# Changes in pro-inflammatory cytokines in association with exposure to moisture-damaged building micr

The biochemical linkage between microbial exposure and the large variety of reported respiratory symptoms has been poorly understood in the past and this article published by the European Respiratory Journal tries to make this linkage more clear cut. (SMH)

Changes in pro-inflammatory cytokines in association with exposure to moisture-damaged building microbes

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Abstract

Several epidemiological studies have described an association between adverse health effects and exposure to mould and microbes present in the indoor air of moisture-damaged buildings. However, the biochemical linkage between microbial exposure and the large variety of reported respiratory symptoms is poorly understood.

In the present study, the authors compared the respiratory symptoms, the production of inflammatory mediators interleukin (IL)-1, IL-4, IL-6, tumour necrosis factor-± (TNF-±) and cell count in nasal lavage fluid and induced sputum samples of subjects working in moisture-damaged and control school buildings. The sampling was performed and the questionnaires were completed at the end of the spring term, at the end of the summer vacation (2.5 months), during the winter term and after a 1-week winter holiday.

The authors found a significant elevation of IL-1, TNF-± and IL-6 in nasal lavage fluid and IL-6 in induced sputum during the spring term in the subjects from the moisture-damaged school building compared to the subjects from the control building. The exposed workers reported sore throat, phlegm, eye irritation, rhinitis, nasal obstruction and cough in parallel with these findings.

The present data suggests an association between microbial exposure, and symptoms as well as changes in pro-inflammatory mediators detected from both the upper and lower airways.

- Cytokines
- induced sputum
- moisture
- mould
- nasal lavage

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Several epidemiological studies have shown an association between adverse health effects and exposure to mould and microbes present in the indoor air in moisture-damaged buildings 1–4. However, little is known about the biochemical link between microbial exposure and the large variety of reported respiratory symptoms. The authors' recent results point to an association between increased levels of pro-inflammatory cytokines in nasal lavage fluid, high prevalence of respiratory symptoms among occupants and exposure to microbes in mouldy buildings 5. These results are supported by the authors' in vitro findings indicating that exposure to spores of mouldy house microbes, such as Streptomyces and Mycobacterium, induce significant time- and dose-dependent production of pro-inflammatory cytokines such as interleukin (IL)-6 and tumour necrosis factor-± (TNF-±) in both mice and human cells 6–9. Moreover, an association between occurrence of asthma or wheezing and skin-prick test reactivity to moisture-indicative moulds has been reported in children attending mouldy school buildings, although skin-test positivity to moulds is relatively rare 10.

Nasal and bronchial epithelium, in addition to acting as a physicochemical barrier, plays a crucial role in initiating and augmenting host-defence mechanisms by synthesizing a variety of inflammatory mediators including pro-inflammatory cytokines. IL-1 and TNF-± are the principal mediators of the host response towards infectious organisms, and they are also involved in repair processes in the nasal mucosa 11. IL-6 has growth regulatory effects on many cells, and it is also an important cofactor in IL-4 dependent immunoglobulin (Ig)-E synthesis. IL-4 is a product of T-helper 2-derived lymphocytes, as well as eosinophils, and basophil and mast cell lineages. High levels of IL-1, TNF-± and IL-6 have been reported in response to viral infections, allergic rhinitis and asthma 12–19. Very low levels of IL-1, IL-6 and TNF-± 15–20 have been detected from samples of healthy subjects.

The present study compared respiratory symptoms, production of inflammatory mediators (IL-1, IL-4, IL-6, TNF-±) and cell counts in nasal lavage fluid and induced sputum samples from subjects working in moisture-damaged and control school buildings during working and vacation periods.

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## Methods

### Subjects

Thirty-seven employees working in a school building with moisture damage and visible mould growth, and 23 employees from a control school building volunteered to participate in this study (table 1Ó). The subjects were teachers and kitchen personnel from the schools. The studied school buildings are included in a larger study project (Finnish Research Programme on Environmental Health, The Academy of Finland), which aims to clarify moisture and mould problems in school buildings and health effects among occupants. The study was approved by the Ethical Committee of Kuopio University Hospital, Kuopio, Finland.

### Study protocol

The subjects were contacted four times during the study. On each visit, they were interviewed and asked to fill in a one-page questionnaire concerning their current health. The first contact was in May, at the end of spring term, to evaluate the effect of a prolonged exposure period (January–May). The second time was in August at the end of summer vacation (June–August) to explore the effects of absence from the school buildings. The third sampling was carried out in February, when the ground in Finland is covered with snow, and thus effects of pollen and outdoor mould could be avoided. The fourth contact was in March after a 1-week winter holiday, to find out whether a short break in the exposure could cause changes in the inflammatory markers in nasal lavage fluid. At visits 1–3 nasal lavage fluid, induced sputum and a venous blood sample for eosinophil cationic protein (ECP), IgE and C-reactive protein (CRP) analyses were collected. On the fourth visit, only nasal lavage fluid was collected (fig. 1).

## Characterization of the microbial exposure in the buildings

Both the moisture-damaged and the control school building 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 moisture-damaged school and the nondamaged status of the control school building. Concentrations of viable fungi, bacteria, and fungal flora were determined in the school buildings. Indoor air, surface and material sampling was performed in 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 a six-stage impactor (Andersen 10–800, Graseby Andersen, Atlanta, Georgia, USA). The fungal samples were plated on 2% malt extract agar and on dichloran glycerol agar, whereas the bacterial samples were plated on tryptone glucose yeast agar. Seventeen samples were taken from the test school and 18 from the control school. The colonies on the incubated agar plates were counted as colony forming units (cfu·m 3), and the fungi were identified morphologically to the genus level using a light microscope. The total concentrations of airborne bacteria and actinomycetes were counted from the bacteria samples. To complete the inspection samples of building material were collected from structures showing visible mould growth, as detected by the technical investigator, where dismantling of the structures was possible. Some surface samples were collected from the kitchens of both schools. These procedures have been described earlier 21, 22.

## Sputum induction

Sputum was induced as previously reported, with inhalation of 4% hypertonic saline for 5–20 min 20. The collected sputum samples were examined within 2 h to avoid cell destruction 23. Total cell count was determined using a haemocytometer and cell viability was analysed with the trypan blue exclusion method. The supernatant was aspirated and frozen at  70°C.

## Nasal lavage

Nasal lavage was performed as described earlier 24 with some modifications 5. Briefly, 4.5 mL of prewarmed Hank's balanced salt solution (37°C) was instilled through a heat-softened catheter into the naris, while the subject held their 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 precursor. The procedure was repeated in the opposite naris. The lavage fluid was collected and placed immediately on ice until processing.

## Biochemical analyses

From the sputum supernatant ECP (µg·L 1) was analysed by radioimmunoassay (RIA, Pharmacia & Upjohn, Uppsala, Sweden). Cytokines were analysed from sputum and nasal lavage supernatants by using human IL-1, IL-4, IL-6 and TNF-± Duoset enzyme-linked immunosorbent assay (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 absorbances of samples to the standard curve.

## Cytospin

The cell pellet of induced sputum was resuspended in phosphate buffered saline (Dulbecco's phosphate buffered saline, D-PBS, Life Technologies Ltd, Paisley, Scotland) to reach a concentration of 1×10E6 cells·mL 1. The cell suspension was centrifuged at 19×g (450 revolutions per minute (rpm) Shandon centrifuge, Life Sciences, International Ltd, Cheshire, UK). 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 included in the analysis 25. The cytocentrifuge preparations of nasal lavage fluid 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 26.

Skin-prick tests

Skin-prick tests were carried out with the ALK skin-prick test system (ALK Laboratories, Copenhagen, Denmark) with 14 common allergens (birch, alder, the three most common types of hay pollen, mugworth, dandelion, horse, dog, cat, cow, Dermatophagoides farinae, D. pteronyssimus, latex) and 13 moulds (Alternaria alternata, Aspergillus fumigatus, A. versicolor, Aureobasidium pullulans, Botrytis cinerea, Chaetomium globusum, Cladosporium herbarum, Geotrichum candidum, Mucor racemosus, Penicillium brevicompactum, P. expansum, Phoma herbarum, Trichoderma viride, Wallemia sebi). A mean wheal diameter of 3 mm or more was regarded as a positive result. At least one positive result with the skin-prick test was considered to mean atopy.

Statistical analyses

Preliminary data analyses revealed that the distributions of cytokine values obtained in this study were not normal, and therefore logarithmic transformations were used in paired t-tests (difference between the time points) and unpaired t-tests (difference between the schools). The Chi-squared test (difference between the schools) and McNemar test (difference between the time points) were used to analyse data concerning symptoms. Based on previous epidemiological data, one-sided significance $p<0.05$ was considered adequate in symptom analyses.

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Results

Microbial exposure

Microbial analyses confirmed the classification of the school buildings into the moisture-damaged test school and control school. There was a significant difference in the geometric means (29 and 6 cfu·m 3, respectively) of the total concentrations of viable airborne fungi between the schools ($p=0.0002$). Microbes indicating moisture damage, such as A. fumigatus, Eurotium, Trichoderma, Ulocladium, Fusarium, Rhodotorula, Phialophora, Exophiala and actinomycetes 27 were found only in the moisture-damaged school.

Symptoms

At the end of exposure I and exposure II, the subjects working in the moisture-damaged school reported more respiratory and nonspecific symptoms than they did at the end of vacation I (table 2Ó). This was not seen within the control group during exposure I, after vacation I, during exposure II nor after vacation II. However, due to the small number of subjects, no significant differences were detected between the subjects from the moisture-damaged and the reference schools.

Production of pro-inflammatory cytokines

Nasal lavage fluid

During exposure I, the concentration of nasal IL-1 ($p=0.037$) (fig. 2Ó) was significantly higher among the personnel of the moisture-damaged school building compared to the subjects 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 TNF-± ($p=0.008$) and IL-6 ($p=0.046$) of exposed workers were significantly higher during exposure I than after vacation I. However, when the measurements of subjects from the moisture-damaged and control schools were compared, the differences in nasal TNF-± and IL-6 did not reach statistical significance during exposure I. In the control group, the level of nasal IL-6 was higher during exposure I than after the vacation I ($p=0.003$). (fig. 2).

IL-4 concentration was significantly higher among the subjects of the control school when compared to the moisture-

damaged school at the end of vacation I (p=0.021) and also during exposure II (p=0.015). However, if atopic subjects were excluded from the analyses, no difference was detected in IL-4 concentrations between the groups at these time points.

After vacation II, no changes in IL-1 concentrations in nasal lavage fluid samples of those working in the moisture-damaged school were detected. TNF-± concentrations were significantly lower after vacation II than before this vacation (p=0.02). In contrast, 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. (table 3).

## Induced sputum

During exposure I, the IL-6 concentration in induced sputum was significantly higher among the subjects working in the moisture-damaged school (p=0.002) than that of the subjects from the control school (fig. 3Ó). After vacation I, IL-1 (p=0.052) and IL-4 (p=0.005) in induced sputum of subjects working in the moisture-damaged school, and TNF-± (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 induced sputum were also significantly elevated (p=0.012) among the control subjects during vacation I, when compared to the subjects 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, levels of both TNF-± (p=0.131) and IL-6 (p=0.110) in the induced sputum of the subjects working in the moisture-damaged school were elevated when compared to the control group, but the difference did not reach statistical significance (table 3).

## Cell-differential count

The relative share of lymphocytes, eosinophils and neutrophils in nasal lavage fluid did not differ between the subjects from the moisture-damaged and the control schools nor within the groups in the different time points, except at the end of vacation I, when 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), However, this difference disappeared when atopic subjects were excluded from the analysis.

The induced sputum 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. In contrast, the number of neutrophils in the end of vacation I was significantly (p=0.026) higher than during exposure I. Again, the statistical significance of the difference was lost when atopic subjects were excluded from the analyses (data not shown).

## Sputum eosinophil cationic protein

ECP values in the subjects from the moisture-damaged school were significantly higher at the end of vacation I (47.7 µg·L 1, p<0.05) when compared to the values during exposure I (26.7 µg·L 1, p<0.05) or exposure II (19.5 µg·L 1). This finding lost its statistical significance when the atopic subjects were excluded from the analyses. No such changes in the ECP levels were found within subjects from the control school, or between the subjects from the two schools. Furthermore, no significant difference was detected between schools in IgE or CRP values during the whole study period (data not shown).

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## Discussion

The present data point to an association between microbial exposure, reported symptoms, and the pro-inflammatory mediators assayed from both the upper and lower airways in subjects from a moisture-damaged school building. This is of particular interest because evidence of an association between moisture-related mould growth in buildings and increased frequency of respiratory symptoms among the inhabitants or occupants was previously based largely on questionnaire studies 3. There has been a serious lack of objective biochemical data concerning the inflammatory changes occurring in the respiratory tract mucosa of subjects exposed to microbial growth. In addition to increased production of pro-inflammatory cytokines in the nasal mucosa of the exposed individuals, the present study reports

elevations in pro-inflammatory cytokine concentrations in induced sputum samples of individuals working in a moisture-damaged school with microbial growth confirmed by both technical building inspection and microbial assessment.

Although both sputum induction and nasal lavage are reproducible methods for studying inflammatory markers in airways 20, the wide variability in cytokine levels, especially during exposure periods, was noticed in samples from the personnel of the moisture-damaged school. This reflects differences in sensitivity against indoor air exposures among exposed subjects. The lower variability among control subjects supports this finding.

In the present study, both IL-1 and TNF-± were elevated in nasal samples during the first exposure period when compared to their levels during the summer holiday. This is in line with the authors' previous results showing the TNF-± response in relation to working in a moisture-damaged building 5. Interestingly, a vacation as short as a 7-day break in the winter term was long enough to significantly decrease the nasal TNF-± levels of exposed subjects. In concordance with the increased nasal TNF-± response, an elevation of TNF-± concentration in induced sputum was also detected during exposure II, although this did not reach statistical significance due to the small number of successful sample pairs. This is in line with the authors' in vitro findings, which demonstrate TNF-± elevation after exposure to actinomycetes and mycobacteria, both in mouse macrophages and a human lung epithelial cell line 6, 8. Low levels of TNF-± were detected at all time points in induced sputum samples from the control group, which agrees with previous findings 28. Chronic, nonasthmatic cough has been reported to increase concentrations of TNF-± in induced sputum 29. However, in this data, cough was frequent during exposure period I among subjects from the moisture-damaged school, but no significant elevation of TNF-± in induced sputum could be detected.

IL-6 was elevated in both nasal and sputum samples in association with prolonged microbial exposure from the moisture-damaged building. Throughout the whole study period, IL-6 in induced sputum was lower in the control group than in the exposed group. This result supports the hypothesis that elevation of IL-1 and TNF-± in nasal lavage fluid as a result of exposure to indoor air microbes activates the production of IL-6 in the bronchial epithelium 16. IL-6 is also known to suppress the inflammatory process by depressing the synthesis of IL-1 and TNF-±, and also by inhibiting their expression and release from macrophages 30–32. After vacation I, the IL-6 concentration in induced sputum among exposed subjects was higher than at other time points. However, the difference did not reach statistical significance.

Interestingly, after vacation I, IL-4 levels were significantly higher among the subjects from the control school in both nasal lavage fluid and induced sputum samples than the values measured from the subjects from the moisture-damaged school. In addition, the TNF-± level in the induced sputum of the control group and the IL-1 levels in induced sputum of the exposed group were higher in the end of vacation I than during exposure II. This may have been due to the relatively high outdoor pollen and mould levels in Finland in August, which may lead to the activation of atopic inflammation in airway mucosa and cause changes in IL-4 and TNF-± levels. The nasal eosinophil count in the exposed group was elevated after vacation I, but the differences disappeared, when atopic subjects of both study groups were excluded from the analysis. These findings point to the important role of eosinophils, IL-1, IL-4 and TNF-± in allergic inflammation 15, 33. There was no significant difference in the prevalence of atopy or positive skin-prick tests between the studied groups. Positive skin-prick test findings to moulds were rare, as reported earlier 10.

According to questionnaire data, few of the participants were living in moisture-damaged homes in either group, no difference between the groups being found. Furthermore, the number of pets did not differ between the groups. Thus, the reason for differences in IL-4 elevation is unlikely to be related to the home environment. The cold winter air as a cause of inflammation has previously been discussed 34, but its effect ought to be the same in both groups. Finally, the elevations in IL-1, IL-6 and TNF-± in nasal lavage fluid during exposure I were not affected by the exclusion of atopic subjects, which confirms that their role in inflammatory reactions was caused by indoor air bioaerosols.

The small number of successful cell counts between time points is likely to have an effect on the cell findings. The number of macrophages in the differential cell count of induced sputum was elevated among exposed subjects during exposure I. This finding also lost its statistical significance when atopic subjects were excluded from the analyses. Since the role of macrophages in allergic inflammation is well known, this observation further supports the view that the atopic subjects were more sensitive to indoor air microbes. However, to confirm this proposal, a larger series of repeated, paired induced sputum samples from the same subjects needs to be assayed.

The higher ECP values in the induced sputum of the exposed group, during exposure I and also after vacation I, are reflections of the elevated reactivity of eosinophils. Although a significant change in eosinophil count was detected only at the end of vacation I, the elevation of ECP most likely was related to the elevated levels of outdoor allergens during exposure I, as well as during vacation I in summer when ECP levels in the exposed group were highest (although there was no work-related mould exposure). Despite the changes in the IL-4 levels, no significant changes in total IgE levels were found at any time point, although IL-4 is known to activate B-lymphocytes and to promote IgE synthesis 35. CRP levels were also low throughout the follow-up. This indicates that changes in pro-inflammatory cytokine concentrations were not attributable to infectious diseases.

The authors conclude that the changes in the concentrations of pro-inflammatory cytokines in nasal lavage and induced sputum samples at different time points provide evidence for an association between exposure to mouldy house

microbes and the symptoms of the exposed subjects.

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Acknowledgments

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