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# Inhalation of (1→3)-β-D-glucan causes airway eosinophilia

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**Background: Moulds are present in a variety of environments and aerosols of fungal spores are generated when mouldy materials are handled. Molds contain (1→3)-β-D-glucan, a polyglucose which is present in the cell wall of fungi, certain bacteria and plants.**
***Aim:* This study was undertaken to investigate the cellular inflammatory response in the lung after inhalation of (1→3)-β-D-glucan and bacterial endotoxin.**
***Methods:* Guinea pigs were exposed daily to an aerosol of pure (1→3)-β-D-glucan and pure endotoxin for five weeks. Lung lavage and lung interstitial cell preparations were done and the inflammatory cells counted. Histological sections were prepared from the trachea.**
***Results:* There was an increase in eosinophil numbers in lung lavage, lung interstitium, and the airway epithelium of animals exposed to (1→3)-β-D-glucan. In animals simultaneously exposed to endotoxin, there was no increase in eosinophils. In the lung interstitium, (1→3)-β-D-glucan exposure caused an increase in lymphocytes, which was not found after endotoxin exposure. Endotoxin exposure caused an increase in neutrophils and macrophages in lung lavage, which was not found after (1→3)-β-D-glucan exposure.**
***Conclusions:* The results support previous findings that (1→3)-β-D-glucan causes a different response in the airways as compared to endotoxin. Endotoxin modulated the increase in eosinophils caused by (1→3)-β-D-glucan exposure, suggesting a complex interaction between the microbial cell wall components.**

**Key words:** (1→3)-β-D-glucan, Endotoxin, Eosinophils, Inflammation

## Introduction

Moulds are present in occupational and general environments. Aerosols of fungal spores are generated when mouldy materials are handled. High amounts have been reported in farm environments where mouldy hay is handled, in areas where mouldy wood is processed and inside humid buildings.[1] Moulds contain a number of bioactive substances that may produce toxic effects and interact with the immune system.[2] Of particular interest is (1→3)-β-D-glucan, a polyglucose compound present in the cell wall of fungi, certain bacteria and plants including pollen.[3] The effects of (1→3)-β-D-glucan on inflammatory and immune competent cells in general have been documented in several *in vitro* and *in vivo* animal models.[4–9]

In comparison with the abundance of information on the effects of (1→3)-β-D-glucan administered by injection using *in vivo* models, data on the effects of inhalation are limited. Previous reports from our laboratory have demonstrated that an acute inhalation of (1→3)-β-D-glucan in guinea pigs prevented an endotoxin induced invasion of neutrophils in the airways.[10] Another study involved a five-week exposure, which caused an increase in the number of neutrophils in the airways when endotoxin was administered alone, but no such effect was found after exposure to (1→3)-β-D-glucan alone.[11] An even greater increase in neutrophil numbers was observed when endotoxin was administered together with (1→3)-β-D-glucan. These data suggest that inhaled (1→3)-β-D-glucan initiates a response that is different from that induced by inhaled endotoxin as there is no increase in the number of neutrophils – the hallmark of endotoxin exposure.

There is also information on other inflammatory cells. In a subchronic study using guinea pigs, an infiltration of eosinophils was seen in the trachea epithelium of animals exposed to (1→3)-β-D-glucan.[12]

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In a mouse model where the animals inhaled ovalbumin, it was found that (1→3)-β-D-glucan potentiated the ovalbumin-induced eosinophilia in the airways.[13]

The present experiments were undertaken to further study the ability of (1→3)-β-D-glucan to induce an inflammatory response in the lungs after a subchronic inhalation exposure and to study the effect of a simultaneous exposure to endotoxin.

## Material and methods

### Animals

Male and female guinea pigs from our own breeding colony with an initial weight of about 700 g were used in the experiments. All animals were kept in cages supplied with filtered air at a slight overpressure. Food and water were supplied *ad lib*. Each exposure group had the same proportion of females and males. The animals were without signs of latent infections in the airways, as evaluated by a low number of inflammatory cells in the lavage fluid of unexposed animals.

### (1→3)-β-D-glucan exposure

For the (1→3)-β-D-glucan exposures, we used grifolan, prepared from the mushroom *Grifolanum commune*, which is non-soluble in water (kindly supplied by Professor N. Ohno, Tokyo). It was rendered water-soluble by suspending it in 0.3 N NaOH and was then diluted with distilled water into 10 μg/ml. An aerosol was generated using a Collison atomizer,[14] and the animals were exposed in a small exposure chamber in a continuous flow system. The animals were exposed for four hours a day, five days a week, for five weeks.

The amount of (1→3)-β-D-glucan in the chamber air was 30 μg/m$^3$, yielding an estimated dose of about 1.5 ng/animal/day, presuming a 50% deposition of the inhaled small-particulate aerosol and using previously published data on ventilation in guinea pigs.[15]

### Endotoxin exposure

For the endotoxin exposure, we used a solution in water containing 25 μg/ml of lipopolysaccharide (LPS, *Escherichia coli* 026 B6, Difco lab) in similar exposure equipment. The animals were exposed for 40 min a day prior to the (1→3)-β-D-glucan exposure, five days/week for five weeks. The amount of LPS in the chamber was 75 μg/m$^3$, yielding a dose of about 4 ng/animal and day.

### Examination

At 24 hours after the last exposure, the animals were anaesthetized with an i.p. injection of sodium pentobarbital (120 mg/ml). The thoracic cavity was opened and the lung vascular bed perfused with chilled Dulbecco's PBS (phosphate-buffered saline) without $Mg^{2+}$ and $Ca^{2+}$ (DPBS, NordCell, Sweden). Perfusion was performed until the lungs were clear white, at which point the aorta was tied off. The right lung was used for determining the number of inflammatory cells in the lavage fluid and in the lung interstitium. The trachea was used for histological examinations.

### Lung lavage cells (LLC)

The right lung was subjected to lung lavage *in situ* after forceps had closed the left main bronchus. A portion of 70 ml saline was used at a body weight of 700–800 g; for each additional 100 g, the lavage volume was increased by 10 ml. The saline was divided into 10 aliquots, which were slowly injected into the lung lobes via a cannula in the trachea. After each instillation, the fluid was withdrawn and collected in 50-ml centrifuge tubes placed on ice. The lavage fluid was centrifuged at 350×*g* for 10 min, and the cell pellet was collected, resuspended and counted in a light microscope. A cell differentiation was done using May–Grünewald–Giemsa stain. These cells are referred to as lung lavage cells (LLC).

### Lung interstitial cells (LIC)

After the lung lavage, the right lung was prepared according to a method previously described.[11,16] The trachea was cut off beneath the larynx, and the whole lung was removed from the thoracic cavity. The upper right lobe was placed on a filter paper and sliced into 0.4 mm thick sections. About 0.8 g of lung tissue was suspended in siliconized flasks containing 10 ml of PBS with 10% inactivated calf serum, 175 units/ml of collagenase (Boehringe, Mannheim, Germany) and 50 units/ml of DNAse (Sigma, St Louis MO, USA). Following an incubation of 90 min in a shaking water bath (180 s.p.m. 37° C), the preparation was agitated and remaining tissue fragments in the cell slurry were removed by filtration through a thin cotton wool plug. The cells were collected by centrifugation, counted and typed. Cell viability was checked by Trypan blue exclusion (the viability range was 80–92%). These cells are referred to as lung interstitial cells (LIC).

### Eosinophils in the tracheal epithelium

The trachea was fixed in 4% buffered formaldehyde and was sectioned longitudinally, including the first bifurcation, and stained with Weigert haematoxylin-eosin. The degree of eosinophil infiltration in the epithelial layer was calculated at three different levels in the trachea. One level was right below the bifurcation (level A), another near the fifth gristle ring

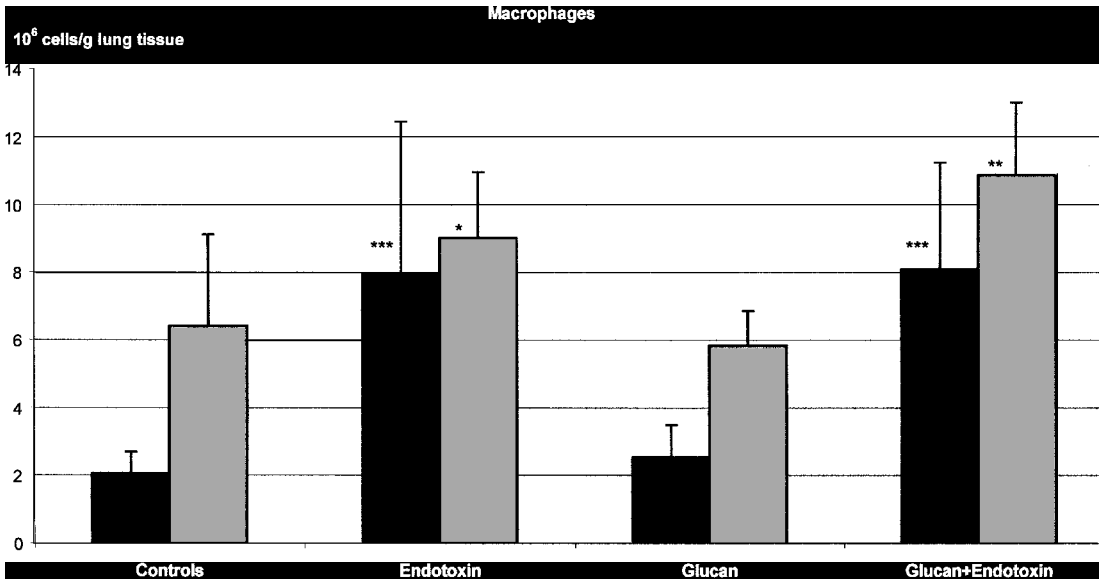


FIG. 1. The numbers of macrophages in lung lavage fluid (black bars) and lung interstitium (grey bars). The results are presented as mean values with standard deviations. *$p$<0.05, **$p$<0.01, ***$p$<0.001 as compared to controls.

(level B) and a third level near the seventh gristle ring above the bifurcation (level C).

### Statistics

The mean and median numbers of each cell type in the LLC and LIC preparations were calculated for each group of animals. In the same manner, the mean and median numbers of eosinophils at each level in the trachea epithelium were calculated for each group of animals. The differences between the groups were evaluated using two-sided Mann–Whitney $U$-test. Differences were considered statistically significant at $p$<0.05.

## Results

The animals tolerated the exposures well and no effect on weight development was found. There were no respiratory distress symptoms i.e. there was no indication of sensitization to either of the agents used. The numbers of different cells in the lung lavage and lung interstitium are shown in Figs 1–4.

In the controls, the macrophage was the dominant cell type both in the lung lavage and the lung interstitium. The proportions of cells in the two compartments varied; macrophages were about three times more numerous per gram lung in the interstitium than in the lung lavage. For the neutrophil and

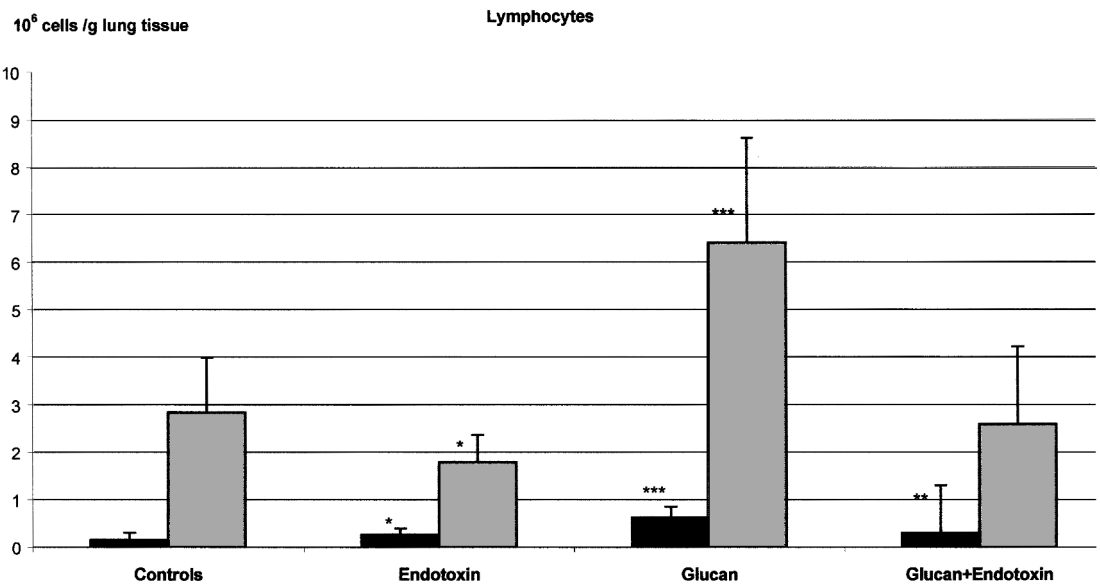


FIG. 2. The numbers of lymphocytes in lung lavage fluid (black bars) and lung interstitium (grey bars). The results are presented as mean values with standard deviations. *$p$<0.05, **$p$<0.01, ***$p$<0.001 as compared to controls.

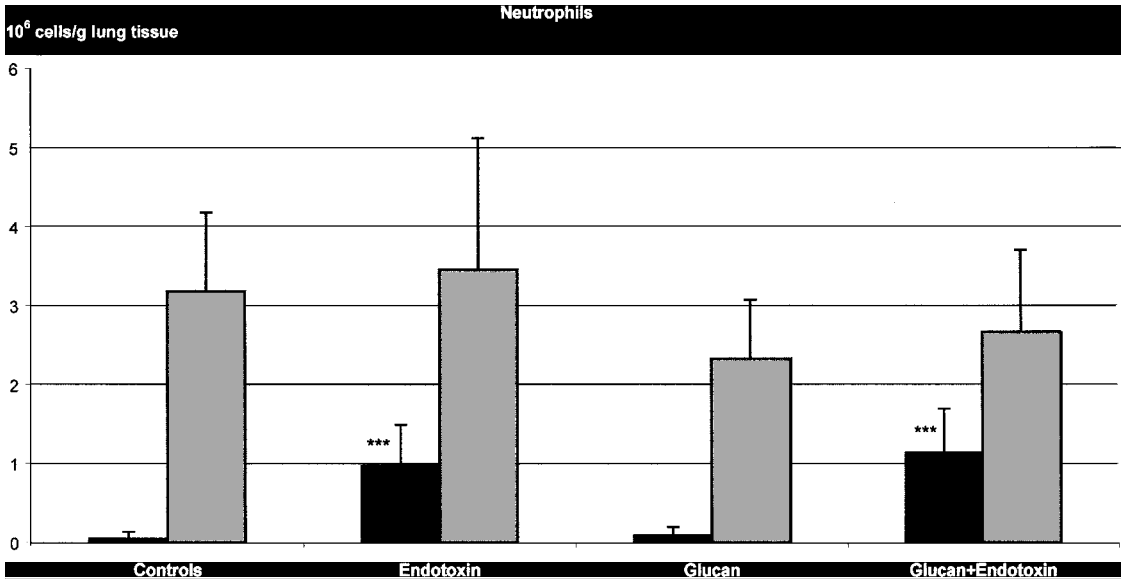


FIG. 3. The numbers of neutrophils in lung lavage fluid (black bars) and lung interstitium (grey bars). The results are presented as mean values with standard deviations. ***$p<0.001$ as compared to controls.

lymphocyte numbers, the differences were even larger with the numbers in the interstitium, almost hundred-fold higher for neutrophils and 20-fold higher for lymphocytes as compared with the lung lavage. For eosinophil numbers there was only a small difference.

The numbers of macrophages in lung lavage fluid and lung interstitium are shown in Fig. 1. After endotoxin exposure, the numbers of macrophages were increased in the lung lavage as well as in the lung interstitium. A similar result was found when (1→3)-β-D-glucan was inhaled together with endotoxin. No effect was found after (1→3)-β-D-glucan exposure alone. The numbers of lymphocytes in lung lavage fluid and lung interstitium are shown in Fig. 2.

After endotoxin exposure, the number of lymphocytes was moderately increased in lung lavage but no increase was found in the lung interstitium. (1→3)-β-D-glucan exposure caused an increase in lymphocyte numbers, both in lung lavage and lung interstitium. In animals simultaneously exposed to endotoxin, the (1→3)-β-D-glucan-induced increase in lymphocyte numbers was not present. The numbers of neutrophils in lung lavage fluid and lung interstitium are shown in Fig. 3.

After exposure to endotoxin, the number of neutrophils was increased in lung lavage but not in

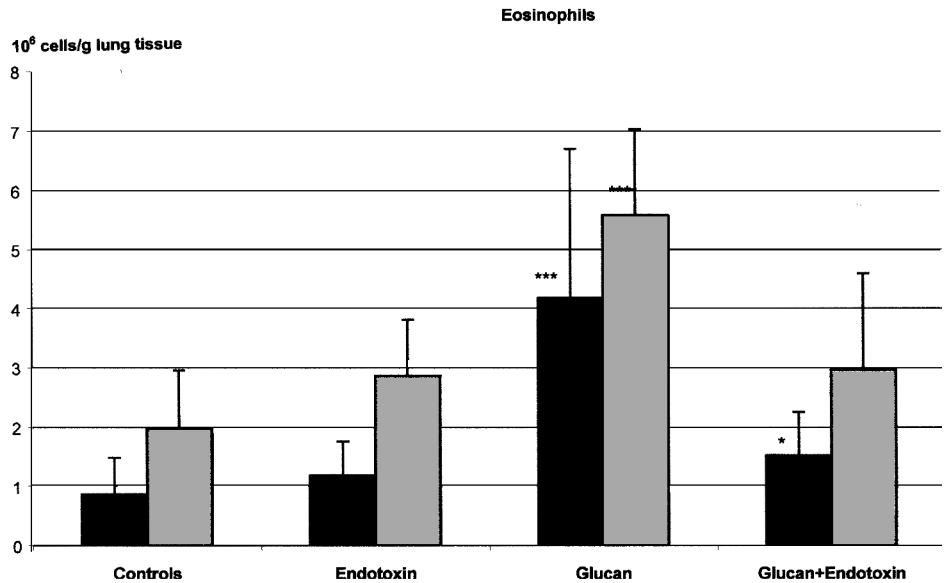


FIG. 4. The numbers of eosinophils in lung lavage fluid (black bars) and lung interstitium (grey bars). The results are presented as mean values with standard deviations. *$p<0.05$, ***$p<0.001$ as compared to controls.

**Table 1.** Eosinophilic leukocytes in tracheal epithelium (cells/field at 40×magnification) of guinea pigs exposed to endotoxin, (1→3)-β-D-glucan (GLU) or endotoxin + GLU

| Exposure | μg/ml | Levels in the trachea | | |
|---|---|---|---|---|
| | | A | B | C |
| Controls | – | 15.1 (6.6) | 32.0 (10.8) | 35.6 (12.4) |
| Median | | 17.5 | 33.0 | 36.0 |
| Endotoxin | 25 | 13.0 (8.2) | 24.0 (14.5) | 22.6 (12.7)* |
| Median | | 12.5 | 24.50 | 23.25 |
| GLU | 10 | 41.1 (22.4)** | 53.4 (30.6)* | 57.0 (39.4) |
| Median | | 39.75 | 47.50 | 55.25 |
| GLU+Endotoxin | 10 + 25 | 6.9 (6.9)** | 9.5 (7.4)*** | 7.6 (6.3)*** |
| Median | | 4.00 | 7.75 | 6.75 |

Means, medians and standard deviations in parentheses. A = a level right below the bifurcation, B = a level near the fifth gristle ring, C = a level near the seventh gristle ring above the bifurcation. Controls $n = 13$, endotoxin $n = 10$ and GLU $n = 10$.
*$p<0.05$, **$p<0.01$, ***$p<0.001$ as compared to controls.

lung interstitium. No effect was found after (1→3)-β-D-glucan exposure. When (1→3)-β-D-glucan was inhaled together with endotoxin, the result was similar as compared with endotoxin exposure alone. The numbers of eosinophils in lung lavage fluid and lung interstitium are shown in Fig. 4.

After endotoxin exposure, the numbers of eosinophils was slightly increased in lung lavage and lung interstitium, but the differences were not statistically significant. After (1→3)-β-D-glucan exposure, the numbers of eosinophils were significantly increased both in the lung lavage and in the lung interstitium as compared with controls. Simultaneously exposure to endotoxin decreased the (1→3)-β-D-glucan-induced increase in both sites. The number of eosinophils in the tracheal epithelium is shown in Table 1. In animals exposed to endotoxin, there was a tendency to a lower number, which was significant at level C as compared with controls. Exposure to (1→3)-β-D-glucan caused an increase in the eosinophil numbers. In animals simultaneously exposed to endotoxin, the (1→3)-β-D-glucan-induced increase in eosinophil numbers was not present, instead a significantly lower number of eosinophils was present as compared with controls.

## Discussion

The animals were exposed by inhalation to doses of endotoxin and (1→3)-β-D-glucan that were similar to amounts found in the environment. On the cellular level, the doses were far inferior to those used in *in vitro* experiments. A calculation of the amount of (1→3)-β-D-glucan in relation to the number of macrophages in the lung[17] showed that the dose at the cellular level in this experiment corresponded to about 0.15 ng/$10^6$ macrophages. In *in vitro* studies, the doses used are often in the order of 0.5 to 10 μg for the same number of macrophages. Against that background, one should be careful when extrapolating reactions in *in vitro* studies to effects found *in vivo* after inhalation.

The major finding in the study was that the exposure to (1→3)-β-D-glucan caused an increase in the number of eosinophils in the airway epithelium. This is consistent with a previous report where another kind of (1→3)-β-D-glucan (curdlan) was used.[12] In another study that used a similar exposure protocol, a large increase in eosinophil numbers was seen after exposure to ovalbumin, but no effect was found after (1→3)-β-D-glucan exposure.[18] The discrepancy between these studies could be a consequence of having examined the animals in the last study 48 hours after the last exposure as compared to 24 hours after exposure in the previous studies.

The increase in eosinophil numbers found after (1→3)-β-D-glucan exposure was decreased by a simultaneous exposure to endotoxin. This effect is consistent with findings in a previous study, where an ovalbumin-induced eosinophilia in lung lavage was decreased by a simultaneous exposure to endotoxin.[18] It is conceivable that the macrophage stimulation brought about by endotoxin induced the secretion of cytokines, e.g. tumour necrosis factor α (TNFα)[19,20] and interleukin (IL)-12 (Th1 promoting pattern), that depressed the release of cytokines involved in eosinophil effects such as IL-5 and IL-10 (Th2 promoting pattern). Unfortunately no measurements of such cytokines could be undertaken in this study due to the lack of suitable reagents in guinea pigs. Guinea pigs normally have a rather high amount of eosinophils, but this could not explain the different responses found after endotoxin and (1→3)-β-D-glucan exposures as they are compared to the same controls.

The results in the present study suggest that endotoxin and (1→3)-β-D-glucan activate different inflammatory mechanisms when inhaled. The endotoxin exposure caused an increase in the numbers of

neutrophils and macrophages without an increase in eosinophil numbers and lung interstitial lymphocytes, while the (1→3)-β-D-glucan exposure caused an increase in eosinophil numbers and lung interstitial lymphocytes, without an increase in neutrophil and macrophage numbers. There are previous studies that further support that (1→3)-β-D-glucan acts through a different mechanism as compared with endotoxin. In a mouse model, there was an increase in the secretion of IL-10 and a decreased secretion of IL-12 after (1→3)-β-D-glucan exposure.[13] A depression of inflammatory cytokines, including TNFα, in mice pretreated with soluble (1→3)-β-D-glucan has recently been reported.[21] In a recent acute inhalation experiment in humans, the subjects inhaled (1→3)-β-D-glucan and saline in a random, double-blind design (manuscript submitted for publication). Inhalation of (1→3)-β-D-glucan decreased the phytohaemagglutinin (PHA) induced TNFα production from blood mononuclear cells. The number of eosinophils in blood was significantly decreased 72 h after exposure, suggesting a recruitment of eosinophils into the lung tissue. No increase in the number of neutrophils was found. These results indicate that exposure to (1→3)-β-D-glucan could induce a Th2 type cytokine secretion pattern.

In a previous experiment involving acute exposures, (1→3)-β-D-glucan was found to block the endotoxin-induced neutrophil recruitment in a dose dependent fashion. While this blocking effect could be explained by a down-regulation of macrophage activity, caused by the secretion of interleukin receptor antagonists induced by (1→3)-β-D-glucan,[22] no such effect could be demonstrated in the present experiment involving a subchronic exposure. This suggests that the blocking effect is a temporary phenomenon and that macrophages regain their capacity to secrete neutrophil attracting cytokines such as TNFα and IL-1 after repeated exposures.

Another study involving subchronic exposures,[11] suggested a synergistic effect between endotoxin and (1→3)-β-D-glucan with respect to neutrophil recruitment into the airways but no such effect was found in the present study. The reason for the discrepancy may be due to the different structures/preparations of the two types of (1→3)-β-D-glucan used in the different experiments. The experiment where a synergistic effect was suggested used the (1→3)-β-D-glucan curdlan. This has a single helix structure and was administered in a saline suspension. The present experiment used grifolan which has a triple helix structure and was suspended in 0.3 N NaOH to render it water-soluble. Regarding the recruitment of eosinophils, however, the two types of (1→3)-β-D-glucan caused similar responses. In this study grifolan caused an increase in eosinophils in the airway epithelium, which is consistent with the results of a previous study where curdlan was used.[12]

In conclusion, the results provide further evidence that (1→3)-β-D-glucan induces a different response in lung inflammatory cells than occurs after exposure to endotoxin. They further suggest that (1→3)-β-D-glucan can cause an eosinophil dominated inflammatory response in the airway epithelium which is abrogated by a simultaneous endotoxin exposure, suggesting a complex interaction between these environmental microbial cell wall components. Finally, the results illustrate the need to evaluate different compartments of the lung – airway spaces, epithelium and lung interstitium – in order to understand the complicated cell dynamics that constitute the response to inhaled microbial cell wall agents.

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