Effects after inhalation of (1→3)-β-d-glucan and relation to mould exposure in the home

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INTRODUCTION

A large number of investigations has reported a higher prevalence of respiratory symptoms and asthma among children and adults living in damp houses.1–7 Damp conditions indoors favour the growth of microorganisms, and these contain several agents that may cause inflammation when inhaled. Moulds contain a polyglucose in their cell wall, defined as (1→3)-β-d-glucan, exhibiting effects on inflammatory cells.

BACKGROUND: Damp conditions indoors favour the growth of microorganisms, and these contain several agents that may cause inflammation when inhaled. Moulds contain a polyglucose in their cell wall, defined as (1→3)-β-d-glucan (grifolan), exhibiting effects on inflammatory cells.

Aim: The aim of the present study was to evaluate whether an inhalation challenge to purified (1→3)-β-d-glucan in humans could induce effects on inflammatory markers in blood, and to evaluate whether the reactions were related to the home exposure to (1→3)-β-d-glucan.

METHODS: Seventeen subjects in homes with high levels of airborne (1→3)-β-d-glucan (G-high) and 18 subjects in homes with low levels of (1→3)-β-d-glucan (G-low) underwent two randomised, double-blind inhalation challenges, one to (1→3)-β-d-glucan suspended in saline and one to saline alone. A blood sample was taken before and after the challenges, and differential cell count, granulocyte enzymes in serum and the secretion of cytokines from peripheral blood mononuclear cells (PBMC) were measured.

RESULTS: Inhalation challenge with (1→3)-β-d-glucan induced a decrease in the secretion of tumour necrosis factor α from endotoxin-stimulated PBMC in the G-high group as well as in the G-low group. In the G-high group, the inhalation of (1→3)-β-d-glucan induced an increase in blood lymphocytes that was significantly different from the saline-induced effect.

CONCLUSIONS: The results suggest that an inhalation challenge to (1→3)-β-d-glucan has an effect on inflammatory cells and this effect may be related to a chronic exposure to moulds at home.

Key words: Moulds, (1→3)-β-d-Glucan, Inflammation, Cytokines, Lymphocytes

Introduction

A large number of investigations has reported a higher prevalence of respiratory symptoms and asthma among children and adults living in damp houses.1–7 Damp conditions indoors favour the growth of microorganisms, and these contain several agents that may cause inflammation when inhaled. The relation between bacterial endotoxin from Gram-negative bacteria and airways inflammation has been described in several studies.8–10 Exposure to high concentrations of moulds may cause inflammation in the airways characterised by an involvement of lymphocytes. Prolonged exposure may lead to a granulomatous pneumonitis (i.e. allergic alveolitis).11,12

Moulds contain a polyglucose in their cell wall, defined as (1→3)-β-d-glucan. Results from in vitro and animal exposure studies demonstrate that this agent can affect the function of the defence system in the lungs by changing the reaction to an inhaled inflammagenic agent, endotoxin, as well as to antigens.13 A previous study demonstrated that persons living in houses with fungal contamination, as measured by the amount of airborne (1→3)-β-d-glucan, had a higher spontaneous secretion of tumour necrosis factor α (TNFα) from peripheral blood mononuclear cells (PBMC), and a reduced proportion of CD8+ T cells in blood, as compared with subjects living in homes with low levels of (1→3)-β-d-glucan.14

The aim of the present study was to evaluate whether an experimental exposure to purified (1→3)-β-d-glucan could induce symptoms and effects on inflammatory markers in blood, and whether the reactions were related to the home exposure of (1→3)-β-d-glucan.
Materials and methods

Subjects

Subjects for the study were recruited from a previous investigation in a rowhouse area and by advertising in the local press, requesting persons suspecting mould growth in their homes. Inclusion criteria were age 19–65 years, non-smokers and without physician-diagnosed asthma or diseases requiring medication. The amount of airborne (1→3)-β-D-glucan in the subject’s homes were measured and two groups were selected: those with lower than 2.0 ng/m³ (G-low), and those with higher than 4.0 ng/m³ (G-high) in their homes. One subject was recruited from each home, except for three homes in the G-low group and two homes in the G-high group, where couples were recruited. The study was approved by the Ethics Committee of the Faculty of Medicine at Göteborg University, and all participating subjects gave their informed consent. Table 1 presents the home levels of (1→3)-β-D-glucan, the number of subjects and their characteristics.

Determination of (1→3)-β-D-glucan

To determine the exposure in the subjects’ homes, airborne dust was created using a machine designed to whirl up dust roughly equivalent to that generated by a few people moving about the room. The dust generator was run for 5 min followed by a 10-min pause. Two such periods were used for the sampling. Air samples were taken during these 30 min by drawing air at a flow rate of 5 l/min through parallel filters (Isopore, ATTP 0.8 μm; Millipore Inc., Bedford, MA, USA), placed 0.8 m above the floor. Two rooms, usually the bedroom and the living room, were investigated in each home.

The filters were shaken for 10 min in 10 ml of pyrogen-free water. Thereafter, 0.3 M NaOH was added and the filters were shaken on ice for 10 min to unwind the triple helix structure of the (1→3)-β-D-glucan and make it water soluble. Filter extract samples of 50 μl were placed in a microwell plate and 50 μl of specific (1→3)-β-D-glucan lysate (Fungitic G Test®; Seikagaku Co, Tokyo, Japan) was added. The plate was incubated in a microplate spectrophotometer, and the kinetics of the ensuing colour reaction was read and compared with a standard curve (Pachyman, T; Seikagaku Co., Tokyo, Japan). The results were expressed as nanograms per millilitre of liquid and the detection limit for this technique is 20 pg/ml. The airborne concentration of (1→3)-β-D-glucan was calculated using the value for the airflow through the filter, and the mean exposure value in a house was based on four filters.

Inhalation challenge

Grifolan, a (1→3)-β-D-glucan extracted from Grifola frondosa, was suspended in 0.3 M NaOH to dissolve the triple helix and to render the substance water soluble. The solution was diluted 840 times with pyrogen-free saline to a final concentration of 6 μg/ml. The inhalation challenge was performed in a chamber, 2 m × 3.5 m × 2.45 m, furnished as a sitting room. The solution was aerosolised and led into the chamber through the ventilation system. Each subject inhaled (1→3)-β-D-glucan in saline or saline alone on two double-blind, randomised occasions. One to three subjects were challenged at the same time. Three Isopore filters were placed in the chamber and the amount of airborne (1→3)-β-D-glucan was determined. The inhalation challenge time was 3 h and the mean concentrations during the (1→3)-β-D-glucan inhalations were 28.1 ng/m³ (range, 17.1–44.9 ng/m³) for the G-low group and mean 27.0 ng/m³ (range, 15.3–44.9 ng/m³) for the G-high group.

Exposure endpoints

Blood samples were taken from each subject for analyses of serum inflammatory markers and cytokine secretion from PBMC. The samples were taken before, immediately after and 24 h after the inhalation. No differences were found in any variable immediately after the inhalations of (1→3)-β-D-glucan or saline.

Table 1. Population characteristics and levels of (1→3)-β-D-glucan (GLU) in the home (ng/m³)

|                  | G-low group | G-high group |
|------------------|-------------|--------------|
| Subjects         | n           |             |
| Home GLU levels  | Median      | 0.9          |
|                  | Range       | 0.2–1.9      |
| Female           | n           | 9            |
|                  | %           | 50           |
| Atopics          | n           | 6            |
|                  | %           | 33           |
| Age (years)      | Median      | 52           |
|                  | Range       | 26–66        |

G-high group, > 4 ng/m³ GLU in their homes; G-low group, < 2 ng/m³ GLU in their homes.
alone as compared with pre-inhalation values; thus, only before and 24 h results are reported

Inflammatory markers

Venous blood was collected in ethylenediamine tetraacetic acid tubes, and a differential cell count was made on smears stained with May–Grünwald–Giemsa and counted in a light microscope. Eosinophilic cationic protein (ECP) was assayed in serum by a fluorescent enzyme immunoassay technique (CAP ECP FEIA; Pharmacia Diagnostics AB, Uppsala, Sweden). Myeloperoxidase (MPO) was assayed by a radioimmunoassay technique (CAP MPO RIA; Pharmacia Diagnostics AB).

Cytokine production

Venous blood was collected in vacutainer tubes containing sodium citrate, a gel barrier and a density gradient fluid (CPT™ tubes; Becton Dickinson, Franklin Lakes, NJ, USA). After centrifugation, the PBMC were collected and washed twice in Hank’s salt buffer supplemented with 10% homologues serum. The PBMC were then suspended in AIM-V medium (Gibco BRL, Life Technologies, UK) supplemented with 2-mercaptoethanol, 4 × 10⁻⁵ M to a concentration of 2 × 10⁶/ml. The cells were incubated with or without phytohaemagglutinin (PHA) (Murex Diagnostics Limited, Dartford, UK) at a final concentration of 250 ng/ml, or lipopolysaccharide (LPS) (Escherichia coli 026:B6; Difco Laboratories, Detroit, MI, USA) at a final concentration of 500 pg/ml. PHA was used as a major T-cell stimulus and LPS as a major monocyte stimulus. After 48 h incubation at 37°C/5% CO₂, the supernatants were collected and stored at -25°C before cytokine analysis. TNFα from unstimulated PBMC was analysed using an enzyme-linked immunosorbent assay (ELISA) kit with a sensitivity of 0.1 pg/ml (Quantikine high sensitive; R&D Systems, Abingdon, UK). Interleukin (IL)-1β, IL-4, IL-10, interferon γ and TNFα from stimulated PBMC were analysed using the PeliKine-compact™ human cytokine ELISA (CLB, Amsterdam, The Netherlands).

Atopy

Atopy was determined by measuring the concentration of specific serum immunoglobulin E antibodies against a pool of 10 airborne allergens using a fluorescent enzyme immunoassay technique (CAP Phadiatop FEIA; Pharmacia Diagnostics AB). The results were expressed as positive (atopic) or negative (non-atopic). Due to the low number of subjects, no separate analyses of atopic and non-atopic subjects could be carried out.

Statistical analyses

The differences in effect variables comparing before values with immediately after and 24 h after the inhalation challenge, as well as the differences between the (1→3)-β-D-glucan and saline challenges within the G-high and the G-low groups were analysed using Wilcoxon’s test. The differences in effects after challenge between the G-high and G-low groups were analysed using the Mann–Whitney U-test. Differences were considered statistically significant at p < 0.05.

Results

Table 2 presents the secretion of TNFα from LPS-stimulated PBMC before and 24 h after inhalation of saline and (1→3)-β-D-glucan in relation to the (1→3)-β-D-glucan exposure in the subjects’ homes. Among G-low subjects, the inhalation of saline or (1→3)-β-D-glucan did not influence the LPS-induced secretion of TNFα from PBMC. Among G-high subjects, the TNFα secretion was not influenced by inhalation of saline. After inhalation of (1→3)-β-D-glucan, the LPS-induced secretion of TNFα was lower than before (p < 0.02). The change (before -24 h after inhalation) induced by (1→3)-β-D-glucan was statistically significant compared with the change after saline (p < 0.01). The change in TNFα secretion after inhalation of (1→3)-β-D-glucan was also significantly different when comparing the G-high group with the G-low group (p < 0.02).
There was a significant negative correlation between the amount of \((1\rightarrow3)\beta-D\)-glucan in the air in the exposure chamber and the LPS-induced TNF\(\alpha\) secretion from PBMC after the challenge \((r_{xy} = -0.40, p < 0.05)\). This correlation was present in the G-low group but not in the G-high group.

Table 3 presents the secretion of IL-10 from PBMC stimulated with LPS before and 24 h after the inhalation of saline and \((1\rightarrow3)\beta-D\)-glucan in relation to the \((1\rightarrow3)\beta-D\)-glucan exposure in the subjects’ homes. The difference before –24 h after inhalation of \((1\rightarrow3)\beta-D\)-glucan was significantly different from that induced by saline \((p < 0.05)\).

Table 4 presents the number of lymphocytes in blood before and 24 h after the inhalation of \((1\rightarrow3)\beta-D\)-glucan and saline in relation to the \((1\rightarrow3)\beta-D\)-glucan exposure in the subjects’ homes. Among G-low subjects, the inhalation of saline or \((1\rightarrow3)\beta-D\)-glucan did not induce any significant changes 24 h after inhalations. Among G-high subjects, the increase induced by the \((1\rightarrow3)\beta-D\)-glucan inhalation was significant as compared with the change induced by the saline inhalation \((p < 0.02)\). The change after inhalation of \((1\rightarrow3)\beta-D\)-glucan in the G-high group was on the borderline of statistical significance as compared with the change in the G-low group \((p = 0.05)\).

No significant differences were found 24 h after inhalation of \((1\rightarrow3)\beta-D\)-glucan or saline regarding the PBMC secretion of IL-1\(\beta\), IL-4, interferon \(\gamma\) or ECP and MPO in serum.

### Discussion

The design of this study, selecting groups with ‘high’ and ‘low’ \((1\rightarrow3)\beta-D\)-glucan exposure at home, was chosen because no information on \((1\rightarrow3)\beta-D\)-glucan-induced dose–response relationships or \((1\rightarrow3)\beta-D\)-glucan levels for triggering inflammatory reactions were available at the start of the study. Using well-discriminated groups, according to the exposure variable, would therefore increase the possibility of demonstrating differences related to the exposure.

A larger number of dwellings was measured, after which the two groups were selected. The limits used for high and low exposure were based on the results from the previous study, in which 30% of the 75 houses examined had a \((1\rightarrow3)\beta-D\)-glucan level above 4 ng/m\(^3\) and 30% of the houses had a glucan level less than 2.0 ng/m\(^3\).

The subjects recruited to the study represent a biased population sample, as they were aware of problems related to mould exposure at home. The assignment into exposure groups was, however, made according to measurements of \((1\rightarrow3)\beta-D\)-glucan in the home, irrespectively of the presence of indoor-related symptoms. It is thus unlikely that selection bias according to symptoms accounted for the differences found in the variables measured in blood between the G-low and G-high groups.

The main result from the study is that the inhalation challenge with \((1\rightarrow3)\beta-D\)-glucan decreased the capacity of PBMC to secrete TNF\(\alpha\) after an in vitro LPS stimulation.

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**Table 3.** IL-10 (pg/ml) secreted from PBMC stimulated with LPS in vitro before and 24 h after saline and \((1\rightarrow3)\beta-D\)-glucan (GLU) inhalations

|                | G-low group | G-high group |
|----------------|-------------|-------------|
|                | Saline inhalation | GLU inhalation | Saline inhalation | GLU inhalation |
| n              | 18          | 18          | 14            | 14            |
| Before exposure| 140 (4–462) | 146 (2–910) | 129 (14–907) | 90 (14–973)   |
| 24 h after exposure | 131 (13–639) | 138 (19–623) | 78 (9–963) | 69 (1–762)   |
| Exposure-induced change | 1.7 (–92 to 377) | –13.7 (–287 to 169) | –10.2 (–123 to 323) | –10.5 (–211 to 107)* |

Data presented as medians (ranges). G-high group, ≥ 4 ng/m\(^3\) GLU in their homes; G-low group, < 2 ng/m\(^3\) GLU in their homes.

* \( p < 0.05\) comparing inhalation of GLU with saline.

**Table 4.** The numbers of lymphocytes \((\times 10^9/l)\) in blood before and 24 h after saline and \((1\rightarrow3)\beta-D\)-glucan (GLU) inhalations

|                | G-low group | G-high group |
|----------------|-------------|-------------|
|                | Saline inhalation | GLU inhalation | Saline inhalation | GLU inhalation |
| n              | 18          | 18          | 17            | 17            |
| Before challenge| 2.2 (1.2–3.6) | 2.6 (0.8–4.6) | 2.1 (1.2–4.0) | 1.9 (1.2–3.9) |
| 24 h after challenge | 2.4 (1.1–4.0) | 2.1 (1.2–3.3) | 1.8 (1.2–4.5) | 2.1 (1.2–4.6) |
| Challenge induced change | 0.3 (–0.9 to 1.0) | –0.2 (–2.2 to 0.7) | –0.3 (–1.4 to 0.9) | 0.1 (–0.3 to 0.9)*† |

Data presented as medians (ranges). G-high group, ≥ 4 ng/m\(^3\) GLU in their homes; G-low group, < 2 ng/m\(^3\) GLU in their homes.

* \( p < 0.05\) comparing inhalation of GLU with saline.
† \( p = 0.05\) comparing the G-high group with the G-low group after GLU inhalation.
stimulation. This effect was found in the group previously exposed to a higher level of (1→3)-β-D-glucan in their homes, indicating an effect of chronic exposure. In the G-low group, the (1→3)-β-D-glucan challenge did not induce a reduced TNFα secretion measured as a group value, but a negative correlation was found between the amount of (1→3)-β-D-glucan in the inhalation chamber and the LPS-induced TNFα secretion from PBMC after the challenge. This (1→3)-β-D-glucan-induced effect was confirmed in a recent study, where a decreased TNFα secretion from PBMC was found in volunteers, without indoor air problems at home, after an inhalation challenge with (1→3)-β-D-glucan via a nebuliser.16

These findings are supported by data from animal models where a down-regulation of the inflammatory response after pretreatment with (1→3)-β-D-glucan has been shown. In mice given (1→3)-β-D-glucan intramuscularly, a decreased TNFα secretion from mononuclear cells after an in vivo stimulation with LPS was demonstrated.17 Repeated inhalations of (1→3)-β-D-glucan prior to an inhalation challenge to LPS decreased the LPS-induced neutrophil invasion into the airways of guinea pigs.18

The effects in blood lymphocytes did not differ significantly in the G-low group after the saline or (1→3)-β-D-glucan challenges, but in the G-high group the (1→3)-β-D-glucan challenge induced an increase in lymphocytes that was significantly different from the saline challenge effect. Previous studies have shown relations between mould exposure and effects on blood lymphocytes. Household waste collectors, exposed to levels of (1→3)-β-D-glucan similar to the levels used in the exposure chamber in this study, had an increased number of blood lymphocytes compared with control subjects, and the lymphocyte numbers were correlated to the exposure levels of (1→3)-β-D-glucan.19 Dales et al. found that children living in more fungal-contaminated homes did not differ in blood lymphocyte numbers compared with children living in less fungal-contaminated homes, but certain subtypes of T cells were significantly increased among the more fungal-exposed children.20 These data suggest that exposure to moulds influences the lymphocyte homeostasis, akin to what is known in allergic alveolitis.

In summary, the present results suggest that an inhalation challenge to (1→3)-β-D-glucan has an effect on inflammatory cells, shown as both increased as well as decreased effects in inflammatory markers. Persons living in houses with levels of (1→3)-β-D-glucan above 4 ng/m³ (indicator of mould growth) show an increased reactivity to an acute inhalation challenge to (1→3)-β-D-glucan.

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