

**Background and aim:** This study was performed to assess the effects of an exposure to a pure (1→3)-β-D-glucan, a cell wall component of fungi, plants and certain bacteria.

**Methods:** Twenty-one healthy subjects inhaled saline or (1→3)-β-D-glucan suspended in saline in a random, double-blind, cross-over design. They were examined before exposure and 24 and 72 h afterwards with spirometry, blood sampling and collection of induced sputum. Differential cell counts and eosinophilic cationic protein (ECP) were determined in blood and sputum, and myeloperoxidase (MPO), tumour necrosis factor-α (TNF-α), and interleukin (IL)-8 and IL-10 were determined in sputum supernatants. TNF-α was determined after cultivation of blood mononuclear cells.

**Results:** In sputum, inhalation of saline caused a significant increase in ECP and TNF-α. (1→3)-β-D-Glucan inhalation caused a further increase in these cytokines, although not statistically significantly different from the increase induced by inhalation of saline alone. In blood, the number of eosinophils was significantly decreased 72 h after the challenge with (1→3)-β-D-glucan. This effect was not found after the inhalation of saline alone. TNF-α production from stimulated blood mononuclear cells was significantly decreased 72 h after the (1→3)-β-D-glucan inhalation as compared with the increase induced by saline inhalation.

**Conclusions:** The results suggest that (1→3)-β-D-glucan causes a different type of response as compared with inflammatory agents such as bacterial endotoxin that cause a neutrophil-dominated inflammatory response.

**Key words:** (1→3)-β-D-Glucan, Inflammatory markers, Cytokines, Induced sputum

## Effects after inhalation of (1→3)-β-D-glucan in healthy humans

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## Introduction

Microbial cell wall components are important constituents in organic dusts.<sup>1</sup> Among the most well known of these are bacterial endotoxins. Endotoxin has been related to several effects in a number of experimental and epidemiological studies.<sup>2–5</sup> A review of the different effects and a suggestion for guidelines has previously been presented.<sup>3</sup>

Glucan, a cell-wall component of fungi, plants and certain bacteria, may also be present in organic dusts. Glucans are composed of glucopyranosyl subunits that are connected by α or β interchain linkages consisting of 1→3, 1→4, or 1→6 bonds.<sup>6–8</sup> Glucans in which subunits are connected by (1→3)-β-D interchain linkages have potent immunobiological effects.<sup>9–10</sup> Results from *in vitro* and *in vivo* experiments suggest that glucans can bind to cells by receptors, stimulate bone marrow, activate macrophages and induce production of cytokines such as interleukin (IL)-1 and IL-2.<sup>9,11–14</sup> *In vitro* studies show

that grifolan, a glucan isolated from *Grifola frondosa*, stimulates the secretion of inflammatory cytokines such as tumour necrosis factor-α (TNF-α) and IL-6 from cultured macrophages.<sup>14</sup> The effects after inhalation are less well known.

In an animal model in which inflammation in the airways was induced by exposure to endotoxin (lipopolysaccharide (LPS)), inhalation of (1→3)-β-D-glucan did not cause an increase in neutrophil numbers, which is the hallmark of LPS exposure. However, a single exposure to aerosolised (1→3)-β-D-glucan reduced the invasion of neutrophils caused by exposure to LPS.<sup>15</sup> A sub-chronic exposure to glucan induced an increased number of eosinophils in the trachea.<sup>16</sup> Repeated exposures have also suggested that (1→3)-β-D-glucan can potentiate the inflammatory effect of LPS and cigarette smoke.<sup>16–18</sup>

Airborne (1→3)-β-D-glucan has been measured in occupational and general environments with levels ranging up to 370 ng/m<sup>3</sup>. As effects have been found in animal inhalation studies at considerably lower

levels,<sup>16</sup> it is of interest to study the effects of inhaled (1 $\rightarrow$ 3)- $\beta$ -D-glucan in humans. In previous investigations in which human subjects inhaled pure (1 $\rightarrow$ 3)- $\beta$ -D-glucan (Curdlan), a slight increase in the intensity of throat irritation and skin symptoms was observed.<sup>19,20</sup>

The purpose of this study was to further assess the effects of an acute exposure to pure (1 $\rightarrow$ 3)- $\beta$ -D-glucan in healthy human subjects and to compare two different effect compartments, blood and induced sputum.

## Materials and methods

### Subjects

The study group comprised twenty-one healthy subjects (eight males and 13 females) recruited among students at the local university. The mean age was 23 years, with a range of 20–31 years. Potential subjects were interviewed using a questionnaire, and inclusion criteria were never smokers, no current colds, no self-reported allergy, no chronic bronchitis and no physician-diagnosed asthma.<sup>21</sup> Questions were posed about the odour of moulds, visible mould growth, dampness and water damage in the subjects homes. None of the subjects reported signs of mould contamination in their homes. They were also non-atopic as defined by a negative reaction in the Phadiatop test.<sup>22</sup>

### Study design

In a random, double-blind fashion, the subjects inhaled saline or grifolan, a pure (1 $\rightarrow$ 3)- $\beta$ -D-glucan suspended in saline using a cross-over design. They were examined before the exposures and 24 and 72 h after. Examinations consisted of spirometry measurements and collection of blood and induced sputum samples. Differential cell counts and eosinophilic cationic protein (ECP) were determined in blood and sputum, and myeloperoxidase (MPO) in sputum. Cytokines were determined in sputum supernatants and in blood mononuclear cells after *in vitro* cultivation with and without stimulation. The wash-out period between the two inhalation challenges was 2 weeks. The sputum inductions for baseline values were performed 1 week before the start of the inhalation challenges (one baseline induction/subject) and blood samples were taken immediately before each exposure.

The Ethics Committee of the Faculty of Medicine in Gothenburg approved the study.

### Inhalation of grifolan

Grifolan, a (1 $\rightarrow$ 3)- $\beta$ -D-glucan extracted from *G. frondosa*, was suspended in 0.5 M NaOH to dissolve the

triple helix and render the substance water soluble. It was then diluted 600 times with pyrogen-free saline to a final concentration of 8.3  $\mu$ g/ml. The final solution contained 0.8 mM NaOH and was adjusted with HCl to pH 7.0. The saline and grifolan in saline were aerosolised using a Pari Boy nebuliser with automatic dosing, giving 3  $\mu$ l per puff. The subject exhaled, placed the nebuliser in the mouth and started to inhale. Each subject inhaled five puffs of grifolan solution to a final dose of 125 ng.

### Differential cell counts in blood

Blood samples were collected in ethylenediamine tetracetic acid (EDTA) tubes, and total leukocyte counts were performed using a microscope. Differential cell counts were made on smears stained with May-Grünwald-Giemsa. The results were expressed as 10<sup>9</sup> cells/l of blood.

### Induced sputum

Sputum induction was performed according to Pin *et al.*<sup>23</sup> with some modifications.<sup>4</sup> Five percent saline was nebulised and inhaled over a period of 20 min. Ten minutes after the start of nebulisation and every 5 min thereafter, subjects interrupted the inhalation and were asked to rinse their mouth and throat carefully and to cough sputum into a sterile plastic container. The total amount of hypertonic saline administered was 20 ml. For safety reasons, spirometry measurements were performed before and immediately after sputum inductions, using standard techniques. A Vitalograph model S with a PFT printer was used and calibrated every morning with a 11 syringe. The subjects performed at least three technically acceptable trials according to ATS (American Thoracic Society) criteria, and the largest values for the forced expiratory volume in 1 sec (FEV<sub>1</sub>) and the forced vital capacity (FVC) were registered and compared with predicted values.<sup>24</sup>

The volume of the collected sputum sample was determined, and an equal volume of Sputasol (Oxoid; Unipath LTD, Basingstoke, UK) was added to obtain a final concentration of 3.25 mM dithiothreitol. The samples were incubated in a shaking water bath at 37°C for 5–10 min to ensure complete homogenisation. The samples were transferred into tubes and centrifuged at 600  $\times$  g for 5 min. The supernatants were aspirated and frozen at -70°C for later ECP, MPO and cytokine determinations. The cell pellets were washed twice in a washing solution (Hanks salt solution + 5% fetal calf serum + 2% EDTA, adjusted to pH 7.2). The total cell count and the cell viability were determined using a Bürker chamber and trypan blue exclusion. Differential cell counts were made on smears stained with May-Grünwald-Giemsa. A cell sample was considered adequate if, on differential cell

counting, it contained less than 50% squamous epithelial cells. The results were expressed as 10<sup>9</sup> cells/l of sputum.

### Inflammatory markers

ECP was assayed in serum and sputum samples by a fluorescent enzyme immunoassay technique (CAP ECP FEIA; Pharmacia Diagnostics AB, Uppsala, Sweden) and expressed as µg/l. MPO was assayed in sputum samples by an enzyme-linked immunoassay (MPO-EIA; R&D Systems, UK) and expressed as µg/l. Prior to the analyses, cell-free sputum samples were treated with equal volumes of 0.4% cetyltrimethylammonium bromide (CTAB) (Calbiochem, CA, USA), diluted in 0.25% phosphate-buffered saline and incubated at room temperature for 1 h. After centrifugation at 1500 × g for 10 min, the supernatants were aspirated and used for ECP and MPO determinations. CTAB was used to decharge the ECP and MPO to optimise the immunoassays.

### Cytokine release from blood mononuclear cells and in induced sputum supernatants

Whole blood was collected in vacutainer cell preparation tubes (CPT tubes; Becton and Dickinson, USA) and centrifuged for 20 min at 1500 × g to collect the mononuclear cell (MNC) fraction. The MNC was washed twice in Hank's salt buffer, supplemented with 10% of homologue serum. The cells, 1 × 10<sup>6</sup>/ml, were suspended in AIM-V medium (Gibco BRL, Paisley, Scotland) supplemented with 2-mercaptoethanol, 4 × 10<sup>-5</sup>M, and incubated without or with phytohaemagglutinin (PHA) (Murex Diagnostics Limited) to obtain a final concentration 0.005 mitogenic units/ml. After 48 h of incubation at 37°C and 5% CO<sub>2</sub>, the supernatants were collected and stored at -70°C until cytokine analysis.

TNF-α and IL-10 from unstimulated blood MNC and in induced sputum were analysed using an enzyme-linked immunosorbent assay (ELISA) kit with a sensitivity of 0.1 pg/ml (Quantikine high sensitive; R&D Systems). TNF-α in PHA-stimulated MNC and IL-8 in induced sputum were analysed using PeliKine-compact™ human cytokine ELISA (CLB, The Netherlands).

### Treatment of data

The differences between effect variables before and 24 and 72 h after the exposures were analysed using non-parametric tests (Wilcoxon's tests) and paired sample tests. The differences between the (1→3)-β-D-glucan and saline challenges were analysed using non-parametric tests (Mann-Whitney U-test and chi-squared test). Differences were considered statistically significant at *p* < 0.05.

## Results

### Inflammatory markers, cytokines and cells in induced sputum

The results of the determinations of ECP, MPO, cytokines and cells in induced sputum before and 24 h after the challenges are shown in Table 1.

The amounts of ECP, MPO, TNF-α and IL-8 were larger after the inhalation of saline and even higher after the (1→3)-β-D-glucan inhalation. The changes (before-24 h after inhalation) induced by the (1→3)-β-D-glucan inhalation as compared with the changes induced by the saline inhalation were not statistically significant. The same was found for the number of neutrophils. The amounts of IL-10 were slightly lower after both inhalations as compared with before.

No significant differences were observed 72 h after the challenges with (1→3)-β-D-glucan and saline as compared with before (data not shown).

**Table 1.** Amounts of ECP, MPO, TNF-α, IL-8 and IL-10 as well as the number of cells (× 10<sup>9</sup>/l) in induced sputum

|                       | Before inhalation | After saline inhalation | After (1 → 3)-β-D-glucan inhalation |
|-----------------------|-------------------|-------------------------|-------------------------------------|
| Subjects ( <i>n</i> ) | 21                | 21                      | 21                                  |
| ECP (µg/l)            | 63 (58)           | 100 (84)*               | 122 (175)*                          |
| Median                | 41                | 88                      | 98                                  |
| Range                 | 14–268            | 10–254                  | 22–800                              |
| MPO (µg/l)            | 2456 (2307)       | 3531 (4436)             | 4030 (4127)                         |
| Median                | 949               | 1244                    | 1937                                |
| Range                 | 208–3740          | 561–8088                | 366–13316                           |
| TNF-α (pg/ml)         | 6.5 (4.7)         | 9.6 (8.9)*              | 12.5 (14.0)**                       |
| Median                | 4.3               | 5.5                     | 5.2                                 |
| Range                 | 1.8–17.2          | 2.1–29.1                | 2.0–52.2                            |
| IL-8 (ng/ml)          | 4.7 (3.2)         | 5.6 (5.2)               | 8.3 (9.5)                           |
| Median                | 3.1               | 3.8                     | 4.4                                 |
| Range                 | 1.3–11.6          | 1.6–12.4                | 0.9–41.7                            |
| IL-10 (pg/ml)         | 3.5 (2.0)         | 2.5 (1.5)               | 2.6 (1.2)                           |
| Median                | 2.8               | 2.2                     | 2.1                                 |
| Range                 | 1.3–9.0           | 0.4–5.6                 | 1.2–5.4                             |
| Eosinophils           | 0 (0.01)          | 0 (0)                   | 0 (0)                               |
| Median                | 0                 | 0                       | 0                                   |
| Range                 | 0–0.1             | 0–0                     | 0–0                                 |
| Lymphocytes           | 0.05 (0.05)       | 0.07 (0.06)             | 0.07 (0.06)                         |
| Median                | 0.05              | 0.08                    | 0.08                                |
| Range                 | 0.01–0.15         | 0–0.17                  | 0.01–0.20                           |
| Macrophages           | 0.8 (0.5)         | 0.7 (0.4)               | 0.9 (0.6)                           |
| Median                | 0.6               | 0.8                     | 0.9                                 |
| Range                 | 0.2–2.1           | 0.1–1.6                 | 0.4–2.6                             |
| Neutrophils           | 0.40 (0.40)       | 0.46 (0.56)             | 0.59 (0.70)*                        |
| Median                | 0.26              | 0.27                    | 0.40                                |
| Range                 | 0.09–1.6          | 0.07–0.7                | 0.07–2.2                            |

Mean values, standard deviations in parentheses, medians and ranges before and 24 h after inhalation of saline or 125 ng (1→3)-β-D-glucan are shown.

\**p* < 0.05, \*\**p* < 0.01.

**Inflammatory markers, cytokines and cells in blood**

In blood, no significant differences in any of the variables were observed 24 h after the challenges as compared with before (data not shown).

The results of the determinations of ECP, TNF- $\alpha$  and cells in blood before and 72 h after the challenges are shown in Table 2.

The amounts of TNF- $\alpha$  from unstimulated and stimulated MNC were slightly higher after the inhalation of saline as compared with before. After the (1 $\rightarrow$ 3)- $\beta$ -D-glucan inhalation, the amounts were instead decreased and significantly different from the increase induced by the inhalation of saline alone in stimulated MNC ( $p < 0.05$ ).

The number of eosinophils was not influenced after the inhalation of saline. After the inhalation of (1 $\rightarrow$ 3)- $\beta$ -D-glucan, however, a significantly lower number of eosinophils were found as compared with before. The change in eosinophil numbers induced by the (1 $\rightarrow$ 3)- $\beta$ -D-glucan inhalation as compared with the change induced by the saline inhalation was on the borderline for statistical significance (-0.07; 95% confidence interval (CI), -0.14-0;  $p=0.06$ ).

**Spirometry**

No significant differences in lung function values were observed 24 h after the challenges as compared with before (data not shown). At 72 h, FEV<sub>1</sub> values were slightly higher after the inhalation of (1 $\rightarrow$ 3)- $\beta$ -D-glucan as compared with before (107%, SD = 15 versus 105%, SD = 14;  $p < 0.05$ ). No significant differences were observed after the challenge with saline (106%, SD = 14 versus 107%, SD = 14; not significant). The change in FEV<sub>1</sub> values induced by the (1 $\rightarrow$ 3)- $\beta$ -D-glucan inhalation as compared with the change induced by the saline inhalation was statistically significant (2.8%; 95% CI, 0.6-4.9;  $p < 0.05$ ).

At all occasions of sputum induction, the FEV<sub>1</sub> and FVC values were significantly lower immediately after hypertonic saline inhalation as compared with before.

**Discussion**

Spirometry measurements, blood and induced sputum samples were collected at 24 and 72 h after the challenges. The first post-exposure time was chosen on the basis of results of previous experiments with

**Table 2.** Amounts of TNF- $\alpha$  in unstimulated and stimulated MNC and ECP as well as the number of cells ( $\times 10^9/l$ ) in blood

|  | Saline      |             | (1 $\rightarrow$ 3)- $\beta$ -D-glucan |              |
|--|-------------|-------------|--|--------------|
|  | Before      | After       | Before                                 | After        |
| Subjects ( <i>n</i> )                        | 21          | 21          | 21                                     | 21           |
| ECP ( $\mu$ g/l)                             | 5.7 (2.6)   | 6.1 (3.6)   | 6.3 (3.9)                              | 5.8 (3.3)    |
| Median                                       | 6.0         | 5.6         | 5.6                                    | 6.3          |
| Range  | 2.0-11.0    | 2.0-13.5    | 2.0-12.9                               | 2.0-12.3     |
| <i>TNF-<math>\alpha</math>, unstimulated</i> |             |             |  |              |
| MNC (pg/ml)                                  | 12.8 (10.1) | 14.1 (10.8) | 12.2 (9.7)                             | 9.2 (8.1)    |
| Median                                       | 7.7         | 8.6         | 7.0                                    | 6.1          |
| Range  | 3.8-40.5    | 2.9-36.2    | 3.1-32.7                               | 2.8-29.4     |
| <i>TNF-<math>\alpha</math>, stimulated</i>   |             |             |  |              |
| MNC (pg/ml)                                  | 3047 (1743) | 4071 (2589) | 4150 (2471)                            | 3694 (1893)  |
| Median                                       | 2783        | 3302        | 3454                                   | 3401         |
| Range  | 1013-7608   | 1774-9796   | 969-9465                               | 1311-7351    |
| Eosinophils ( <i>n</i> )                     | 0.12 (0.09) | 0.13 (0.12) | 0.15 (0.11)                            | 0.09 (0.07)* |
| Median                                       | 0.11        | 0.11        | 0.15                                   | 0.08         |
| Range  | 0-0.36      | 0-0.58      | 0.02-0.48                              | 0-0.27       |
| Lymphocytes ( <i>n</i> )                     | 2.5 (0.6)   | 2.6 (0.8)   | 2.8 (0.6)                              | 2.6 (0.7)    |
| Median                                       | 2.7         | 2.4         | 2.7                                    | 2.6          |
| Range  | 1.4-3.5     | 1.4-4.3     | 1.5-4.2                                | 1.2-3.8      |
| Monocytes ( <i>n</i> )                       | 0.18 (0.13) | 0.17 (0.08) | 0.15 (0.09)                            | 0.14 (0.08)  |
| Median                                       | 0.17        | 0.17        | 0.15                                   | 0.15         |
| Range  | 0.03-0.46   | 0.03-0.34   | 0.06-0.37                              | 0-0.27       |
| Neutrophils ( <i>n</i> )                     | 3.4 (1.3)   | 3.4 (1.4)   | 2.8 (0.9)                              | 3.4 (1.8)    |
| Median                                       | 3.5         | 2.8         | 2.7                                    | 3.0          |
| Range  | 1.5-6.0     | 1.9-7.2     | 1.2-4.8                                | 1.7-8.8      |

Mean values, standard deviations in parentheses, medians and ranges before and 72 h after inhalation of saline or 125 ng (1  $\rightarrow$  3)- $\beta$ -D-glucan are shown.

\* $p < 0.05$ .

LPS that showed respiratory and general effects at that time.<sup>4</sup> The post-exposure time of 72 h was chosen on the basis of a few preliminary results where symptoms after exposure to glucan persisted after 3 days.<sup>20</sup> The (1→3)-β-D-glucan dose was selected as it corresponded to the threshold for inducing symptoms such as throat and nose irritation, sore throat and headache in a pilot experiment involving two subjects. As a comparison with the 125 ng dose used in this study, (1→3)-β-D-glucan levels up to 366 ng/m<sup>3</sup> have been measured in occupational environments and up to 106 ng/m<sup>3</sup> in homes where symptoms have been reported.<sup>25,26</sup>

The induced sputum technique requires inhalation of hypertonic saline. As this may influence the response to (1→3)-β-D-glucan, the sputum inductions for baseline values were performed 1 week before the start of the inhalation challenges. To minimise the risk of an influence of the hypertonic saline on the endpoints used, the sputum inductions were performed at least 48 h apart.<sup>4,27</sup> At all occasions of sputum induction, the FEV<sub>1</sub> and FVC values were significantly lower immediately after hypertonic saline inhalation as compared with before. This confirms that hypertonic saline can induce a bronchoconstriction, as has been reported in previous studies.<sup>4,28</sup> At 72 h, the FEV<sub>1</sub> values were slightly higher after the inhalation of (1→3)-β-D-glucan as compared with the saline inhalation. These results indicate that (1→3)-β-D-glucan does not induce a bronchoconstriction at the dose level used.

The results suggest a significant increase in the amounts of ECP and TNF-α and a tendency to an increased level of MPO in induced sputum after the saline inhalation, indicating that saline has an inflammatory effect. Increased levels of these inflammatory markers have been found in previous studies on airway inflammation and after organic dust/LPS exposures.<sup>25,29</sup> As (1→3)-β-D-glucan was suspended in saline, the increase in these variables after (1→3)-β-D-glucan inhalation was probably partly due to saline. Although the changes were higher after the (1→3)-β-D-glucan inhalation, the differences to the saline inhalation alone were not statistically significant. Whether (1→3)-β-D-glucan as such can induce an additional inflammatory response has to be investigated in future studies.

In blood, the secretion of TNF-α was slightly increased after the saline inhalation both in unstimulated and stimulated mononuclear cells 72 h after exposure. After exposure to (1→3)-β-D-glucan, the secretion was lower as compared with before. This indicates that inhaled (1→3)-β-D-glucan can induce a depression of the TNF-α-producing capacity of blood mononuclear cells. This agrees with results from previous studies. In a guinea-pig model, the neutrophil response in the airways to inhaled endotoxin was abrogated in a dose-dependent way by a previous

exposure to (1→3)-β-D-glucan.<sup>15</sup> In mice, there was a depression of inflammatory cytokines such as TNF-α after pretreatment with (1→3)-β-D-glucan.<sup>30</sup>

The number of eosinophils in blood was significantly decreased 72 h after the (1→3)-β-D-glucan challenge as compared with before. This effect was not observed after the saline inhalation. The eosinophil number in induced sputum was not increased, suggesting that there was a recruitment of eosinophils from the blood to the lung tissue. This is in accordance with findings observed after exposure to (1→3)-β-D-glucan in an animal model where an increased number of eosinophils was found in the airway epithelium.<sup>31</sup>

As (1→3)-β-D-glucan, we used grifolan. There are several other forms of glucans, with different bonds and branches, that have been investigated in animal models. At this point, no comparisons have been made of the relevance of different (1→3)-β-D-glucans or different (1→3)-β-D-glucan preparations in human subjects. Thus, no conclusions of the effects on humans of glucans in general can be drawn from the results in this study.

In conclusion, the results suggest that (1→3)-β-D-glucan in the dose used does not cause an acute neutrophil-dominated inflammatory response, in contrast to inflammatory agents such as bacterial endotoxin. The decreased eosinophil numbers and decrease in TNF-α production from stimulated mononuclear cells in blood after the (1→3)-β-D-glucan challenge indicate a different type of response.

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