



EXPOSURE to dust may involve co-exposure to agents which are allergens, together with those which are pro-inflammatory. To study the effects of such a co-exposure, the humoral and inflammatory responses were studied in guinea pigs inhaling the T-cell-dependent antigen ovalbumin (OVA) and the inflammatory agents (1 → 3)-β-D-glucan and lipopolysaccharide (LPS). The effects were evaluated as inflammatory cells in the lung and serum antibodies to OVA. LPS caused a stimulation of the OVA-induced antibody production which was abolished by simultaneous exposure to (1 → 3)-β-D-glucan. An increase of eosinophils after OVA exposure was decreased by co-exposure to (1 → 3)-β-D-glucan. The results demonstrate a complex interaction between adaptive and innate immune mechanisms in the lung, determined by exposure to common contaminants in airborne dust.

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

## (1 → 3)-β-D-Glucan and endotoxin modulate immune response to inhaled allergen

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### Introduction

The lung is continuously exposed to environmental particles which can potentially cause local tissue damage. These agents usually occur as complex mixtures, of which microbial cell wall (MCW) components such as bacterial endotoxin and (1 → 3)-β-D-glucan from moulds are common components. Both agents are present in organic dusts, which are prevalent in many occupational settings<sup>1</sup> and also in buildings with indoor air related symptoms.<sup>2</sup> Protein antigens from pollens, house dust mites, animal danders, etc., are also often present in these dusts.

Endotoxins are lipopolysaccharide compounds on the outer cell wall of Gram-negative bacteria and they cause an inflammatory response in the airways and the lung tissue after inhalation<sup>3–5</sup> (1 → 3)-β-D-glucans are present in the cell wall of fungi and some bacteria and are potent inducers of inflammation as well as modulators of the immune system.<sup>6</sup>

The current experimental literature on the mechanism(s) underlying lung tissue damage resulting from inhalation exposure to irritants, relates to a large extent to models employing single agents and quite often single acute exposures. While this approach has yielded valuable information on the potential pathways involved in lung inflammatory reactions, it has left unanswered questions relating to possible interactions which may occur when exposure to different pro-inflammatory agents occurs simultaneously, and over longer time periods, as is usually the case *in vivo*.

Recent work from our laboratory provides an illustration of these points. An acute inhalation exposure to endotoxin causes a massive neutrophil invasion into the airways, whereas in a chronic exposure situation, this response is almost absent. (1 → 3)-β-D-glucan in itself does not cause a neutrophil inflammation, but the endotoxin-induced neutrophil invasion into the airways was depressed by the simultaneous inhalation of (1 → 3)-β-D-glucan.<sup>7</sup> In chronic exposure experiments, the adaptation to endotoxin which normally occurs, was hampered by co-exposure to (1 → 3)-β-D-glucan, resulting in the persistence of an increased number of neutrophils in the lung and the formation of granulomas.<sup>8</sup>

The present experiments extend these investigations to analysis of how the reaction to a T cell dependent antigen was influenced by the simultaneous exposure to endotoxin and (1 → 3)-β-D-glucan. As an antigen we choose the soluble protein antigen ovalbumin (OVA), which is widely used in experimental models for induction of immuno-inflammatory lung damage in terms of antibody production and eosinophilia.

### Material and Methods

#### Experimental rationale

The experiment was set up to fulfil the criteria of a realistic environmental exposure. The exposure was by inhalation and the doses given by the MCW agents were similar to those encountered in industrial

environments. Guinea pigs were chosen in view of the previous experience on the cellular reactions in airways and lung tissue of this species.

### Animals and exposure

Adult male and female Duncan Harley guinea pigs from our own breeding unit were exposed in inhalation chambers to aerosols of the different agents used, as previously described.<sup>5,7,8</sup> Ovalbumin (OVA, Chicken egg no A5503, Sigma) was suspended in saline at 10 mg/ml. (1  $\rightarrow$ 3)- $\beta$ -D-glucan, ('Curdlan') Waiko Pure Chemical Ind, Tokyo, Japan) was suspended in saline in a concentration of 100  $\mu$ g/ml. Endotoxin (*Escherichia coli* O26:B6, LPS, Sigma) was suspended in saline at 10  $\mu$ g/ml. The liquids were aerosolized using a Collison spray, generating particles less than 0.5  $\mu$ m in diameter as measured with a Royco optical particle counter.

The animals were placed in a 180 litre aluminum exposure chamber and exposed to a continuous flow of the aerosols, the dose being 2 ng LPS and/or 8 pg (1  $\rightarrow$ 3)- $\beta$ -D-glucan. Animal exposure took place during 4 h/day, 5 days/week for 5 weeks.

At that time, certain animals were in addition exposed to an aerosol of OVA (30 mg/m<sup>3</sup>, 40 min/day, 1 day/week) for 3 weeks together with a continued LPS or/and (1  $\rightarrow$ 3)- $\beta$ -D-glucan exposure. The animals were examined 48 h after the last exposure to LPS/(1  $\rightarrow$ 3)- $\beta$ -D-glucan and 24 h after the last exposure to OVA for animals receiving this exposure. Six animals were exposed to (1  $\rightarrow$ 3)- $\beta$ -D-glucan, nine to OVA, five to OVA and (1  $\rightarrow$ 3)- $\beta$ -D-glucan, eight to OVA + LPS, nine to OVA + LPS + (1  $\rightarrow$ 3)- $\beta$ -D-glucan and 15 animals were unexposed controls.

The concentrations of LPS and (1  $\rightarrow$ 3)- $\beta$ -D-glucan were chosen on the basis of previous experience using the same animal model.<sup>7,8</sup> By and large, they correspond to concentrations which can be encountered in occupational and environmental settings with organic dusts exposure, e.g. handling of mouldy hay.<sup>1,2</sup> There are several types of (1  $\rightarrow$ 3)- $\beta$ -D-glucan—curdlan was chosen as a representative for a straight single helix, water insoluble form.

There was no exposure to LPS only or to LPS and (1  $\rightarrow$ 3)- $\beta$ -D-glucan as these effects have been described previously.<sup>8</sup>

### Antibody determination

Cardiac blood was drawn into a syringe, centrifuged and the serum was freeze-dried using standard methods. Antibodies to OVA in the serum samples were determined with a standard haemagglutination technique, and confirmed as IgG on the basis of 2-mercaptoethanol intensity and expressed as reciprocal titres.<sup>9</sup>

### Lung cell determinations

After a lethal i.p. dose of pentothal, the vascular lung bed was flushed with chilled Dulbecco's PBS, introduced via cannulation of the heart, the aorta being first severed in the abdominal cavity. Perfusion was performed until the lungs were clear white, at which point the heart was immediately tied off.

The right lung was used for collecting the inflammatory cell population. It was subject to lung lavage using a standardized volume of saline adjusted to body weight. A body weight of 400–500 g corresponded to 50 ml and for each additional 100 g, the lavage volume was increased by 10 ml. The lavage fluid was slowly injected into the lung in 10 ml portions and withdrawn. After each instillation, the fluid was withdrawn and collected in 50 ml centrifuge tubes placed on ice. The cells were centrifuged, the cell pellet collected and resuspended in saline whereafter the cells were counted and typed using May-Grünwald-Giemsa stain. These cells are referred to as lung lavage cells (LLC).

The lung was further prepared using a modification of a technique previously described.<sup>5,10</sup> It was excised and the upper right lobe including bronchi was placed on a filter paper. The lung was sliced in 0.4 mm thick slices and about 0.8 g was suspended in siliconized flasks containing 10 ml of PBS with 10% inactivated fetal calf serum (FCS), 175 units per ml of collagenase (Worthington) and 50 units per ml of DNase (Sigma). Following an incubation of 90 min in a shaking water bath (180 shakings per minute 37°C), the preparation was agitated and remaining tissue fragments in the cell slurry were removed via filtration through a thin cotton wool plug. The cells were collected as for LLC. Viability was controlled by Trypan blue exclusion (the range was 80–92%). These cells are referred to as lung wall cells (LWC).

### Data analysis and statistical treatment

The mean values for different cell types were calculated for all exposure regimes and differences in lung cell populations were evaluated using the Student's *t*-test. Differences in antibody titres were evaluated using the Wilcoxon test. The total number of cells recruited to the lung by the different exposures was calculated by adding the average numbers of LLC and LWC in each group, and subtracting the values found in the controls.

## Results

### General observations

The animals tolerated the different exposures without overt signs of distress. However, in the group exposed to OVA, occasional animals, irrespective of the expo-

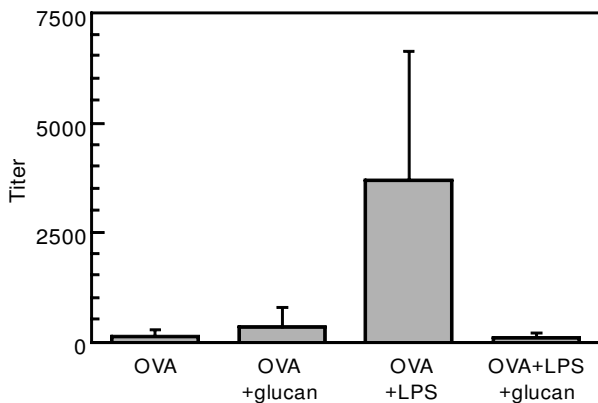


FIG. 1. IgG ovalbumin antibody titres in blood of guinea pigs exposed to ovalbumin (OVA), OVA + glucan, OVA + LPS. Mean and SD.

sure type, showed marked respiratory difficulties after the second exposure. Following this observation, all animals were given an antihistamine (Tavegyl®, 50 µg/kg) 15 min prior to the OVA exposure which inhibited the reactions.

### OVA antibodies

Determinations of OVA-antibodies showed that control animals all had titres less than 2 (background values). The same results were found in the group of animals exposed to (1 → 3)-β-D-glucan alone. The results from the groups exposed to OVA, OVA + (1 → 3)-β-D-glucan, OVA + LPS and OVA + LPS + (1 → 3)-β-D-glucan, are reported in Fig. 1.

The figure shows that exposure to OVA + (1 → 3)-β-D-glucan did not cause a significant increase in OVA antibody titre. In animals exposed to OVA + LPS, the titres were significantly higher ( $P < 0.001$ ). In animals which received OVA + LPS + (1 → 3)-β-D-glucan, the titres were not significantly different from those from animals exposed to OVA only or to OVA + (1 → 3)-β-D-glucan and significantly lower than in the group exposed to OVA + LPS ( $P < 0.001$ ).

### Lung cells

The number of different cell types recovered by lung lavage is shown in Table 1.

The exposure to OVA increased the number of macrophages, neutrophils and particularly eosinophils compared with controls ( $P < 0.01$ ,  $P < 0.05$  and  $P < 0.001$ , respectively). The exposure to (1 → 3)-β-D-glucan by itself did not influence the number of different lung lavage cells, apart from a slight decrease in neutrophils (NS). When (1 → 3)-β-D-glucan was added to the OVA exposure, there was a slight but non-significant reduction of the OVA-induced increase in neutrophils and eosinophils.

When LPS was added to the OVA exposure, there was an increase in macrophages and neutrophils, compared with animals exposed to OVA only ( $P < 0.001$  and  $P < 0.01$ , respectively). When (1 → 3)-β-D-glucan was added to the OVA + LPS exposure, the number of neutrophils and eosinophils was slightly lower (NS), mimicking the tendency found by (1 → 3)-β-D-glucan on the OVA exposure.

The absolute numbers of LWC are shown in Table 2. When compared with the number in the lung lavage, a striking difference in proportion was present for lymphocytes and neutrophils. In the lung wall, the average numbers of these cells were 20–30-fold higher than in lavage, whereas macrophages and eosinophils were only two to three times higher.

The changes in LWC numbers caused by the different exposures were overall smaller than those found in lung lavage. The OVA exposure caused an increase in all cell types (macrophages  $P < 0.05$ , lymphocytes  $P < 0.02$ , neutrophils NS and eosinophils  $P < 0.001$ ). The exposure to (1 → 3)-β-D-glucan did not by itself influence the number of lung wall cells. When (1 → 3)-β-D-glucan was added to the OVA exposure, the number of all cell types was lower ( $P < 0.04$  for macrophages and lymphocytes).

When LPS was given with OVA, the number of macrophages, lymphocytes and eosinophils was somewhat lower than in the group which had been exposed to OVA alone ( $P < 0.04$  for lymphocytes). The addition of (1 → 3)-β-D-glucan to this exposure,

**Table 1.** Lung lavage cells ( $\times 10^6$ /g lung) in animals exposed to different combinations of endotoxin, (1 → 3)-β-D-glucan and ovalbumin for 5 + 3 weeks. Average and  $\pm$ SD

Exposure	<i>n</i>	M	L	N	E
Control	15	2.8 ± 1.4	0.2 ± 0.2	0.2 ± 0.3	2.0 ± 1.7
Ovalbumin	9	6.0 ± 3.6	0.5 ± 0.2	0.6 ± 0.4	13.0 ± 8.4
Glucan	6	3.7 ± 1.4	0.1 ± 0.2	0.01 ± 0.02	1.9 ± 1.2
Ovalbumin + glucan	5	4.5 ± 2.0	0.4 ± 0.2	0.3 ± 0.1	8.5 ± 5.5
Ovalbumin + LPS	8	12.9 ± 5.8	0.9 ± 0.5	2.9 ± 2.2	7.4 ± 3.4
Ovalbumin + LPS + glucan	9	15.2 ± 4.1	1.0 ± 0.5	1.7 ± 1.4	5.5 ± 2.5

M = macrophages, L = lymphocytes, N = neutrophils, E = eosinophils, *n* = number of animals in each group.

**Table 2.** Lung wall cells ( $\times 10^6/\text{g}$  lung) in animals exposed to different combinations of endotoxin, (1  $\rightarrow$ 3)- $\beta$ -D-glucan and ovalbumin for 5 + 3 weeks. Average and  $\pm$  SD

Exposure	n	M	L	N	E
Control	15	7.9 $\pm$ 2.9	5.8 $\pm$ 3.3	4.3 $\pm$ 2.8	3.7 $\pm$ 2.0
Ovalbumin	9	19.4 $\pm$ 11.1	12.2 $\pm$ 8.8	6.7 $\pm$ 4.7	17.0 $\pm$ 10.0
Glucan	6	7.7 $\pm$ 2.8	5.7 $\pm$ 2.6	4.1 $\pm$ 0.9	3.6 $\pm$ 1.8
Ovalbumin + glucan	5	9.9 $\pm$ 2.6	4.7 $\pm$ 1.2	4.1 $\pm$ 2.5	10.9 $\pm$ 8.1
Ovalbumin + LPS	8	12.5 $\pm$ 5.6	5.6 $\pm$ 2.1	5.3 $\pm$ 3.4	11.1 $\pm$ 2.3
Ovalbumin + LPS + glucan	9	15.9 $\pm$ 4.7	7.0 $\pm$ 1.8	6.5 $\pm$ 2.6	7.8 $\pm$ 3.2

M = macrophages, L = lymphocytes, N = neutrophils, E = eosinophils, n = number of animals in each group.

caused a decrease in the number of eosinophils ( $P < 0.02$ ).

Table 3 presents a summary of the LLC and LWC migration of different cell types into the lung (values from exposed less than controls).

It is seen that exposure to OVA caused an increase of all types of cells as did exposure to LPS. There was no effect of (1  $\rightarrow$ 3)- $\beta$ -D-glucan alone. When (1  $\rightarrow$ 3)- $\beta$ -D-glucan was given together with OVA, there was a lower accumulation of all cell types as compared to animals exposed to OVA only. LPS given together with OVA, completely abolished the lymphocyte increase and decreased eosinophil accumulation induced by OVA and increased the number of neutrophils. When both (1  $\rightarrow$ 3)- $\beta$ -D-glucan and LPS were given together with the OVA, the attenuation effect of each agent on the OVA induced accumulation of eosinophils, seemed to be additive.

## Discussion

The study was designed to test the interactions between two microbial cell wall components on the response to a T cell protein antigen. The IgG antibody response after OVA exposure is a model which has previously been used by several authors. Bachelet *et al.*<sup>11</sup> presensitized guinea pigs by subcutaneous

**Table 3.** Net increase in individual inflammatory cell types in lung following exposure to different agents ( $10^6$  cells/g lung). Figures are lung wall and lung lavage cells in exposed animals less values in controls. Figures are rounded to whole numbers and values  $<1$  are shown as 0

Exposure	M	L	N	E
OVA	15	7	3	24
LPS	8	9	2	11
Glucan	0	0	0	0
OVA + glucan	4	0	0	14
OVA + LPS	18	0	7	13
OVA + LPS + glucan	20	2	4	8

M = macrophages, L = lymphocytes, N = neutrophils, E = eosinophils. Data on LPS from ref 7.

injection of OVA and Karol *et al.*<sup>12</sup> injected the OVA i.p., prior to aerosol exposure. While deliberate parenteral pre-immunization ultimately produces immune responses of higher titre than those achieved here, the protocols used in the present experiments where exposure was via inhalation alone, more closely approximate the situation in industry and the general environment.

The analysis of lung lavage and lung wall preparations was restricted to cell quantitation. The information is thus partly incomplete as functional tests are required for a full understanding of the observed reactions. Nevertheless, experimental exposures leading to large and consistent increases in specific cell types, particularly in the context of chronic exposure, are likely to be of pathological significance.

Exposure to aerosolized LPS exerted a stimulatory effect on the IgG response to inhaled OVA. This is consistent with its well-known adjuvant activity in relation to B-cell activation.<sup>13</sup> LPS inhalation has also been shown to increase the traffic of airway antigen-bearing dendritic cells (DC) to regional lymph nodes (RLN),<sup>14</sup> where they initiate immune responses via presentation of their sequestered stores of inhaled antigens to T cells.<sup>15,16</sup> The principal stimulus for DC migration from the intraepithelial tissue sites to RLN appears to be TNF $\alpha$ ,<sup>17</sup> the major source of which in the LPS-stimulated lung is alveolar macrophages (AM),<sup>18,19</sup> and we suggest the latter as a likely mechanism for LPS enhancement of the anti-OVA antibody response in these experiments.

When (1  $\rightarrow$ 3)- $\beta$ -D-glucan was given together with OVA and LPS, there was a pronounced depression of the adjuvant activity of the LPS concerning the antibody response. The mechanism behind this effect was not investigated in this study but the following hypothesis can be presented, based on experience from previous studies.

It has been shown that (1  $\rightarrow$ 3)- $\beta$ -D-glucan is phagocytosed by macrophages and that it decreases the endotoxin-induced secretion of TNF $\alpha$  from these cells.<sup>20</sup> It also attenuates the acute endotoxin-induced migration of neutrophils into the lung,<sup>7</sup> but increases the endotoxin-induced inflammatory response in the

lungs after chronic inhalation.<sup>8</sup> These observations suggest that (1 → 3)-β-D-glucan interferes with AM function. As noted above, TNFα is likely to be the principal stimulus for migration of DC from the airway epithelium to RLN, and interference in TNFα production would thus be expected to interrupt the flow of 'processed' antigen to the T helper cells in RLN, which are responsible for IgG production against inhaled OVA.<sup>15,16</sup>

The (1 → 3)-β-D-glucan exposure did not *per se* alter the number of inflammatory cells in the airways or in the lung wall. This is consistent with previous data from acute as well as chronic inhalation experiments.<sup>7,8</sup> There was, however, an alteration in the response to OVA in glucan-exposed animals, demonstrable as a general tendency to a reduction in number of inflammatory cells recruited into the lung, as shown in Table 3.

In the airways, (1 → 3)-β-D-glucan caused a tendency to a decrease in the number of OVA induced neutrophils and eosinophils. In the lung wall there was also a decrease in the number of lymphocytes.

These changes also suggest an effect of (1 → 3)-β-D-glucan on the regulatory function of macrophages and agrees with previous studies where a pre-exposure to (1 → 3)-β-D-glucan attenuated the migration of neutrophils into lungs of animals exposed to LPS.<sup>7</sup> Macrophages are known to control the migration of neutrophils into the airways after an inflammatory challenge, e.g. LPS.<sup>21</sup> Regarding eosinophils, their migration into the airways is likely to be due to local IL-5 release from OVA-reactive T cells of the T helper-2 (Th-2) phenotype.<sup>22,23</sup> It is known that AM, particularly activated AM, suppress T cell activation both *in vitro* and *in vivo*,<sup>24</sup> principally through release of nitric oxide.<sup>25–27</sup>

While we have no data on production from this experiment, it is relevant to note that (1 → 3)-β-D-glucan has been shown to inhibit LPS-induced release by macrophages of reactive oxygen radicals,<sup>28</sup> which are generally co-produced with NO. Also, as shown in Table 3, the presence of glucan markedly reduces the number of macrophages recruited in response to OVA. Given that one of the important roles of macrophages is to 'switch off' local immune responses in the lung to inhaled antigens,<sup>15,16,22,24,26</sup> the reduction in the macrophage response induced by glucan might contribute to prolongation of the overall anti-OVA response.

In summary, these results show that inhalation of LPS leads to an increased antibody response to 'bystander' antigen (OVA), with a concomitant small (and probably insignificant) reduction in the accompanying large eosinophil response to the antigen. In contrast, glucan exposure did not affect the humoral response. It exerted only a small effect on the OVA-induced eosinophilia, but almost entirely abrogated the large attendant macrophage response. Further-

more, co-exposure to both agents re-established the macrophage response while further reducing the eosinophil response to one-third of controls. It is important to emphasize that none of these complex interactions involving environmental exposure to environmental levels of airborne MCW products are predicted by the results of single exposures to either agent alone. The magnitude of the effects on host response are so pronounced that they are likely to have relevance to human exposure to the same agents.

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ACKNOWLEDGEMENTS. The work was supported by the Swedish Work Environment Fund (contract 91–0325) and the Swedish Heart Lung Foundation (grant 30019).

**Received 12 November 1997;  
accepted 20 January 1998**



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