

AIRWAY irritation effects after single and repeated inhalation exposures to aerosols of β -glucan (grifolan) were investigated in mice. In addition, the effects on serum total immunoglobulin E (IgE) production and histopathological inflammation in the respiratory tract were studied. The β -glucan aerosols provoked slight sensory irritation in the airways, but the response was not concentration dependent at the levels studied. Slight pulmonary irritation was observed after repeated exposures. No effect was found on the serum total IgE levels, and no signs of inflammation were seen in the airways 6 h after the final exposure. The results suggest that, irrespective of previous fungal sensitization of the animals, inhaled β -glucan may cause symptoms of respiratory tract irritation but without apparent inflammation. Respiratory tract irritation reported after inhalation of fungi may not be entirely attributed to β -glucan.

Key words: Fungal cell-wall component, Grifolan, Inhalation exposure, Mice

Slight respiratory irritation but not inflammation in mice exposed to (1 \rightarrow 3)- β -D-glucan aerosols

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Introduction

Occupants in buildings with moisture or mold damage often complain of eye and upper respiratory tract irritation. Based on epidemiological studies, fungi (molds) have been suggested to be responsible for many of the irritative symptoms. Fungi contain several specific agents such as microbially produced volatile organic compounds, mycotoxins, and the polyglucose cell-wall component (1 \rightarrow 3)- β -D-glucan.^{1–4} Airborne β -glucan levels in indoor air have been used as indicators of total fungal cell mass and related to symptoms of irritation, cough, tiredness and headache.^{5,6} Using the pure substance, a slightly increased prevalence of throat irritation and cough has been demonstrated in healthy, non-sensitive persons after a 4 h inhalation exposure to β -glucan at 210 ng/m³.⁷

The sensation of irritation experienced by persons exposed to molds or to other agents in their homes may originate either from an inflammatory reaction in the tissue or from a direct stimulation of the nerve

endings; the two processes do not have to be related. A standardized mouse bioassay [American Society for Testing and Materials (ASTM)⁸] is used to assess the latter by determining changes in the breathing pattern of mice during inhalation of aerosols, gases or vapors. Thus, effects on various parts of the respiratory system (i.e. upper and lower respiratory tract and conducting airways) can be recorded. With the head-out body plethysmography, we have previously shown that inhalation of *Stachybotrys chartarum* extract aerosols that contained β -glucan provoked sensory irritation in the airways of mice, whereas aerosols of ovalbumin or phosphate-buffered saline (PBS) did not cause this effect.⁹

In view of this, we undertook experiments to investigate the potency of β -glucan to cause upper respiratory tract irritation and inflammation. Mice were exposed to an aerosol of β -glucan at different concentrations, and the effects on the respiratory pattern were recorded. Furthermore, the reaction in mice pre-exposed to a fungal extract was evaluated during repeated exposures to β -glucan.

Materials and methods

Animals

A total of 30 female, 6-week-old (13 weeks old at the end) BALB/cJ Bom mice (M&B A/S, Ry, Denmark) and 24 male 5-week-old Ico:OF1 mice (IOPS Caw; Iffa Credo, Saint-Germain sur L'Arbresle, France) were used in the experiments (Table 1). To study the potency of β -glucan to provoke sensory irritation, the OF1 strain was used as this strain has been widely used in the ASTM method.¹⁰ For another purpose (i.e. to illustrate immunological changes due to repeated exposures in mice pre-sensitized to a fungal extract), we chose the BALB/c mice. The animals were housed in a carefully controlled environment in an animal room that operated on a 12-h dark/light cycle at 19.5–20.5°C and at 60–65% relative humidity. Groups of four mice were housed in stainless steel cages (42 cm deep \times 24 cm wide \times 13 cm high) bedded with non-autoclaved cellulose wadding (Katrinn Care Wadding; Metsä Tissue, Mänttä, Finland). Food (Labfor R36; Lactamin AB, Stockholm, Sweden) and tap water were provided to the animals *ad libitum*.

Immunization of mice with a fungal extract

For fungal immunizations of two mouse groups (groups Sc and Sc/glu), the fungal extract was prepared from a non-toxic strain of *S. chartarum* (Sc) (American Type Culture Collection 208877, previously stored as University of Kuopio strain 10) according to Korpi *et al.*⁹ This extract has been shown to contain β -glucan, endotoxin and ergosterol for 6.7 ng/ml, 0.02 ng/ml, and 6.4 μ g/ml, respectively.⁹ These concentrations were determined per milliliter of the dialyzed and sterile-filtered solution before lyophilization.

Mice were either immunized intraperitoneally with 50 μ g *S. chartarum* extract in adjuvant twice with a 2-week interval, or treated with equal amount of PBS (200 μ l), or not injected at all (see Table 1). Commercial Imject Alum (Pierce, Rockford, IL, USA) was used as an adjuvant according to the manufacturer's instructions. The experiments were performed in agreement with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 18 March 1986, adopted in Finland 31 May 1990). The study was approved by the Animal Care and Use Committee of the University of Kuopio, and by the provincial government.

Generation of glucan aerosols

We used one of the available forms of fungal β -glucans, grifolan, which is a gel-forming 1,6-

Table 1. Description of the mouse groups

Mouse group	Mouse strain	n	Immunization/injection 12 and 26 days before inhalation exposure	Inhalation exposure to β -glucan	Number of animals analyzed for:		
					Respiratory parameters	Histopathological samples	Serum total IgE
PBS	BALB/c	8	With 200 μ l PBS	–	–	2	8
PBS/glu	BALB/c	8	With 200 μ l PBS	Seven exposures within 22 days	4	8	8
Sc	BALB/c	6	With 50 μ g <i>S. chartarum</i>	–	–	–	6
Sc/glu	BALB/c	8	With 50 μ g <i>S. chartarum</i>	Seven exposures within 22 days	4	8	8
naïve/glu	OF1	6*4	–	Each group exposed to single and different concentrations	6*4	2	–

branched (1 \rightarrow 3)- β -D-glucan isolated from liquid-cultured mycelium of *Grifola frondosa* (kindly provided by Prof. Ohno, Tokyo University of Pharmacy and Life Science, Tokyo, Japan). A water-soluble form of β -glucan was chosen because the *S. chartarum* extract studied earlier (with its β -glucan moiety) was readily soluble in water and PBS. The resulting grifolan has been analyzed to contain 91% sugar (glucose as a component sugar) and 0.3% protein.^{11,12} Two β -glucan batches were used in this experiment. At first, the glucan was dissolved in 0.5 N NaOH to give 0.5% (w/v) solution, and the consecutive working solutions were diluted in sterile water (Medipolar, Oulu, Finland). From the glucan solution (or from sterile water), polydisperse aerosols were generated with an atomizer as described previously.⁹ For safety precautions, all the exposure apparatuses were held in a safety hood and the exhaust air was filtered prior to leading it into exhaust air duct.

To determine the glucan concentration of aerosols in the exposure chamber and to ascertain absence of bacterial contamination by determining endotoxin concentration, air samples (2 l/min) were collected at the outlet of the exposure chamber on a polycarbonate filter (ATTP03700, 0.8 μ m, 37 mm; Millipore, Molsheim, France). The β -glucan and endotoxin concentrations were determined from a selection of the filters using specific Limulus lysates.¹³ A side-stream from the outlet of the chamber was led via a dilutor to the optical particle counter (Hiac/Royco model 5000; Pacific Scientific, Silver Springs, MD, USA) to register the number and size distribution of the generated aerosols continuously during the experiments. This device separates particles into six different classes: from 0.3 to 0.5 μ m, from 0.5 to 1.0 μ m, from 1.0 to 3.0 μ m, from 3.0 to 5.0 μ m, from 5.0 to 10 μ m, and particles over 10 μ m. This generator produces also particles < 0.3 μ m.⁹

Exposure and mouse bioassay

The six groups of naïve/glu mice were each exposed once to different glucan concentrations (from 37 to 1189 μ g/m³) or to sterile water (control experiment) (Table 1). In the immunized mice, the first aerosol exposure to glucan was performed 12 days after the second immunization of the PBS/glu and Sc/glu mice. The immunized mice were exposed to glucan aerosol on days 0, 1, 7, 8, 14, 15, and 21 (Table 1).

Before each aerosol exposure, there was initially a 15 min acclimation period followed by a 15 min control (baseline) period when baseline respiratory parameter values were obtained for each mouse. The exposures lasted for 15–20 min and were followed by a 15 min recovery period. During the acclimatiza-

tion, control and recovery periods, filtered compressed air was fed into the exposure chamber.

The mouse bioassay followed the ASTM E 981-84 standard,⁸ as described by Korpi *et al.*⁹ Briefly, the respiratory function of the four simultaneously exposed animals was monitored. The baseline values of different respiratory parameters collected during the control period are presented in Table 2. Based on the baseline values [mean and standard deviation (SD)], each breath was then classified either as normal or as indicating sensory irritation, pulmonary irritation, pulmonary irritation in phase 1, airflow limitation within the conducting airways of the lungs (i.e. bronchoconstriction), or their combinations with each animal acting as its own control.^{14–16} Due to the high number of individual breaths analyzed and the low coefficients of variation, using four mice per group is sufficient to establish limits of detection. The smoothing polynomial spline program¹⁴ was used to determine changes in each breath variable or category, and mean responses of four simultaneously exposed mice were calculated. This time series analysis was used to define time–response relationships for each group of mice and each exposure regimen. The 95% confidence intervals were also calculated for each smoothed curve.

The animals were observed daily. The body weights of the animals were also recorded on every exposure day. Six hours after the final aerosol exposure, mice were sacrificed with pentobarbital (Mebunat®), and serum samples as well as tissue samples from the respiratory tract were collected from the PBS/glu and Sc/glu mice that were exposed repeatedly to β -glucan. Serum samples were collected also from the groups PBS and Sc to measure antibody levels without inhalation exposure. Tissue samples were collected also from two naïve/glu mice 6 h after their exposure to 1189 μ g/m³ β -glucan (see also Table 1).

Measurement of total IgE concentrations and histopathology of nasal cavity and lungs

The serum total IgE levels were determined with an enzyme-linked immunosorbent assay as described in Korpi *et al.*⁹

The mouse skull with nasal cavity and lungs was collected and fixed with 4% formaldehyde solution. The mouse skull was decalcified for 6 h in 5% nitric acid solution (Riedel-de Haën KG, Seelze, Germany). After that, multiple sections were cut to reveal the distribution of epithelial types and submucosal glands in the nasal cavity (modified after reference 17). The resulting three or four blocks of the tissue were embedded in paraffin, sectioned at a thickness of 5 μ m, and stained with hematoxylin and eosin (HE). The left and right lung (all lobes) including samples from hilar lymph nodes of the lungs were

Table 2. Respiratory parameters calculated from airflow signals

Parameter	Abbreviation	Definition	Unit	BALB/cJBom (n = 8) (value ± SD)	OF1 (n = 24) (value ± SD)
Tidal volume	VT	Volume of air inhaled/exhaled per breath	ml	0.20 ± 0.02	0.22 ± 0.03
Respiratory frequency	F	Number of breaths per minute	BPM	255 ± 38	262 ± 52
Mid-expiratory flow	VD	Airflow at 50% VT during expiration	ml/sec	2.20 ± 0.43	2.53 ± 0.45
Time of inspiration	TI	Duration from minimum to maximum VT	sec	0.09 ± 0.02	0.09 ± 0.02
Time of expiration	TE	Duration from maximum to minimum VT	sec	0.14 ± 0.03	0.14 ± 0.02
Time of braking	TB	Duration of break after inspiration, prior to expiration	sec	0.02 ± 0.003	0.02 ± 0.004
Time of pause	TP	Duration of pause between expiration and inspiration	sec	0.02 ± 0.003	0.03 ± 0.005

Approximately 3600 breaths were measured for each mouse over a baseline (control) period of 15 min for the average value and SD.

routinely processed, embedded in paraffin, sectioned at 5 µm, and stained with HE. An experienced pathologist examined the stained sections of nasal cavity and lungs with light microscopy.

Statistical analysis

The serum total immunoglobulin E (IgE) levels were compared between the mouse groups by the Mann–Whitney U-test (SPSS for MS Windows, release 9.0) to detect the immunological changes induced by β-glucan aerosol inhalation. The body weights of the mice at the first and last exposure day were compared with the Wilcoxon signed Rank Test to detect changes due to repeated exposures. Differences with $p < 0.05$ were considered significant.

Results

Glucan aerosols

During repeated exposures, the number of produced particles (of diameter from 0.3 to > 10 µm) was approximately 2×10^9 particles/m³ except for the fifth and the sixth repeated exposure experiments, when the counts were approximately 7×10^8 parti-

cles/m³ due to the change of the nozzle plate. The diameter of the majority of aerosols produced from solutions of β-glucan was in the range from 0.3 to 3.0 µm when analyzed in the scale from 0.3 to > 10 µm with the optical particle counter. The proportion of particles of 0.5–3.0 µm and the total number of generated aerosols became higher when the amount of β-glucan in the solution was increased (i.e. 425 or 1163 µg/ml). Endotoxin was not detected on any of the nine analyzed filters (detection limit is 10 pg/ml¹³).

Effects of glucan aerosol inhalation on the murine respiratory pattern

When naïve/glu mice were exposed to various β-glucan concentrations, a distinct increase in the time of braking (TB) was observed (Table 3). However, the proportion of the sensory irritation (SI) breaths did not clearly reflect the increase in TB values (Table 3). This is mainly due to the filtering of the data and the prerequisite of three consecutive breaths in the same abnormal category. The TB (and SI) response did not show a clear dependence on β-glucan concentration, and the respiratory response was of the same magnitude at the concentration range from 37 to 883 µg/m³ (Table 3). In the repeated exposures,

Table 3. Respiratory effects of single β-glucan inhalation exposure on OF1 mice

Concentration of β-glucan (µg/ml)	Concentration of β-glucan (µg/m ³)	% of control TB	Maximum% of total breaths classified as SI
0	0	124	< LOD
61	37	127	12
125	n.a.	137	6
205	252	128	11
425	883	135	< LOD
1163	1189	150	20

Groups were naïve/glu, four mice in each exposure. Diagnosis of SI requires an increase in time of braking (TB) greater than 2.0 times the SD of the mean baseline value for that mouse. An increase in TB of 20% compared with the value during control period represent the limit of detection (LOD) in this bioassay, and the LOD of the breath classification for SI 2%^{18,19}. n.a., not analyzed.

a slight SI response was also observed (Table 4). Time–response plots of increases in TB and SI breaths due to exposure to β -glucan are illustrated in Figs. 1 and 2, respectively. (The 95% confidence intervals for each smoothed curve are not included in Figs. 1 and 2 to preserve clarity as they were very narrow around the smoothed mean curves.) The SI response started immediately after the onset of the exposure. During the control periods, no SI was

observed. During the recovery periods of some of the experiments of repeated exposures, breaths indicating SI (< 4% of breaths) were detected; however, in the majority of experiments, no SI during the recovery period was observed.

Slight pulmonary irritation was noticed occasionally in the repeated exposure experiments at a β -glucan level of $118 \pm 53 \mu\text{g}/\text{m}^3$ (mean \pm SD), as evidenced by an increase in breaths classified as

Table 4. Respiratory effects of seven β -glucan inhalation exposures on four BALB/cJBom mice

Mouse group	Order of repeated exposure	% of control period of the respiratory parameter		Maximum% of total breaths classified as:	
		TB	TP	SI	PI
PBS/glu	1	123	< LOD	3	< LOD
	2	< LOD	119	< LOD	< LOD
	3	< LOD	119	3	< LOD
	4	< LOD	132	3	11
	6	< LOD	142	4	6
Sc/glu	1	125	< LOD	5	< LOD
	2	< LOD	124	< LOD	< LOD
	3	< LOD	< LOD	< LOD	< LOD
	4	< LOD	120	< LOD	< LOD
	5	< LOD	125	3	< LOD
	6	< LOD	130	< LOD	3
	7	121	116	8	3

The dilution of β -glucan solution for the aerosol generator was $125 \mu\text{g}/\text{ml}$ and the mean β -glucan concentration \pm SD was $118 \pm 53 \mu\text{g}/\text{m}^3$. The respiratory parameter data from the fifth and seventh exposures of the group PBS/glu could not be processed due to computer program failure. Diagnoses of SI or PI require an increase in TB or time of pause (TP), respectively, greater than 2.0 times the SD of the mean baseline value for that mouse. An increase in TB of 20%, and an increase in TP of 12% compared with the values during control period represent the limit of detection (LOD) in this bioassay, and the LODs of the breath classifications for SI and PI are 2% for both^{18,19}. n.a., not analyzed.

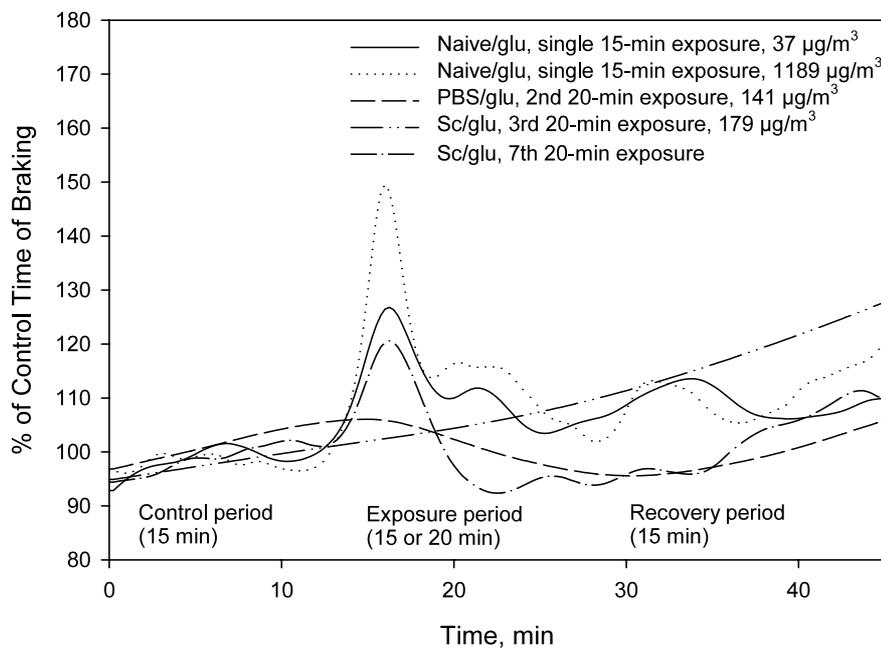


FIG. 1. Average time–response plots of four mice of time of braking (TB) during inhalation exposure to β -glucan aerosol. Mouse group (immunization/inhalation exposure), the order of exposure, and exposure concentration are indicated. The naïve/glu mice were male OF1 mice, and the rest of the groups consisted of female BALB/c mice. The average TB value calculated from all the breaths collected during the 15 min control period was set equal to 100%, and all the data collected during the whole experiment (in each 15 sec collection period) was then presented as a percentage of this value. Plots from the low and high single-exposure concentrations and from repeated exposures experiments are included. During the control and recovery periods, filtered air was fed into the breathing zone of the animals.

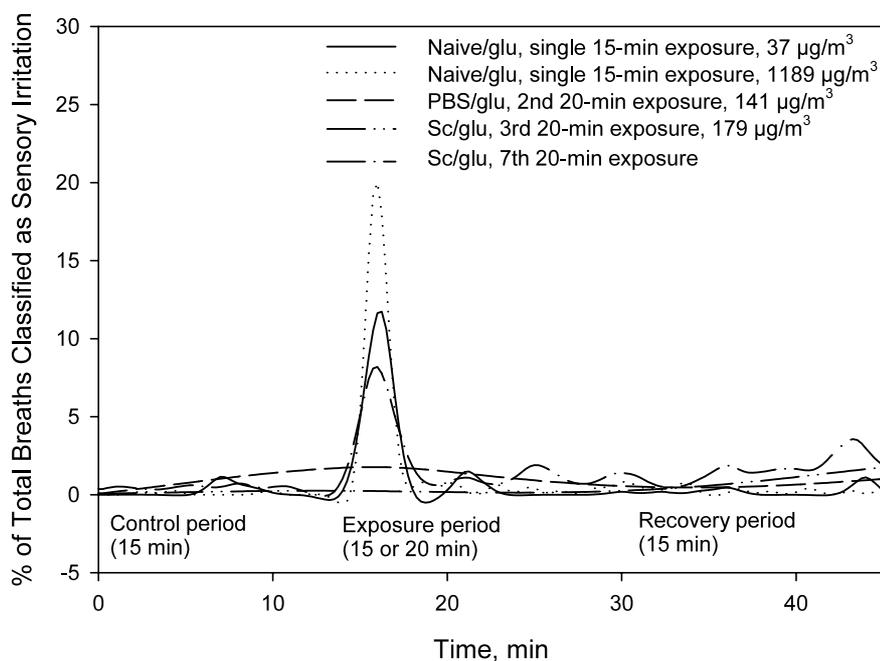


FIG. 2. Average time-response plots of breaths indicating sensory irritation (SI) upon inhalation exposure of four mice to β -glucan aerosol. Mouse group (immunization/inhalation exposure), the order of exposure, and exposure concentration are indicated. The naïve/glu mice were male OF1 mice, and the rest of the groups consisted of female BALB/c mice. Diagnosis of SI requires an increase in time of braking greater than 2.0 times the standard deviation of the mean baseline value for that mouse. The time-response plots were prepared by plotting SI breath classification as a percentage of the total number of breaths collected during each 15 sec period. Unless three or more consecutive breaths were classified as SI, the program reclassified the breath as normal. This was done to reduce the number of abnormal breaths created by body movements¹⁴. Plots from the low and high single-exposure concentrations and from repeated exposures experiments are included. During the control and recovery periods, filtered air was fed into the breathing zone of the animals.

pulmonary irritation (PI) (Table 4). This PI effect was not observable during the single exposures. When the maximum SI effect occurred within 3 min after the onset of the exposure, the maximum PI effect occurred later (i.e. 6–20 min after the onset of the exposure). Slight β -glucan-induced pulmonary irritation in phase 1 (rapid shallow breathing) (< 12% of breaths) was observed on few exposure occasions of the groups Sc/glu and naïve/glu. β -glucan aerosols did not cause bronchoconstriction in any of the experiments.

Serum total IgE levels and tissue samples

Immunization with the fungal extract increased the serum total IgE levels in mice significantly ($p = 0.002$) (mean \pm SD, serum total IgE level in the PBS group was 373 ± 123 ng/ml and in the group Sc was $11,883 \pm 7234$ ng/ml). However, no statistically significant differences ($p > 0.4$) in the serum total IgE levels were caused by seven β -glucan aerosol exposures (group PBS/glu, 358 ± 71 ng/ml and group Sc/glu, 9848 ± 6305 ng/ml) compared with the mean serum total IgE levels of the groups PBS and Sc, respectively.

The bodyweights of the mice did not change significantly after repeated exposures in either of the groups PBS/glu or Sc/glu ($p > 0.1$) (data not shown).

The lung specimens of the seven PBS/glu and seven Sc/glu mice were normal (i.e. no signs of inflammation or fibrosis) at histopathological examination (data not shown). In one PBS/glu and one Sc/glu mouse, there was a slightly increased number of perivascular/peribronchial lymphocyte aggregates in the lungs. The lung specimens of the two heavily-exposed naïve/glu mice were normal.

The histopathological analysis of the nasal cavity did not reveal any signs of inflammation or tissue damage in any of the Sc/glu mice, the PBS/glu mice, or either of the two naïve/glu mice.

Discussion

The primary objective was to study whether β -glucan aerosols were capable of stimulating the nerve endings directly and whether the response was dependent on previous fungal sensitization. The results showed that aerosolized form of water-soluble β -glucan at concentrations from 37 to $1189 \mu\text{g}/\text{m}^3$ induced a minor SI response, and that the effect was not affected by preceding fungal exposure. SI response is not a general response to any type of inhaled material. For instance, saline solution of approximately $55 \text{ mg}/\text{m}^3$ or ovalbumin solution of approximately $109 \text{ mg}/\text{m}^3$ does not induce the SI effect.⁹ However, inhalation of the fungal (*S. char-*

tarum) extract showed a distinct SI response. Based on the β -glucan content of the extract, working dilutions used, the air volume and the volume of the working solution aerosolized during an exposure, we can estimate that the *maximum* β -glucan concentration in the air was $10 \mu\text{g}/\text{m}^3$ in those experiments.⁹ In the present experiments, β -glucan concentrations from 0 to $1189 \mu\text{g}/\text{m}^3$ were tested for their SI potency, and these concentrations were found to exert lower increases in time of braking and SI breaths than the *S. chartarum* extract. Thus, β -glucan does not contribute significantly to the SI response provoked by aerosols of fungal extract.

The reason for the fact that the SI response was not observed at every exposure level or at every occasion of repeated exposure remains unexplained. The generated particles reached the murine airways, as the majority of the particles were—at each concentration level—in the range from 0.3 to $3 \mu\text{m}$.²⁰ Since the biological effect probably depends on the mass rather than the particle count number, we transformed the results from the optical particle counter from the count number into mass fraction, assuming the same density as that of NaCl for all particles. Even for the 6×10^9 particles/ m^3 generated from the most concentrated β -glucan solution ($1163 \mu\text{g}/\text{ml}$), the mass median diameter and geometric SD were low ($2.5 \mu\text{m}$ and 1.9). This suggests that the particles were respirable.²¹

In indoor environments, microbial growth on moist building materials has also been related to the lower respiratory tract symptoms, such as cough, wheezing and dyspnoea.^{22–26} When the amount of β -glucan was used as an indicator of mold growth in buildings, increased levels of β -glucan (i.e. $> 5–10 \text{ ng}/\text{m}^3$) in the air were reported to be associated with cough and general symptoms.²⁷ The ability of β -glucan to increase the prevalence of cough was also verified experimentally, when healthy non-sensitive persons were exposed for 4 h but to much higher levels of β -glucan ($210 \text{ ng}/\text{m}^3$) than normally present in the environment.⁷ In the present study, after repeated exposures to soluble β -glucan only slight signs of pulmonary irritation were noticed in mice. Thus, the results from this study suggest that these sensory and pulmonary irritation responses reported after inhalation of fungi are probably not attributed to β -glucan.

Inhalation of aerosols of different mold species for 5 weeks results in a massive cell influx in the lungs of guinea pigs, akin to the reaction after inhalation of bacterial endotoxin.^{28,29} In this study, repeated inhalation exposures to β -glucan within 22 days did not induce notable inflammatory changes in the lungs of the mice. Histological changes have both been detected and been absent after acute or chronic inhalation exposures to β -glucan.^{29–34} The reasons for different observations regarding the degree of cell

infiltration may be due to different glucan delivery protocols as well as different β -glucan preparations. It should be noted, however, that any particular form of β -glucan has not been systematically proven the most potent when assessing both inflammatory cell influx^{31,34} and other inflammatory markers (e.g. cytokine and nitric oxide production in *in vitro* studies).^{11,35–38} Taken together, these results suggest that the inflammation caused by inhalation of fungi is not caused by the β -glucan component in the fungal cell wall.

So far, no evidence of β -glucan being an allergen by the inhalation route has been presented. This is supported by the results from this study, since repeated β -glucan exposure did not increase the serum total IgE levels in mice, irrespective of whether the mice were pre-exposed to a fungus. This corroborates the previous suggestions according to which β -glucan may act as an adjuvant exerting additive or synergistic effects only when combined with other agents, such as allergens (ovalbumin) either via inhalation exposure or via injection.^{39–41} However, various gel-forming β -glucans have different effects on balancing helper T-cell activity (i.e. on the production of Th1 or Th2 antibody subclasses and cytokines).⁴²

In conclusion, a single inhalation of β -glucan (grifolan) aerosol provoked a slight sensory irritation response in mice without a dose–response relationship at β -glucan levels from 37 to $883 \mu\text{g}/\text{m}^3$. Mice repeatedly exposed to an average β -glucan level of $118 \pm 53 \mu\text{g}/\text{m}^3$ developed a minor pulmonary irritation response in addition to sensory irritation. A previous sensitization to a fungal extract did not affect the response. No inflammatory changes in the nasal cavity or in the lungs could be demonstrated after the exposures. Inhalation of β -glucan did not increase the production of serum total IgE.

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