al. 1999).

2.4.1 Induced sputum

Bronchoalveolar lavage (BAL) and bronchial biopsies with fiberoptic bronchoscope are used in assessing inflammatory changes in lower airways. However, these procedures are invasive and time consuming, and not appropriate for monitoring airway inflammation repeatedly for example, to study the time courses of inflammatory processes. (Holz et al. 2000) Already at the end of 19th century, the presence of inflammatory cells in the sputum of asthmatic subjects was proposed to have found diagnostic significance (Gollasch 1889). IS was taken into use again as a non-invasive diagnostic tool in studying Pneumocystis carini infections of HIV patients during the 1990s (Shimomoto et al. 1995). It was also reintroduced in asthma research (Pin et al. 1992b), and since then it has been widely used.

Sputum induction with ultrasonically nebulized hypertonic saline has been proved to be a direct, non-invasive, valid and repeatable method for evaluating inflammatory changes in the lower airways. (Fahy et al. 1993, Pizzichini et al. 1996a) Bacci et al. have shown that hypertonic saline does not alter sputum cell composition in single sampling when compared to inhalation of isotonic saline. (Bacci et al. 1996) The inflammatory markers determined
from IS of asthmatic subjects are at the same level as in their spontaneous sputum sample, also supporting the minor effect of hypertonic saline on inflammatory markers in asthmatic airways (Pizzichini et al. 1996b). However, in repeated sampling within 24 hours, the time interval between induction is critical because the loss of macrophages as well as an excess of neutrophils has been reported by several authors (Nightingale et al. 1998, Holz et al. 1998). Inflammatory mediators determined from the sputum correlate best with bronchial washings and more variably, but reasonably, with bronchial biopsies and BAL (Fahy et al. 1995a, Grootendorst et al. 1997, Keatings et al. 1997, Pizzichini et al. 1998). This refers to the different origin of samples; sputum and also bronchial washings reflect the inflammation in more central airways whereas BAL provides information about the more distal airways and alveoli. Though inhalation of hypertonic saline may cause bronchial obstruction, sputum induction has been found to be safe even in subjects with moderate to severe asthma (de la Fuente et al. 1998).

In addition to cell counts, several other parameters have been reliably determined from IS to view different aspects in airway inflammation. These consist of various cytokines (i.e. TNFα, IL-4, IL-5, IL-6, IL-8) (Keatings et al. 1996, Michel et al. 1997, Shi et al. 1998, Nightingale et al. 2000), cytokine receptors (RANTES) (Gelder et al. 1995), biochemical markers of neutrophils (MPO, HNL) and eosinophils (ECP, EPO, EPX, MBP) (Keatings and Barnes 1997, Koller et al. 1997, Metso et al. 2001), markers of microvascular leakage (albumin, fibrinogen, α2-macroglobulin) (Pizzichini et al. 1996a, in ’t Veen et al. 1996, Greiff et al. 1999b), NO (Kanazawa et al. 1997), cysteinyl leukotrienes and prostaglandins (Brightling et al. 2000) and substance P (Tomaki et al. 1995). At present, normal values have been proposed only for cell counts of healthy subjects (Belda et al. 2000, Spanevello et al. 2000).

2.4.2 Measurement of NO

Measurement of NO in biological specimens is difficult both because of the minute quantities present and the lability of NO in the presence of oxygen. Several biochemical assays reflect indirectly the presence of NO. Expression of iNOS protein with Western blot (Hirvonen et al. 1996) and mRNA of iNOS (Robbins et al. 1996) have been determined. Measurement of cyclic GMP assesses the effect of NO on guanulate cyclase, and measurement of nitrite accumulation is indicative of NO oxidation. Citrulline, which is a co-product of the action of NOS on L-arginine, can also be determined. However, none of these
measurements is a specific assay for the gaseous molecule, NO. NO and its oxidation products can be reliably detected by using one of the following three strategies: 1) NO is “trapped”, forming a stable adduct with hemoglobin or nitroso compounds and detected with electron paramagnetic resonance, 2) reduced hemoglobin is oxidised by NO yielding methemoglobin and detected with spectrophotometry, or 3) NO is mixed with ozone producing chemiluminescence. (Archer 1993)

When measuring NO from gas samples such as exhaled air from the bronchial tree or nasal cavities, the measurement with chemiluminescence method is both easy and economical to perform. The NO contained in the gas sample reacts with an excess of ozone (O₃) to produce NO₂ with an electron in an excited state (NO₂*). NO₂* changes back to the ground state (NO₂) while emitting electromagnetic radiation ranging from 600-3000 nm. The chemiluminescence is detected by a photomultiplier tube that proportionally converts the intensity of luminescence into an electrical signal for display. NO can be detected at a concentration of approximately 1 part per billion (ppb). (Palmer et al. 1987)

For single breath measurement of NO, the subject inhales through the mouth to his/her total lung capacity, and then exhales to the collecting tube. Since NO measurement is highly dependent on flow, it is crucial that flow is maintained constant (Silkoff 1999). When measuring NO production of the lower airways, exhalation pressure does not affect the NO plateau. It should be maintained over 5 cmH2O to keep the soft palate closed during exhalation, thus preventing the contamination of the sample with nasal NO (Silkoff et al. 1997). NO-free air is recommended, when ambient NO levels are high (Kharitonov et al. 1997). NO may also be measured during normal tidal breathing e.g. during exercise testing (Bauer et al. 1994). However, even the normal exhalation rate dilutes NO concentrations, and the maintenance of the constant flow may be difficult (Lundberg et al. 1996a). Expiratory lung function measurements may elevate the exhaled NO level, and this should be taken into account when planning study protocols (Silkoff 1999). Also short acting beta-agonists can influence levels of NO (Silkoff et al. 1999).
2.4.3 Nasal lavage

Nasal mucosa serves as a primary barrier to inhaled irritants. NAL by physiological salt solution through soft catheter via naris has been found to be a minimally invasive and well tolerated method without any side effects (Steerenberg et al. 1996). Though the variability in cell counts and cytokine concentrations between subjects is quite high, the low intrapatient variability supports its utility in investigating nasal cavity responses to inhaled air pollutants (Hauser et al. 1994). In addition, low intrapatient variability and high reproducibility of both NO and cytokine measurements without seasonal variation in the markers, have been shown in our laboratory by Roponen et. al. (unpublished data). Previous studies have shown that nasal mucosa produces cytokines during viral infection (Proud et al. 1994, Linden et al. 1995), during various occupational exposures (Åhman et al. 1995, Dahlqvist et al. 1996, Diaz-Sanchez et al. 1996, Senthilselvan et al. 1997) and in allergic diseases (Gosset et al. 1993, Hiltermann et al. 1997). Cytokine concentrations as well as markers of endothelial leakage (Keman et al. 1998, Greiff et al. 1999a) and biochemical markers of inflammatory cells, e.g. ECP and MPO (Noah et al. 1995, Wålinder et al. 2001), can also be measured from supernatant of nasal lavage fluid. Total cell count (Blaski et al. 1996) and differential cell count of inflammatory cells such as neutrophils (Larsson et al. 1997) and eosinophils (Prat et al. 1993) can be reliably used in assessing upper airway inflammation. It has also been suggested that NAL could be a valuable tool in evaluating lower respiratory tract inflammation in asthma (Noah et al. 1995) and also after exposure to inhaled irritants (Graham and Koren 1990).
3 AIMS OF THE STUDY

The purpose of the present study was to determine the usefulness of the non-invasive sampling methods, and to identify inflammatory markers which could be used in evaluating the possible inflammatory changes in the airways in association with exposure to microbes present in moisture-damaged buildings.

The specific aims of the study were:
1. To determine the reproducibility of the exhaled NO, cell count and cytokine measurements in IS repeated 48 h apart.

2. To evaluate if inflammatory markers determined from NAL can be used in assessing the inflammation of the lower respiratory tract.

3. To encounter the possible changes in pro-inflammatory cytokines in NAL and IS samples after exposure to moisture-damaged building microbes.

4. To assess the value of NO measurements in determining airway inflammation after exposure to microbes present in moisture-damaged building.
4 SUBJECTS AND METHODS

4.1 Subjects

Thirty-one healthy volunteers were recruited from the staff of the Department of Respiratory Medicine and the Department of Clinical Chemistry to take part in study I. The volunteers in studies II-IV consisted of 37 employees working in a school building with moisture damage and visible mould growth, and 23 employees from a control school building. (Table 3.)

Table 3. Subject characteristics in studies I-IV.

<table>
<thead>
<tr>
<th>Study</th>
<th>Building characteristics</th>
<th>Number of subjects</th>
<th>Male/Female</th>
<th>Age (years)</th>
<th>Atopy# (n)</th>
<th>Smoking (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>-</td>
<td>31</td>
<td>8/23</td>
<td>43 (25-58)</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>II-IV</td>
<td>Moisture-damaged</td>
<td>37</td>
<td>8/29</td>
<td>48 (34-61)</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>II-IV</td>
<td>Control</td>
<td>23</td>
<td>6/17</td>
<td>46 (31-56)</td>
<td>5</td>
<td>0</td>
</tr>
</tbody>
</table>

# mean wheal diameter ≥ 3 mm in skin prick test (SPT) to at least one common allergen (II-IV); in study I questioned.

4.2 Protocols

In study I the subjects attended the laboratory twice, with an interval of 48 h at the same time of the day. On the first visit they completed the health questionnaire (Susitaival et al. 1996) concerning their current health, previous inflammatory diseases, possible respiratory symptoms, occupational and housing conditions and smoking habits. The peak expiratory flow rate (PEFR) (Wright Peak Flow meter; Airemed Ltd., Harlow, UK) and exhaled NO were measured, and sputum was induced. On the second visit the same measurements were repeated.

For studies II-IV, the subjects were contacted four times. On the first visit they completed the same questionnaire as subjects in study I, and on each visit, they were interviewed and asked to fill in a one page questionnaire concerning their current health, e.g.
respiratory symptoms. The first contact was in May 1998 at the end of spring term to evaluate the effects of a prolonged exposure period (January-May). The second time was in August 1998 at the end of the summer vacation (June-August) to explore the effects of absence from the school buildings. The third sampling was carried out in February 1999, when the ground in Finland was covered with snow, and thus the effects of pollen and outdoor mould could be avoided. The fourth contact was in March 1999 after a one week winter holiday to find out whether a short break in the exposure could cause changes in the inflammatory markers in NAL. At visits 1 - 3 NAL, IS and a venous blood sample for ECP, IgE and CRP analyses were collected, and exhaled NO was measured. On the fourth visit, only NAL was sampled (Figure 3.).

![Study protocol (II-IV)](image)

**Figure 3.** – Study protocol (II-IV). Questionnaires (Q) were completed and induced sputum (IS), exhaled nitric oxide (eNO) and nasal lavage (NAL) samples were collected at the end of exposure and vacation periods (arrows).

**4.3 Detailed description of methods**

**4.3.1 Characterisation of the microbial exposure in the buildings (II-IV)**

Both school buildings were inspected for visible signs of moisture and mould growth by a civil engineer using a checklist and a surface moisture recorder. These assessments verified the damage history of the index school and the non-damaged status of the reference school building. Concentrations of viable fungi and bacteria, and fungal flora were determined in the school buildings. Indoor air, surface and material samplings were performed in the winter when the ground was covered with snow, and during working hours, when the buildings were occupied. Microbial samples from indoor air were collected with an Andersen six-stage impactor (Andersen 10-800, Graseby Andersen, Atlanta, Georgia, USA); the fungal samples on 2 % malt extract agar and on dichloran glycerol agar, and bacteria samples on tryptone glucose yeast agar. Seventeen samples were taken from the index
school and 18 from the control school. The colonies on the incubated agar plates were counted as colony forming units (cfu/m³), and the fungi were identified morphologically to genus using a light microscope. The total concentrations of airborne bacteria and actinomycetes were counted from the bacteria samples. To complete the information of the damage observations and microbial status of the school buildings, samples of building materials were collected from structures where visible mould growth could be detected by the technical investigator and where dismantling of the structures was possible. Some surface samples were collected from the kitchens of both schools. These procedures have been described earlier in detail (Hyvärinen et al. 1993, Nevalainen et al. 1998, Meklin et al. 2002).

4.3.2 Sputum induction (I-IV)

The subjects inhaled two puffs of salbutamol (Buventol Easyhaler®, 100 μg/dos), followed by inhalation of 4% hypertonic saline for 5-20 minutes. Saline solution was nebulized by an ultrasonic nebulizer, particle size 7.5μm (Omron U1; Omron Healthcare GmbH, Hamburg, Germany). The collected sputum samples were examined within two hours to avoid cell destruction (Popov et al. 1994). Sputum plugs originating from the lower respiratory tract were separated and weighed. Freshly prepared dithiotreitol (DTT, Sputolysin, Calbiochem corp., San Diego, CA, USA) was diluted in distilled water (1:10), and this solution was added to the sputum in a volume (in ml) equal to two times the weight of the sputum portion (in mg). Sputum was then shaken in a water bath at 37°C for 15 min. To ensure homogenisation, the sputum-DTT mixture was shaken by Vortex every 5 min for 15 s. After this the suspension was further diluted with phosphate buffered saline (Dulbecco's phosphate buffered saline, D-PBS, Life Technologies Ltd., Paisley, Scotland) in a volume equal to the sputum plus DTT. Then the suspension was filtered through a 41 μm nylon gauze (Millipore corporation, Badford, MA, USA) to remove mucus and the sample was centrifuged at 790 G for 10 min. Total cell count (TCC) was counted with a haemocytometer and cell viability was analysed with the trypan blue exclusion method. The supernatant was aspirated and frozen at -70°C.
4.3.3 Nasal lavage (II-IV)

Nasal lavage was performed as described earlier by Graham et al. (1990) with minor modifications (Hirvonen et al. 1999). Briefly, 4.5 ml of prewarmed (+37°C) Hank's balanced salt solution (HBSS) (Gibco, Paisley, Scotland) was instilled through a heat-softened catheter into the nares, while the subject held his/her chin down towards the chest and held the catheter in place by pinching the nares closed. Then the fluid was refluxed three times, and the cartilaginous bridge of the nose was vibrated by a paediatric percussion (NeoCussor™, General Physiotherapy, Inc., MO, USA). The procedure was repeated on the opposite nares. The sample was centrifuged (425 G, 10 min), and the cells were resuspended in 2 ml of the supernatant, incubated for 24 h at +37°C and centrifuged (425 G, 10 min). The supernatant and the cells were frozen at -70°C.

4.3.4 Biochemical analyses (I-IV)

From the sputum supernatant and serum, ECP (μg/l) was analysed by radioimmunoassay (RIA, Pharmacia & Upjohn, Uppsala, Sweden). Cytokines were analysed from sputum and NAL supernatants by using human IL-1, IL-4, IL-6 and TNFα Duoset ELISA-kits obtained from Genzyme (Cambridge, MA, USA), and read by ELISA reader (iEMS Reader MF, Labsystems, Helsinki, Finland) at a wavelength of 450 nm. Cytokine concentrations of samples were calculated by interpolating the absorbances of samples to the standard curve.

NO in the supernatants of NAL and IS were assayed with the Griess reagent (Green et al. 1982) which together with the stable NO oxidation product nitrite, produces a spectrophotometrically quantified chromophore as described earlier (Hirvonen et al. 1996). Expression of iNOS protein in the cells of NAL and IS was determined with Western blot analysis (Hirvonen et al. 1996).

From serum samples, IgE was determined by EIA (Immulite total IgE, Diagnostic Products Corporation, Los Angeles, CA, USA) and C-reactive protein by immunoturbidimetry (Hitachi 717 Automatic Analyzer, Hitachi Ltd., Tokyo, Japan). (Table 4.)
Table 4. Analyses in studies I-IV. Inflammatory markers were determined from nasal lavage fluid (NAL), induced sputum samples (IS), exhaled air and serum.

<table>
<thead>
<tr>
<th>Analysed parameter</th>
<th>Method</th>
<th>Sample</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFα, Interleukin(IL)-1, IL-4, IL-6</td>
<td>enzyme-linked immunosorbent assay (ELISA)</td>
<td>NAL, IS</td>
<td>I (no IL-1), II, III</td>
</tr>
<tr>
<td>Nitric oxide</td>
<td>Griess-reaction chemiluminescence</td>
<td>NAL, IS exhaled air</td>
<td>II, IV I, IV</td>
</tr>
<tr>
<td>Inducible nitric oxide synthase (iNOS)</td>
<td>Western blot</td>
<td>NAL IS</td>
<td>IV I, IV</td>
</tr>
<tr>
<td>Eosinophil cationic protein</td>
<td>radioimmunoassay (RIA)</td>
<td>IS serum</td>
<td>I, III</td>
</tr>
<tr>
<td>Immunoglobulin E</td>
<td>enzyme immunoassay (EIA)</td>
<td>Serum</td>
<td>III</td>
</tr>
<tr>
<td>C-reactive protein</td>
<td>Immunoturbidimetry</td>
<td>Serum</td>
<td>III</td>
</tr>
<tr>
<td>Cells</td>
<td>-viability</td>
<td>Trypan blue MGG, microscope</td>
<td>IS NAL, IS I I, III</td>
</tr>
<tr>
<td></td>
<td>-count</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

4.3.5 Cytospin (I-III)

The cell pellet of IS was resuspended in phosphate buffered saline (Dulbecco’s phosphate buffered saline, D-PBS, Life Technologies Ltd., Paisley, Scotland) to reach a concentration of 1 x 10^6 cells per ml. The cell suspension was centrifuged (Shandon, Life Sciences, International Ltd., Cheshire, England) at 450 rpm for 6 min. The slides were fixed in ethanol and stained with May Grünwald Giemsa (MGG) for cell differential count from 500 cells. Only samples with cell viability > 50 % and squamous cell contamination < 20 % were considered adequate (Popov et al. 1995). The cytocentrifuge preparations of NAL were made by using 100 μl of resuspended cell suspension, in which the mucus was broken by 0.5 % dithiotreitol / 0.1 % bovine serum albumin. The solution was centrifuged and the slides were also stained with MGG (Prat et al. 1993).

4.3.6 Measurement of exhaled NO (I, IV)

Exhaled NO was measured by a chemiluminescence analyser (Sievers Model 280 NOA; Sievers Instruments Inc., Boulder, CO, USA) according to the European Respiratory Society (ERS) guidelines (Kharitonov et al. 1997). Subjects performed a slow vital capacity
manoeuvre for 30 s against a fixed expiratory resistance. The relative standard deviation between three measurements was expected to be < 10%. The detection limit for NO was 1 part per billion (ppb). Measurements were made in the same laboratory under constant conditions.

4.3.7 Skin prick tests (II-IV)

Skin prick tests were carried out with the ALK skin prick test (SPT) system (ALK Laboratories, Copenhagen, Denmark) with 14 common allergens (birch, alder, 3 of the most common hay pollens, mugworth, dandelion, horse, dog, cat, cow, *Dermatophagoides farinae*, *Dermatophagoides pteronyssinus*, latex) and 14 moulds (*Alternaria alternata*, *Aspergillus fumigatus*, *Aspergillus versicolor*, *Aureobasidium pullulans*, *Botrytis cinerea*, *Chaetomium globosum*, *Cladosporium herbarum*, *Geotrichum candidum*, *Mucor racemosus*, *Penicillium brevicompactum*, *Penicillium expansum*, *Phoma herbarum*, *Trichoderma viride*, *Wallemia sebi*). Histamine hydrochloride (10 mg/mL) was used as a positive control and 50% glycerol was used as a negative control. A mean wheal diameter of 3 mm or more was regarded as a positive result. At least one positive result in SPT was considered to represent atopy.

4.3.8 Statistical analyses

The statistical tests used are shown in table 5. In study I, Bland-Altman plots (Bland and Altman 1986) were used to visualise reproducibility and intrapatient correlation of measurements. It was expected that 95% of the differences between measures would be < 2SD. Exploratory data analyses revealed that cell counts and TNFα obtained in study I, all cytokine concentrations and cell counts in studies II-III and NO in NAL and IS in studies II and IV, were not normally distributed and therefore, logarithmic transformations were used in the statistical analyses. The statistical analyses were performed by using the SPSS/PC+ software package versions 8.0 and 9.0 (SPSS inc., Chicago, USA).
Table 5. Statistical tests used in studies I-IV.

<table>
<thead>
<tr>
<th>Study</th>
<th>Test</th>
<th>Use of the test</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Reliability coefficient</td>
<td>Reproducibility and intrapatient correlation of measurements</td>
</tr>
<tr>
<td>II</td>
<td>Pearson’s correlation coefficient&lt;br&gt;Chi square, Fisher’s exact test&lt;br&gt;Linear regression analysis</td>
<td>Correlation between exhaled NO and nitrate in IS&lt;br&gt;Contingency tables&lt;br&gt;Comparison of concentrations within NAL and IS sample pairs</td>
</tr>
<tr>
<td>III</td>
<td>Chi square&lt;br&gt;McNemar</td>
<td>Questionnaire; difference between schools&lt;br&gt;Questionnaire; difference between time points</td>
</tr>
<tr>
<td>III, IV</td>
<td>Paired t-test&lt;br&gt;Unpaired t-test</td>
<td>Difference between time points&lt;br&gt;Difference between schools</td>
</tr>
</tbody>
</table>

Note. In linear regression analysis markers measured from IS represent the dependent variable. If the R square value is high, the variable (value in NAL) is a good predictor of the dependent variable (value is IS). If \( y = a + bx \), \( y \) presents nasal concentration and \( x \) sputum concentration of the studied marker; \( B = b \), see results 5.2.3.

4.3.9 Ethics

The protocols were approved by the Ethics Committee of the University of Kuopio.
5 RESULTS

5.1 Reproducibility of measurements in induced sputum and exhaled NO

5.1.1 Sputum induction (I)

Twenty of the 31 healthy volunteers (64%) were able to produce sputum sample within 48 hour interval. One sample was rejected because of the low number of cells (< 500 cells per cytospin) and one because of the high number of squamous cells (> 20 %). Thus, statistical analyses of total cell count, cell differentials and biochemical markers were performed with 18 sample pairs. No significant variability was noticed between PEFR values before and after sampling. The mean weight of sputum on the first measurement day was 356 ± 78 mg and on the second 360 ± 61 mg. The mean cell viability was 86 % and 82 % on time points 0 and 48 hrs.

5.1.2 Reproducibility of measurements of cell count, cytokine concentrations and ECP in induced sputum, and exhaled NO (I)

5.1.2.1 Cell counts, cytokines and ECP

With the exception of lymphocytes, all cell types were highly reproducible, p-value being <0.001 for all of them. Measurements of IL-4, IL-6, TNFα and ECP in IS samples were reproducible as well. Their concentrations and reliability coefficients are shown in table 6.
Table 6. Cell counts and interleukin (IL)-4, IL-6, tumour necrosis factor (TNF)α and eosinophil cationic protein (ECP) in induced sputum collected at 0 and 48 h of healthy subjects (study I).

<table>
<thead>
<tr>
<th></th>
<th>0 h</th>
<th>48 h</th>
<th>r</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total cell count</strong></td>
<td>680±114.5</td>
<td>524±103.5</td>
<td>0.836</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>Lymphocytes</strong></td>
<td>10.2±2.02</td>
<td>11.3±1.20</td>
<td>0.253</td>
<td>ns</td>
</tr>
<tr>
<td><strong>Macrophages</strong></td>
<td>234±26.6</td>
<td>235±19.1</td>
<td>0.752</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>Eosinophils</strong></td>
<td>10.1±5.0</td>
<td>8.50±3.37</td>
<td>0.966</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>Neutrophils</strong></td>
<td>198±30.1</td>
<td>175±22.5</td>
<td>0.762</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>IL-4</strong></td>
<td>178±81.2</td>
<td>189±65.7</td>
<td>0.398</td>
<td>0.046</td>
</tr>
<tr>
<td><strong>IL-6</strong></td>
<td>113±107</td>
<td>65.1±56.1</td>
<td>0.567</td>
<td>0.006</td>
</tr>
<tr>
<td><strong>TNFα</strong></td>
<td>9.97±15.0</td>
<td>5.24±5.39</td>
<td>0.658</td>
<td>0.014</td>
</tr>
<tr>
<td><strong>ECP#</strong></td>
<td>80.3±94.0</td>
<td>59.4±100</td>
<td>0.501</td>
<td>0.003</td>
</tr>
</tbody>
</table>

Cytokine and cell count data presented as mean ± SEM, the cytokine units are pg/ml unless otherwise stated. #: units pg/l r = reliability coefficient. ns = not significant.

5.1.2.2 Exhaled NO

Exhaled NO was analysed from 29 volunteers twice, 48 h apart. The mean±SEM levels on the two study days were 18.5 ± 2.6 ppb and 19.3 ± 2.8 ppb, respectively (r = 0.976, p = 0.0000). Inducible nitric oxide synthase (iNOS) enzyme was not detectable by Western blot analyses in the induced sputum samples of healthy volunteers.

5.2 Inflammatory responses in airways and exposure to moisture-damaged building microbes

5.2.1 Microbial exposure (II-IV)

Microbial analyses confirmed the classification of the school buildings into the moisture-damaged and reference schools. There was a significant difference in geometric means (29 and 6 cfu/m³ respectively) of total concentrations of viable airborne fungi between the schools (p = 0.0002). Microbes indicating moisture damage were found only in the moisture-damaged school. (Table 7.)
Table 7. The presence of indicator microbes in air, surface and material samples collected from schools (II-IV)

<table>
<thead>
<tr>
<th>Fungi</th>
<th>Moisture damaged school</th>
<th>Control school</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeasts</td>
<td>xx0</td>
<td>xx</td>
</tr>
<tr>
<td>Wallemia</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td><em>Aspergillus versicolor</em></td>
<td>xo</td>
<td></td>
</tr>
<tr>
<td>Eurotium</td>
<td>xo</td>
<td></td>
</tr>
<tr>
<td>Sphaeropsidales-group</td>
<td>xo</td>
<td></td>
</tr>
<tr>
<td>Trichoderma</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>Ulocladium</td>
<td>o</td>
<td>x</td>
</tr>
<tr>
<td>Tritirichium</td>
<td>xo</td>
<td></td>
</tr>
<tr>
<td>Fusarium</td>
<td>o</td>
<td></td>
</tr>
<tr>
<td>Scopulariopsis</td>
<td>o</td>
<td></td>
</tr>
<tr>
<td>Rhodotorula</td>
<td>o</td>
<td></td>
</tr>
<tr>
<td>Phialophora</td>
<td>o</td>
<td></td>
</tr>
<tr>
<td>Exophiala</td>
<td>o</td>
<td></td>
</tr>
</tbody>
</table>

**Bacteria**

| Actinomycetes              | xx0                     |

x = in less than five air samples i.e. rooms
xx = in five or more air samples
o = found in material samples

5.2.2 Symptoms (III)

At the end of exposures I and II, the subjects working in the moisture-damaged school reported more respiratory and non-specific symptoms than they reported at the end of vacation I (Table 8.). This was not seen within the reference group during exposure I, after vacation I, during exposure II nor after vacation II. Due to the small number of subjects, no statistically significant differences in symptom prevalence were detected between the personnel of the moisture-damaged and the reference schools.
Table 8. Statistically significant differences in the number of reported respiratory and non-specific symptoms among subjects from the moisture-damaged school after periods of exposure. (II-IV)

| Symptom          | Exposure I |  | Vacation I |  | Exposure II |  |
|------------------|------------|  |------------|  |------------|  |
|                  | Yes (n)    | No (n) | p#         | Yes (n) | No (n) | Yes (n) | No (n) | p# |
| Sore throat      | 8          | 25    | 0.035      | 2       | 30     | 8       | 17     | 0.063 |
| Phlegm           | 12         | 25    | 0.000      | 9       | 23     | 9       | 23     | 0.031 |
| Eye irritation   | 11         | 26    | 0.031      | 4       | 28     | 8       | 17     | 0.063 |
| Rhinitis         | 16         | 21    | 0.019      | 6       | 26     | 9       | 23     | 0.046 |
| Nasal Obstruction| 19         | 18    | 0.029      | 5       | 27     | 11      | 14     | 0.055 |

#: p < 0.05 was considered statistically significant.

5.2.3 Comparison of measurements in nasal lavage and induced sputum (II)

From the studied cytokines (IL-1, IL-4, IL-6, TNFα) only the concentration of IL-4 in NAL predicted its concentrations in IS. R square was 0.078, B 0.241 and p = 0.014, when 76 sample pairs were compared. In addition, NO production assessed as nitrite level in NAL predicted statistically significantly the nitrite level in the sputum sample. The R square was 0.069, B 0.203 and p = 0.026 (74 sample pairs). Differential cell counts in NAL did not predict those in the IS samples. The number of sample pairs varied from 12 to 15.

5.2.4 Cytokines in nasal lavage and induced sputum (III)

5.2.4.1 Nasal lavage

During exposure I, the concentration of nasal IL-1 was significantly higher (p = 0.037) among the personnel of the moisture-damaged school building compared to the personnel from the control school. This difference could also be detected, when atopic subjects of both schools were excluded from the analyses.

The concentrations of nasal TNFα (p = 0.008) and IL-6 (p = 0.046) of exposed workers were significantly higher during exposure I than after vacation I. When the measurements of personnel of the moisture-damaged and control schools were compared to each other, the differences of nasal TNFα and IL-6 did not reach statistical significance during exposure I. The IL-4 concentration was significantly higher among the personnel of the control school at the end of the vacation I (p = 0.021) and also during exposure II (p = 0.015), when compared
to those of the personnel of the moisture-damaged school. However, if atopic subjects were excluded from the analyses, no statistically significant difference was detected in IL-4 concentrations between the groups at these time points. (Figure 4.)

After vacation II, no changes in IL-1 concentrations in NAL samples of those working in the moisture-damaged school were detected. TNFα concentrations were significantly lower after vacation II than before this vacation (p = 0.02). On the contrary, IL-4 concentration increased during this period (p = 0.037). However, after exclusion of atopic subjects, the differences were not statistically significant. In the control group, IL-6 increased significantly during vacation II, and this was not affected by the presence of atopy. No significant differences between the schools were detected after vacation II in any cytokine concentration.
Nasal cytokines

Figure 4. – Concentrations of nasal cytokines (arithmetic mean) at the end of exposure I, vacation I and exposure II in the personnel of moisture-damaged and control schools. Unpaired t-test was used to analyse differences between schools and paired samples t-test between different time points. Logarithmic transformations were used in t-tests. * p < 0.05, ** p < 0.01. n = number of successful samples. (Modified from paper III.)
5.2.4.2 Induced sputum

During exposure I, the IL-6 concentration in IS was significantly higher among the subjects working in the moisture-damaged school (p = 0.002) than that of the subjects from the control school. (Figure 5.) After vacation I, IL-1 (p = 0.052) and IL-4 (p = 0.005) in IS of subjects working in the moisture-damaged school, and TNFα (p = 0.022) and IL-4 (p = 0.000) of the subjects from the control school were higher than those measured during exposure II. IL-4 concentrations in IS were also significantly elevated (p = 0.012) among the control subjects during vacation I, when compared to the personnel of the moisture-damaged school. No difference in IL-4 was detected between the groups at the end of vacation I, when atopic subjects were excluded from analyses. During exposure II, both TNFα (p = 0.131) and IL-6 (p = 0.110) in IS of the personnel of the moisture-damaged school were elevated when compared to the control group, but the difference did not reach statistical significance.

Figure 5. – Concentrations (logarithmic values) of interleukin (IL)-6 in induced sputum from the personnel of moisture-damaged and control schools at different time points. ** p<0.01. Horizontal lines through the bars represent the median value. n = number of successful samples. (Modified from paper III.)
5.2.5 Inflammatory cells in nasal lavage and induced sputum (III)

The relative proportion of lymphocytes, eosinophils and neutrophils in NAL did not differ between the subjects from the moisture-damaged and the control schools nor within the groups at the different time points with one exception. At the end of vacation I, the nasal eosinophils in the personnel of the moisture-damaged school were significantly higher than during exposure II (arithmetic means 4.40 and 2.70 respectively, p = 0.04). The IS of the subjects working in the moisture-damaged school had a significantly higher number of macrophages (p = 0.024) during exposure I than after vacation I. On the contrary, the number of neutrophils in the end of vacation I was significantly (p = 0.026) higher than during exposure I. All these results lost their statistical significance, when atopic subjects were excluded.

5.2.6 ECP, IgE and CRP (III)

ECP values in the personnel of the moisture-damaged school were significantly higher at the end of vacation I (47.7 μg/l, p < 0.05) when compared to the values during exposure I (26.7 μg/l, p < 0.05) or exposure II (19.5 μg/l). This finding lost its statistical significance, when the atopic subjects were excluded from the analyses. No such changes in ECP levels were found within subjects from the control school, or between the personnel from the two schools. Furthermore, no significant difference was detected between the two schools in IgE or CRP values during the whole study period.

5.2.7 Nitric oxide (IV)

5.2.7.1 Exhaled NO

There were no statistically significant differences in the levels of exhaled NO between subjects exposed to microbes of moisture-damaged building and control subjects at any of the time points. Furthermore, there were no statistically significant differences within exposed or control groups between different time points. (Table 9.)
Table 9. Levels of exhaled nitric oxide in different time points. (IV)

<table>
<thead>
<tr>
<th>School</th>
<th>n</th>
<th>Exposure I</th>
<th>n</th>
<th>Vacation I</th>
<th>n</th>
<th>Exposure II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture-damaged school</td>
<td>27</td>
<td>16.5 ± 1.4</td>
<td>31</td>
<td>19.6 ± 1.7</td>
<td>20</td>
<td>20.5 ± 3.5</td>
</tr>
<tr>
<td>Control school</td>
<td>11</td>
<td>18.3 ± 1.8</td>
<td>20</td>
<td>22.7 ± 2.7</td>
<td>19</td>
<td>23.5 ± 4.0</td>
</tr>
</tbody>
</table>

NO data expressed as mean±SEM (ppb). No statistically significant differences between groups at any time point, or between exposure and vacation periods in either of the studied groups were detected.

5.2.7.2 NO and iNOS in nasal lavage and induced sputum

Production of NO, assessed as nitrite in NAL and IS, did not vary between exposure and vacation periods among the studied personnel. Furthermore, no statistically significant changes in nitrite levels in NAL or IS between the exposed and control groups were found at any time point. (Figure 6 a. and b.) There was no statistically significant correlation between the subjects’ nitrite level in IS and their level of exhaled NO in different time points or in pooled data.

iNOS was determined from NAL and IS cell pellets of 20 exposed subjects. These cell pellets were collected during exposure I and vacation I, each NAL and IS sample pair on the same day. No expression of iNOS was detected in any of these samples.
Figure 6. Nitric oxide determined as nitrite in a) IS and b) NAL samples. No statistically significant differences between groups at any time point or between exposure and vacation periods in either of the studied groups were detected. Logarithmically transformed values are used in the statistical analyses of NAL and IS results. Median, range and outliers (O) are shown. n = number of successful samples. (Modified from paper IV.)
6 DISCUSSION

6.1 Reproducibility of measurements in induced sputum and exhaled NO (I)

The success rate in sputum sampling in healthy, non-atopic subjects was somewhat lower than reported in previous studies concerning asthmatic subjects and their controls (Pizzichini et al. 1996a). One reason for the current result may be the strict time interval used to determine the effects of repeated sampling. When repeated induction sessions on various days have been accepted, the success rate has been shown to improve (in 't Veen et al. 1996). Pin et al. (1992a) reported that 20% of healthy volunteers failed to produce sputum after three attempts, and in a population that consisted of asthmatic and healthy subjects, 34 attempts produced 26 samples and only 19 (56%) of these were acceptable.

Several authors have reported that there are changes in sputum cellularity after repeated sampling within 24 hours (Holz et al. 1998, Nightingale et al. 1998). The results reported here (article I) indicate that this problem can be avoided when sputum induction is performed at a 48 hour interval. Reproducibility of differential cell counts was high except for that of lymphocytes, which agrees with previous findings (Spanevello et al. 1997). Concentrations of studied cytokines, i.e. TNFα, IL-4 and IL-6, and ECP in sputum supernatant were also reproducible. ECP levels of studied subjects were low which was in line with the low eosinophil counts detected in the sputum samples.

Measurement of exhaled NO with the chemiluminescence method was found to be highly reproducible. Expression of iNOS was not detected in sputum cells of healthy subjects. This is in concordance with the earlier finding of Hamid et al. (1993) who reported iNOS expression in bronchial epithelium of asthmatic subjects but not in that of healthy controls. TNFα is known to be an inducing factor in iNOS expression, and the low TNFα level in sputum samples of healthy volunteers also agrees with the present finding (Robbins et al. 1994). Thus, the origin of exhaled NO among healthy subjects may be the cells of bronchial epithelium which possess eNOS (Shaul et al. 1994).
6.2 Inflammatory responses in airways and exposure to moisture-damaged building microbes (II-IV)

6.2.1 Evaluation of study population and confounding factors

From the occupants of the moisture-damaged school 53 % (37 out of 70) and from the control school 51 % (23 out of 45) took part in this study (articles II-IV). The age distribution as well as housing conditions, number of pets and smoking habits were similar in these groups. The number of sick leaves also did not vary between the groups. Only one person in the control school reported herself having asthma but was not on permanent medication. In the moisture-damaged school six (16 %) and in the control school three (13 %) subjects reported having allergic rhinitis. During the pollen season in the spring term (exposure I) and in August (vacation I), one person in both schools used nasal glucocorticoids and three persons used occasionally oral anti-histamines. Atopy to common allergens and some moulds was determined by SPTs, but there were no significant differences between the study groups. The number of positive SPTs for moulds was low. This finding must be viewed with caution because only a few fungal allergens have been precisely characterised due to the high variability in their morphology and antigenicity as well as prevalent antigenic cross reactivity (Koivikko et al. 1991, Malling 1992, Shen et al. 1998). Moreover, it is almost impossible to grow consecutive cultures with similar antigenic properties even under laboratory conditions (D'Amato and Spieksma 1995). However, the low serum IgE levels, as well as the non-variability between exposure and vacation periods in total IgE level among the subjects supports the SPT result.

At the beginning of the project, the subjects did not know the status of their school. Some of the control subjects may have suspected that their perceived symptoms were related to a potential moisture problem in the work place, and this may have led to a selection bias. It is possible that some of the non-symptomatic subjects in the control school did not take part in the study. This may have influenced the results by diminishing the differences between the studied groups. The studied schools are both in the same town (Kuopio). Thus, climatic factors, such as outdoor pollen, moulds and temperature, are common to both sets of subjects. In May 1998 the mean outdoor temperature was +8.0, in August it was +13.5 C°. In February and March 1999 the mean temperatures were −10.7 and −3.7 C°, and the thickness of snow cover was 39.6 and 48.6 cm, respectively (Climate Service, Finnish Meteorological Institute, personal communication).
6.2.2 Building characteristics and exposure related symptoms (II-IV)

Technical building inspection and microbial assessment confirmed the classification of the school buildings into the moisture-damaged and control schools. The thick snow cover during the winter months suggests that microbial levels in indoor environment have been reliably determined without contamination by outdoor bacteria. Moisture indicative bacteria and fungi were present mainly in the index school. The mean concentrations of the microbes were lower than those previously reported in residences (Reponen et al. 1992, Hyvärinen et al. 1993), but similar to those reported in Norwegian schools (Dotterud et al. 1995). According to questionnaire data, few of the subjects were living in a moisture-damaged home in either group, no statistically significant difference between the groups being found.

The exposed subjects reported more symptoms of the upper and lower airways as well as eye irritation during working periods compared to vacations. This is in line with previous reports (Platt et al. 1989, Koskinen et al. 1999a). The symptoms were at their highest during spring term, probably in relation to prolonged exposure, as also reported in the study of Meklin et al. (2002) in which the health status of pupils of 32 thoroughly investigated school buildings was studied by questionnaire.

6.2.3 Comparison of measurements in nasal lavage and induced sputum (II)

Several authors have proposed that due to the similarities in the nasal and bronchial mucosa, inflammatory mediators determined from nasal samples could be used in predicting inflammation in the lower airways caused by inhaled irritants and allergens (Graham and Koren 1990, Noah et al. 1995). The present findings indicate that of the many inflammatory mediators assayed in this study, only NO (determined from NAL and IS samples as nitrite) and IL-4 predicted statistically significantly their levels in sputum samples. This highlights the importance of both selection of mediators to be assayed and validation of assay methods, when evaluating the effects of different exposures on the respiratory tract.

The positive correlation between NO levels in NAL and IS is of special interest since significantly more NO is produced and measured from nasal cavities than from bronchi (Lundberg et al. 1996b). Hirvonen et al. (1999) have shown elevated NO level as well as expression of iNOS in nasal lavage after occupational exposure to microbes present in moisture-damaged buildings. At present it is unclear which cells and NOS isoforms produce the NO response in this context in the lower respiratory tract. IL-4 in NAL predicted slightly
(7.8 %) the levels found in IS. When atopic and non-atopic subjects were analysed as separate groups, no correlation in cytokine levels between NAL and IS results was found. This is in line with the recent report of Roponen et al. (2001a) showing elevation of IL-4 during the working period in a moisture-damaged building without connection to atopy among occupants. The role of IL-4 in relation to exposure to bioaerosols in moisture infested building needs further investigation.

The lack of clear correlation in measurements of pro-inflammatory cytokines in NAL and IS is in concordance with the report of Lönkvist et al. (1999) concerning exposure to animal dander, but not with the reports of Larsson et al. (1997) and Wang et al. (1997) showing significant elevation of inflammatory markers in NAL and BAL after exposure to dusts from a swine house. The extent and composition of exposure may have a significant influence on concentrations of pro-inflammatory cytokines in NAL and IS/ BAL samples. The low number of successful sample pairs may be one reason for the lack of correlation between differential cell counts in NAL and IS samples though others have found a correlation after subjects were exposed to ozone (Graham and Koren 1990, Liu et al. 1999, Nightingale et al. 1999) or allergens (Noah et al. 1995). It may be that during exposure to microbes and their metabolites in the indoor air of a moisture-damaged building, the compounds which are attending the nasal mucosa differ from those reaching the bronchial mucosa and this accounts for the different inflammatory patterns in nose and bronchi. In addition, exposure to indoor air bioaerosols is likely to be more changeable and more complicated than experimental exposure e.g. to ozone in an inhalation chamber.

6.2.4 Biochemical markers and inflammatory cells (III)

In NAL the concentrations of pro-inflammatory cytokines, such as IL-1, TNFα and IL-6, were elevated during the prolonged exposure period among exposed subjects which is in line with the previous report of Hirvonen et al. (1999). Interestingly, an absence from work as brief as one week decreased the TNFα concentration in NAL significantly. In parallel to these findings, the level of IL-6 concentration in IS was statistically significantly elevated during the working period. To our knowledge, this is the first study to show objective data of lower respiratory tract inflammation after occupational exposure in a moisture-damaged building, and to utilise induced sputum in this context. There also was a trend towards to elevation of TNFα in IS during exposure period II, but it did not reach statistical
significance probably due to the low number of IS samples. These changes in cytokine levels in NAL and IS were not affected by the exclusion of atopic subjects, which is likely to confirm their role in the inflammatory reactions caused by indoor air bioaerosols. Both IL-1 and TNFα are known to activate IL-6 production in the bronchial epithelium (Cromwell et al. 1992). The fungal allergens are also known to lead to both morphological changes and cytokine production, e.g. IL-6 and IL-8, in epithelial cells (Kauffman et al. 2000). On the other hand, IL-6 suppresses inflammatory processes by inhibiting the production and release of pro-inflammatory cytokines from macrophages (Schindler et al. 1990, Ulrich et al. 1991a, 1991b). These results may point to both activation of the cytokine network and its feedback mechanisms, and its heightened state of readiness due to prolonged exposure to indoor air bioaerosols.

As recently reported, Roponen et al. (2001a) have shown a connection between elevated IL-4 concentrations in NAL and occupational exposure to microbes in moisture-damaged building, but the function of IL-4 in this context is unknown. It has been reported that IL-4 is capable of promoting the growth of eosinophils and basophils (Favre et al. 1990), and it also induces lymphocyte maturation to Th2-like CD4 cells (Le Gros et al. 1990, Swain et al. 1990). IL-4 inhibits production of pro-inflammatory cytokines and iNOS in macrophages, and induces fibroblast chemotaxis and activity (Postlethwaite and Seyer 1990, Postlethwaite et al. 1992). The changes in IL-4 concentrations in NAL and IS samples between exposure I and vacation I in both index and control groups may have been affected by the pollen season. Though there was no statistically significant difference in prevalence of atopy between the two study groups, nasal eosinophils were elevated only in the exposed group during vacation I.

Despite the changes in IL-4 concentrations in NAL and IS, and its well known role in activating B-lymphocytes in IgE production (Vercelli et al. 1989), no changes were noted in the serum levels of total IgE between the different time points. This is in line with previous findings among children (Taskinen et al. 2001, Immonen et al. 2000). Since the ECP level in IS was at its highest during vacation I, it is more likely that the pollen season and allergic reactions are responsible for the elevation rather than the occupational exposure to indoor air bioaerosols. However, the role of ECP in moisture and mould problems in indoor air environment needs further investigation, because Wåhlender et al. (2001) have reported elevated levels of ECP in NAL after exposure to indoor air impurities in a damp office building. Serum CRP levels were low during the study period indicating that changes in cytokine concentrations were not attributable to infectious diseases. Due to low number of
successful cell counts between time points, it is not possible to reliably evaluate the effect of microbial exposure in indoor environment on NAL and IS cell findings.

6.2.5 NO measurements (IV)

In the present study there were no statistically significant differences in NO levels between exposed and control subjects at any of the four time points, or between exposure and vacation periods in either of the studied groups. This is in contrast with previous studies which have shown an association between increased inhalation exposure to microbes, airway symptoms and inflammation among office (Roponen et al. 2001a) and compost workers (Douwes et al. 2000). Elevated levels of NO have also been measured in exhaled air in various inflammatory airway diseases (Kharitonov et al. 1994, 1995b, 1995c, Maziak et al. 1998), as well as from IS of asthmatic subjects (Kanazawa et al. 1997) and NAL after exposure to bioaerosols in indoor air of moisture-damaged building (Hirvonen et al. 1999).

In parallel to our present finding, Nightingale et al. (1999) were not able to discern any changes in NO levels in NAL or exhaled air, though exposure to ozone did induce an increase in the number of neutrophils in IS as a sign of inflammation, and provoked a decrease in FEV1.

The discrepancy in results between the present findings and the report of Hirvonen et al. (1999) which showed an elevation of nitrite in NAL fluid and expression of iNOS in NAL cells during occupational exposure in moisture-damaged school, may be due to the exposure to different microbial flora detected in these buildings, and thus also to different metabolic products of the microbes. Furthermore, in vitro and in vivo studies have demonstrated that the concentrations and combinations of detected inflammatory markers vary significantly depending on which different microbes are present (Hirvonen et al. 1997a, Jussila et al. 1999, Huttunen et al. 2000). This phenomenon has been found to be affected by the growing conditions such as the growth medium and building materials (Hirvonen et al. 2001, Murtoniemi et al. 2001, Roponen et al. 2001b). The low TNFα levels in the NAL and IS samples (article III) together with the low NO level and the lack of iNOS, are in agreement with previous reports showing that pro-inflammatory cytokines are needed to increase both NO production and the increase of iNOS in human airway epithelial cells (Asano et al. 1994, Robbins et al. 1994).
6.2.6 Utility of non-invasive sampling methods in association with exposure to bioaerosols

Sputum induction by hypertonic saline is a well-tolerated, safe, reproducible and objective method for the determination of inflammatory mediators in the lower airways (de la Fuente et al. 1998, Pizzichini et al. 1996a, Lemiere et al. 1999). However, there are some factors limiting its use in outpatient clinics and field studies. Firstly, the success rate in sputum production, especially with healthy subjects, does vary. (Articles I-IV) Secondly, the sputum needs to be processed within 2 hours after induction to avoid cell destruction, and the processing of the samples before biochemical analyses is both time and labour consuming. In addition, the possibility that hypertonic saline may cause bronchial obstruction means that supervision by a clinician is recommended during the sampling. Further development of the sample processing is needed to improve the utility of the induced sputum method, before it can be fully evaluated as a diagnostic tool for the practising physician (Holz et al. 2000, Jayaram et al. 2000). Despite these problems, assays of pro-inflammatory cytokines in IS may have an important role in the future in investigations into airway inflammation in association with occupational and indoor air exposures.

Similarities in signalling events in different inflammatory diseases suggest that elevated levels of NO could also be the result of exposure to microbes in moisture-damaged buildings. Measurement of NO from exhaled air by chemiluminescence analyser has been found to be reproducible and non-invasive and can be used for both children and adults (Kharitonov et al. 1997, Korhonen et al. 2002). It may become a valuable tool in assessing lower airway inflammation in this context, especially from symptomatic subjects, when used together with cytokine measurements from induced sputum.

Collecting NAL and determining inflammatory mediators from the samples has been shown to be a well-tolerated and reproducible method (Steerenberg et al. 1996). The levels of inflammatory markers, such as pro-inflammatory cytokines and NO, seem to be unaffected by climatic changes (Roponen et al., unpublished data). NAL is a valuable tool in investigating upper airway inflammation also in large populations even in field studies in association with occupational exposure to indoor air bioaerosols (Hirvonen et al. 1999, Wålinder et al. 2001). However, NAL has a low predictive value in assessing inflammation of the lower airways due to the microbes and their metabolites present in moisture-damaged building. This suggests that NAL needs to be used in combination with other sampling methods, if one wishes to determine the inflammatory status of the entire respiratory tract.
7. CONCLUSIONS

1. Measurements of pro-inflammatory cytokines, ECP and differential cell counts in IS are reproducible when sputum is induced at 48 hours intervals. Further improvements in the IS method are needed to improve the utility of this kind of sampling in both clinical work and epidemiological studies.

2. Nasal lavage is alone an insufficient technique in assessing the inflammatory state of lower airways after exposure to moisture-damaged building microbes.

3. Changes in IL-1, TNFα and IL-6 concentrations between exposure and vacation periods in NAL and in IL-6 concentration in IS samples of subjects working in a moisture-damaged building indicate an association between the symptoms of the occupants and the exposure to indoor air bioaerosols.

4. NO measurements from NAL, IS or exhaled air are not alone sufficiently sensitive markers of airway inflammation, when evaluating the health effects of a moisture-damaged indoor environment.

5. The present data reveal that measurements of inflammatory mediators in IS and NAL can be used to assess the airway inflammation related to exposure to different impurities in a moisture-damaged indoor environment.
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